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Original Research Article

Abilities of *Bacillus Cereus* CPOU13 in Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs)

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ABSTRACT

In the present study the biodegradation of three PAHs (phenanthrene, anthracene and pyrene) and effects of different factors (PAHs concentrations, pH and incubation time) on PAHs biodegradation using a novel bacterial strain, Bacillus cereus CPOU13 have been reported. Results revealed that growth of the strain decreased with increasing PAHs concentrations from 10 ppm to 250 ppm in MSM. In concern to the incubation period of 13 days, the strain grew gradually from 0 to 3rd day and became consistent between 3rd and 7th days. Growth of the strain was dropped from 7th day onwards. Growth of the strain was tested at different pH and found that it was grown better from pH6 to pH8. Biodegradation of PAHs was studied in vitro for 13 days using HPLC and rate of PAHs degradation (%) was determined at regular time intervals. The strain degraded phenanthrene about to 73.46% and its initial concentration declined from 216.32 µg to 56.57 µg. The strain degraded anthracene to 85.76% and its initial concentration reduced from 209.20 µg to 32.63 µg. Degradation of pyrene was recorded up to 47.88% and its initial concentration reduced from 230.14 µg to 119.95 µg.

Keyword: PAHs; pH; incubation time; *Bacillus cereus* CPOU13; MSM; biodegradation

INTRODUCTION

Now a day, pollution that arises due to the contamination of polycyclic aromatic hydrocarbons (PAHs) has been becoming a global issue and is increasing very rapidly in urban areas [1-2]. Very recent and

comprehensive report on worldwide PAHs emissions, sources and concentrations reported high level presence of PAHs (from 1960 to 2008) in the environment around the globe [3]. As the PAHs are carcinogens and teratogens major national and international health organizations warned about their hazardous effects on human health and considered them as one of the most important pollutants.

The Agency for Toxic Substances and Disease Registry (ATSDR) has grouped 17 PAHs which cause deleterious health effects [4]. The United States Environmental Protection Agency (US-EPA) also placed 16 PAHs among the 28 priority pollutants. The International Agency for Research on Cancer (IARC) was classified PAHs as carcinogenic (group 1) or suspected carcinogenic (group 2A) pollutants in humans [5].

Human activities via the industrialization for the production of electricity and energy have been release high amounts of PAHs into the environment. Major sources for the PAHs emissions are combustion of coal, oil, gas, wood materials for energy supply, waste incineration processes, petroleum processing, aluminium sintering, residential heating, electricity, heat generation etc. [6]. Due to the deleterious effects of PAHs these pollutants are emergent to degrade and ameliorate earliest as possible in all environments.

Degradation of PAHs by diversified organisms in the nature is known from a long back [7]. Intensified work was carried out in the studies of PAHs degradation and many researchers were succeeded in the identification of many PAHs degrading life forms that belongs to bacteria, fungi, and algae [8-10]. Among the all life forms bacteria are more effective in biodegradation studies due to their short time lifecycles and easy multiplication in any environment. The present study is aimed at degradation of phenanthrene, anthracene and pyrene by a novel bacterial strain, *Bