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ISOLATION AND CHARACTERIZATION OF PYRENE DEGRADING BACTERIA FROM PETROLEUM OIL CONTAMINATED SOIL FROM CHANDIGARH

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ABSTRACT

Poly Aromatic Hydrocarbons (PAHs) is a class of organic compounds that are hydrophobic, recalcitrant and carcinogenic in nature. Due to their long persistence and toxicity they pose serious threat to human health and environment. Pyrene, with four cojoined benzene rings is a very stable high molecular weight PAH. The hydrophobic nature of pyrene makes it difficult to undergo biodegradation. Few indigenous microorganisms are reported to degrade pyrene to some extent albeit at low concentrations with slower degradation rates. Therefore, in the present study pyrene degrading bacteria were screened for their degradation potential at higher concentration (100 mg/l). Using pyrene as the only carbon source, a total of 32 microorganisms were screened and isolated from petroleum oil contaminated sites around Chandigarh, India. Out of these, 12 isolates were capable of utilizing different concentrations of pyrene and further three strains (Bj4, Nfl2 and Bt1) showing maximal growth were selected. Identification of the strains was done on the basis of their morphology and biochemical characteristics. All the three isolates were Gram-negative, rod-shaped, non-spore forming bacteria, having the same optimal temperature (30°C) for growth. Two out of the three (Bj4 and Nfl2) belonged to the genus of *Acinetobacter* and one (Bt1) to *Enterobacter*. This is the first study to report degradation of pyrene by *Acinetobacter* species. HPTLC analysis revealed 60-66% fall in the concentration of pyrene in the cultures of *Acinetobacter*

after 24 days of incubation. This is the highest degradation achieved in shortest duration so far. Hence the organism is most suitable and can be exploited for bioremediation of these recalcitrant pollutants.

Keywords: *Acinetobacter*, Degradation, *Enterobacter*, Poly Aromatic Hydrocarbons, Pyrene

INTRODUCTION

Poly Aromatic Hydrocarbons (PAHs) have been identified as priority pollutants by the United States Environmental Protection Agency (USEPA). These constitute one of the largest group of chemical mutagens and carcinogens that are released in the environment primarily due to anthropogenic activities, such as fossil fuel combustion, oil spills and waste incineration [1–3]. Their ubiquitous presence posses serious health and environmental concerns owing to their toxicity, mutagenicity and carcinogenicity [4, 5]. These have been categorized as low molecular weight (LMW) and high molecular weight (HMW) compounds. LMW-PAHs with less than four aromatic rings are considered to be less recalcitrant and hydrophobic in nature as compared to the HMW-PAHs that are more persistent, toxic and difficult to degrade. The hydrophobic and recalcitrant nature of HMW-PAHs is the major limiting factor for their remediation from the soil or aqueous media [6, 7]. Potential of indigenous microorganisms to degrade the PAH's is a major cause of concern because of extremely slow utilisation of HMW-PAHs as a carbon source. Increased toxicity of these contaminants in the environment has been studied by [8, 9].

Very few indigenous microorganisms such as *Pseudomonas*, *Mycobacterium*, *Rhodococcus* have been reported to partially degrade pyrene and other PAHs at a very slow rate [10, 11]. Recent report with a halophilic bacteria *Thalassospira* sp. TSL 5-1 showed that addition of pyrene with an initial concentration of 20 mg/l as sole carbon source, metabolized only 8.28 mg/l (41.4%), over a period of 25 days [12]. Another study using strain F14, constructed through fusing protoplast of *Sphingomonas* sp. GY2B and *Pseudomonas* sp. GP3A, showed 18% degradation of pyrene in 10days [13]. [14] reported more than 90% degradation of phenanthrene and pyrene with a very low initial concentration of 5 mg/kg after 42 days of incubation with *Psedomonas* species. However in another report with *Pseudomonas aeruginosa,* 93% degradation of pyrene (50 mg/l) over a

period of 7 days was observed [11]. Further pyrene degradation studied with extracellular polymeric substances (EPS) in minimal medium demonstrated 80% utilisation of the compound in 35 days [15]. This showed slow metabolism of PAHs even at lower concentrations.

To enhance the biodegradation rate of these recalcitrant's in soil or aqueous medium different strategies such as addition of solvents, surfactants and other carbon or nitrogen sources has been exploited [16–18]. When studied with bacterial (*Pseudoxanthomonas)* or fungal cultures (*Bjerkandera adusta*), addition of solvents and surfactants showed improved degradation of low molecular weight PAHs, such as anthracene and phenanthrene [19–21]. Reactor studies with PAHs dissolved in biocompatible vegetable oils and solvents also showed enhanced rate of enzymatic degradation [21, 22]. However in natural environment, higher concentrations of these recalcitrant's exist and without any additional carbon source, there is negligible biodegradation of these contaminants.

Thus require intensive research for screening and identification of species having good potential of degrading these recalcitrant's in a short span. In the present study, screening of the organisms with capability of transforming pyrene has been attempted, with further optimisation of various factors affecting the process, for enhancing the efficiency of the strains for faster degradation of one of the most potent carcinogen, pyrene.

METHODOLOGY:

1) ***Screening and Isolation of pyrene degrading bacteria***

Pyrene degrading microorganisms were screened on minimal medium containing [(g/l) of KH_2PO_4 1.0, K_2HPO_4 1.0, NH_4SO_2 1.0, $MgSO_4.7H_20$ 0.02, $FeSO_4.7H_2O$ 0.007, $MnSO_4.7H_2O$ 0.0001], by enrichment culture technique. Petroleum oil contaminated soil samples were collected from four different locations around Chandigarh, India. 2 gm of soil was added to 50 ml of minimal medium containing pyrene (50 mg/l) as sole source of carbon and were incubated at 37°C on a rotary shaker at 100 rpm. Sub-culturing at regular intervals was done with pyrene as sole substrate in the minimal medium. Morphologically different bacteria showing consistent growth with pyrene were selected and maintained on minimal agar plates with pyrene. These selected strains were acclimatized in the laboratory for 6 months with pyrene in minimal medium and were used further for degradation studies.

2) ***Identification of the selected bacteria:***

2 (a) Morphological and Biochemical characterisation:

Selected strains (Bj4, Bt1 and Nfl2) were characterised by colony morphology, Gram staining and visualizing under light microscope at (100X) and Field Emission-Scanning Electron Microscope (FE-SEM). Additional biochemical characterization and motility tests were performed using microbes from exponential phase of growth according to Bergey's manual of Determinative Bacteriology [23]. For biochemical characterization, the tests such as Indole, nitrate, oxidase, MR-VP, citrate, hydrogen sulphate, gelatin liquification, carbohydrate and amino acid utilization were performed.

3) Optimization of growth parameters:

Growth of the strains under different pH (5.5, 6, 6.5, 7 and 7.5), temperature (28, 30, 32, 35, 37 and 40°C) and pyrene concentration (10, 50, 100, 150 and 200 mg/l) was investigated. From stock of pyrene (25 mg/ml) in ethyl acetate 100 µl was transferred into sterilized 100 ml Erlenmeyer flask. After complete evaporation of the solvent, 25 ml of minimal medium was added into the flask and inoculated with 1 ml of the inoculum. Cultures were incubated at different temperatures (28-40°C) and CFU's were determined, after 7 days of incubation. For pH optimization, the above experiment was performed in minimal medium with adjusted pH (5.5-7.5) at their optimized temperature. Effect of different concentrations of pyrene (10-200 mg/l) on growth of the strains was determined at their optimum pH and temperature by determination of colony forming units (CFU's).

4) Biodegradation of Pyrene:

Degradation studies were done with 100 mg/l concentration of pyrene in minimal medium having no other carbon source. Flasks were inoculated by transferring 1 ml inoculum of the acclimatized strains and were incubated at optimized temperature (30°C) in rotary incubator at 100 rpm. After every 6^{th} day growth was determined by CFU's for 24 days and degradation was detected by HPTLC. For this residual pyrene was extracted from the whole flask twice with ethyl acetate by sonication for 30 min, followed by centrifugation at 10,000 rpm for 10 min at 4°C for phase separation. Then the extract was vaporised in rotary evaporator to 1 ml. Different concentrations of standard of pyrene (20, 40, 60, 80 and 100 mg/l) were spotted onto the chromatographic plate and chromatograms were developed by methanol:water (8:2). These were observed under illumination at λmax of 338nm. Concentration of pyrene in test samples

was calculated by comparing the peak area of sample with the peak area of standard.

RESULTS AND DISCUSSION:

1) ***Identification of the strains:***

A total of 32 bacterial isolates were screened for their growth on minimal agar plates with pyrene. Twelve strains that showed good growth with increased concentration of pyrene in minimal medium were studied further and out of these three isolates (Bj4, Nfl2 and Bt1) with highest OD values were selected, as shown in Fig 1. These three strains were identified by their morphological and physiological properties, shown in Table (1 & 2) and Fig 2 (a & b). All the selected strains are Gram negative and non-spore forming bacteria. Colonies of Bj4 and Nfl2 were pale yellow in colour, convex with glistening surface and entire margin. Whereas, colonies of the Bt1 are cream in colour, flat, mucoid with irregular form. Bt1 can utilize nitrate, citrate, glucose, lactose but couldn't produce oxidases and hydrogen sulphide. However Bj4 and Nfl2 couldn't utilize nitrate and lactose but can produce oxidases and hydrogen sulphide. On the basis of their morphology and biochemical characterization, Bj4 and Nfl2 showed close relatedness to *Acinetobacter* sp. and Bt1 to *Enterobacter* sp. Same biochemical characters were reported for genus of *Acinetobacter* and *Enterobacter* by [13, 24–26].

2) ***Effect of temperature:***

Effect of temperature on growth of these strains was studied at 28, 30, 32, 35, 37, 40°C. For all these strains (Bj4, Bt1 and Nfl2) temperature of 30°C was found to give optimum growth. However temperatures above and below 30°C were inhibitory to the growth of strains. As shown in Fig. 3, rise in temperature from 30-37°C reduced the cell count of Bt1 from $5.2x10^7$ to $4.0x10^6$, similar pattern was observed for Bj4 and Nfl2. Rise in temperature increases the solubility and availability of the compound, but it decreases the oxygen solubility that reduces the metabolic activity of strictly aerobic microorganisms. In a study by [13] same growth patterns were reported for *Pseudomonas* sp..

Bishnoi et al. [14] checked degradation efficiency of *Pseudomonas* sp. at different temperatures and maximum was found be at its optimum temperature (30°C). Similar studies when carried out with *Pleurotus pulmonarius* F043 at different temperatures, showed direct co-relation between growth and degradation, as reported by [27].

3) ***Effect of pH:***

Growth was checked at different pH (5.5-7.5) in minimal medium with pyrene (50

mg/l) as sole carbon source. For Bj4, growth was found to be maximum at pH 7.0, with a CFU of $2.88x10^7$, whereas for Bt1 and Nfl2, it was at pH 6.5 and 7.0, respectively, Fig 4. Three strains showed marked reduction in growth when pH was more towards the acidic range (pH 5.5). However, gradual fall in cell number of Bt1 and Nfl2 was observed above pH 6.5 and 7.0, respectively. With change in pH from 7.0 to 7.5 there was reduction in cell count from $2.88x10^7$ to $4.8x10^6$ for Bj4. Difference in their optimum value may be because of the enzymes that are released for metabolism of pyrene, have their maximum activity at that pH. [12] also showed higher degradation of pyrene in alkaline conditions as compared to acidic, when studied with an initial concentration of 20 mg/l.

4) ***Effect of pyrene concentration on growth:***

To determine the effect of increased concentration of pyrene on growth of the isolates, different initial concentrations (10, 50, 100, 150 and 200 mg/l) were added to the media. An increased growth with increase in concentration was observed with all the three strains, as shown in Fig 5. Two strains 'Bj4 and Bt1' got their maximum CFU ($3.1x10^6$ and $2.6x10^6$), at 200 mg/l, whereas the third strain (Nfl2) had its maximum ($1.88x10^6$) at 100 mg/l. In comparison to Bj4 and Bt1, Nfl2 showed less growth ($9.0x10^5$) at 200 mg/l, however when compared with other reported bacteria it was found to be much higher. Less growth at lower concentrations may be explained by low carbon supply to the organism and at higher concentrations growth was less due to toxicity of the compound [28]. Though, with Bj4 and Bt1 10 fold increase in growth when concentration increased from 10 to 200 mg/l reflects no such toxic effect on these isolates. This exhibits broader range of substrate utilization by all the three isolates.

5) ***Biodegradation of Pyrene:***

Chromatograms for different concentrations of pyrene were obtained using HPTLC, as explained in methodology. Standard curve was prepared for concentrations (20-100 mg/l), as shown in Fig (6-7). All the three acclimatized strains (Bj4, Nfl2 and Bt1) when incubated with pyrene (100 mg/l) at their optimized conditions (temperature: 30°C and pH: 7.0, 7.0 and 6.5) showed an increased degradation rate with increase in growth (Fig 8-10). Maximum degradation (66 %) and CFU value ($6.8x10^7$) were observed with Bj4, followed by Bt1 (65 %) and least with Nfl2 (60 %), respectively. In case of Bt1, growth in terms of CFU was lower than Nfl2 after 24 days, but degradation rate was found to be more, indicating

inherent ability of the strain to degrade pyrene. Within 12 days, Nfl2 and Bj4 and metabolized more than 50 mg/l of the compound followed by further increase in degradation to 60% and 66% respectively till 24th day, as shown in Fig (9 & 8). However with Bt1, pyrene concentration decreased from 100 to 35 mg/l in 24 days, Fig 10. The degradation rate and absolute amount metabolized within 12 days in present studies with the isolates is much higher than reported previously [12, 13]. Difference in degradation pattern may be due to different enzymes that are released by strains of two diverse families. The degradation profile of two strains belonging to *Acinetobacter* genus showed almost similar results, indicating fast mineralization of pyrene than the other genus reported. This study reflects higher potential of the isolates (Bj4, Nfl2 and Bt1) to metabolise pyrene with a faster degradation rate.

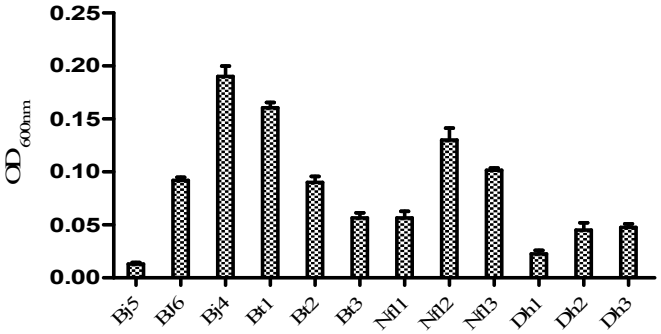

Fig1: Growth of the isolates with pyrene as the only carbon source in minimal medium

Table1: Morphological characteristics of the Isolates

BACTERIAL STRAINS	GRAM STAIN	SHAPE	COLOUR	SPORE STAIN	MOTILITY
Bj4	**Negative**	**Short Rod (Coccobacilli)**	**Pale Yellow**	**Negative**	**Negative**
Bt1	**Negative**	**Rod**	**Cream**	**Negative**	**Positive**
Nfl2	**Negative**	**Short Rod (Coccobacilli)**	**Pale Yellow**	**Negative**	**Negative**

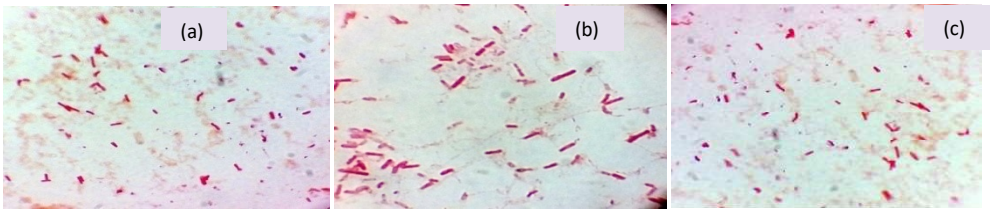

Fig2 (a): Light microscopy at 100X of (a) Bj4, (b) Bt1and (c) Nfl2.

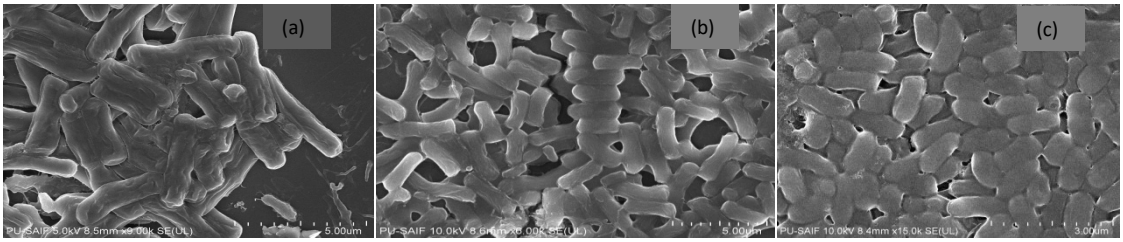

Fig2 (b): Field Emission-Scanning Electron Microscopy of (a) Bj4 (9.00K), (b) Bt1 (6.00K) and (c) Nfl2 (15.0K).

Table2: Biochemical characteristics of the Isolates

Isolates → Characteristic↓	Bj4	Nfl2	Bt1	Isolates→ Characteristic↓	Bj4	Nfl2	Bt1
Oxidase reduction	--	--	--	Esculin hydrolysis	--	+	--
Nitrate Reduction	--	--	+	Arabinose	--	--	--
Lysine utilisation	+	+	+	Xylose	+	--	+
ONPG	--	+	--	Adonitol	--	--	+
Indole test	--	--	--	Rhamnose	--	+	--
Ornithine utilisation	+	+	+	Cellobiose	+	+	--
PA Deamination	--	--	--	Melibiose	+	--	--
Malonate utilisation	+	--	+	Saccharose	--	+	+
Hydrogen sulphide Production	+	+	--	Raffinose	--	--	+
Citrate Utilization	+	+	+	Trehalose	--	--	+
Methyl Red test	+	+	--	Glucose	+	--	+
Voges Proskauer	--	--	+	Lactose	--	--	+
Catalase	+	+	+	Macconkey Agar	+	+	+

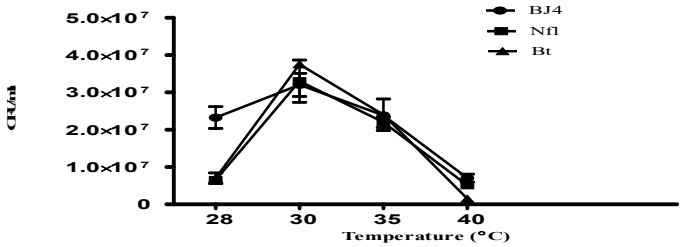

Fig 3: Effect of temperature on the growth of the isolates (Bj4, Bt1 and Nfl2) in minimal medium supplemented with pyrene (100 mg/l), after 7 days of growth.

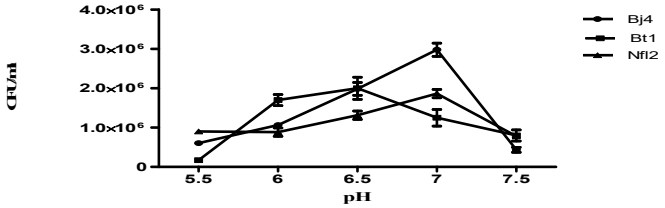

Fig4: Effect of pH on the growth of the isolates (Bj4, Bt1 and Nfl2) in minimal medium supplemented with pyrene (100 mg/l), after 7 days of growth at 30°C.

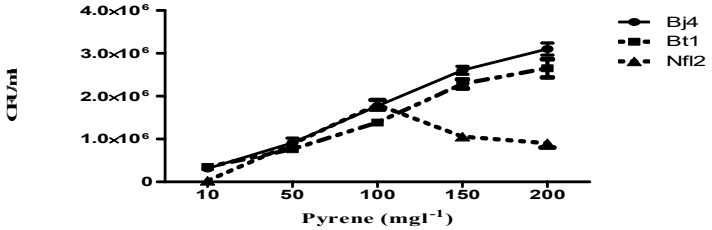

Fig5: Effect of pyrene concentration on the growth of the isolates (Bj4, Bt1 and Nfl2) after 7 days of incubation in minimal medium, at 30°C.

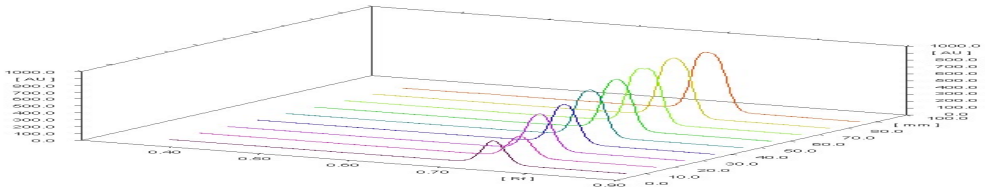

Fig6: Chromatogram of standard pyrene (20, 40, 60, 80 and 100 mg/l) at 338 nm.

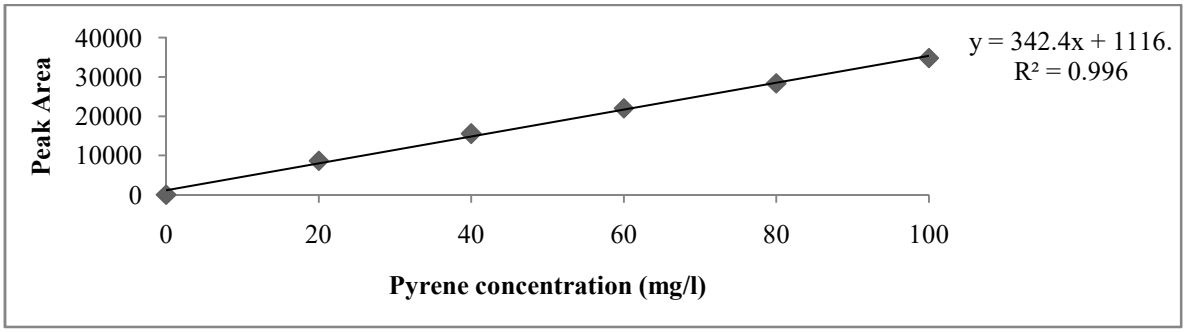

Fig7: Standard curve for pyrene.

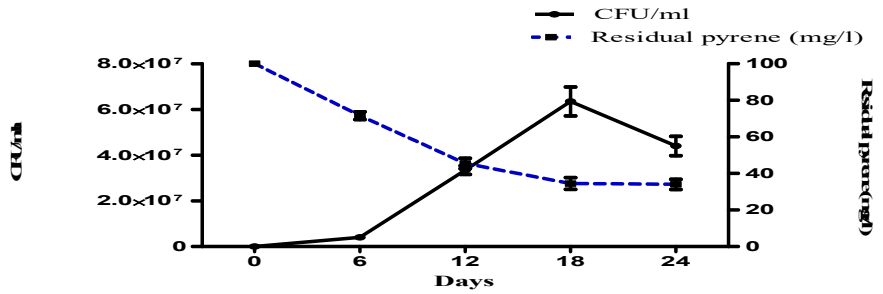

Fig8: Growth and degradation profile of Bj4 at 100 mg/l concentration of pyrene in minimal medium (pH 7) studied at 6, 12, 18 and 24th day.

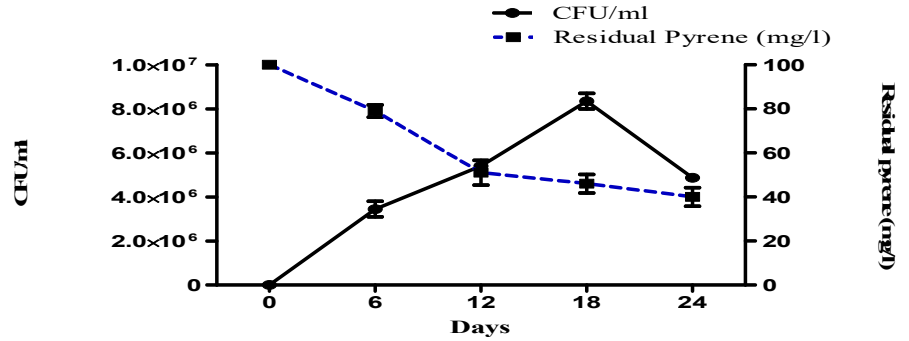

Fig9: Growth and degradation profile of Nfl at 100 mg/l concentration of pyrene in minimal medium (pH 7) studied at 6, 12, 18 and 24th day.

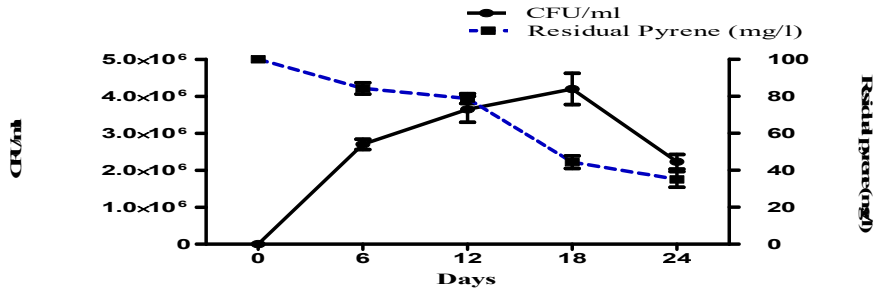

Fig10: Growth and degradation profile of Bt1 at 100 mg/l concentration of pyrene in minimal medium (pH 6.5) studied at 6, 12, 18 and 24th day.

CONCLUSION:

The novel strains isolated in the present study from the oil spilled areas near Chandigarh, possess the ability to degrade pyrene, when grown at a step wise higher concentration (10-200 mg/l) and in a short span than the previous reports. The isolates could metabolize 60-66 mg/l of pyrene within 24 days of incubation, under the conditions devoid of any other carbon source except pyrene. To the best of our knowledge, genus *Acinetobacter* has not been reported for degradation of pyrene. Moreover this genus exhibited excellent potential of degrading HMW-PAH. Based on these observations it is concluded that these acclimatized strains can be exploited further for efficient bioremediation of these contaminants in the natural environment.

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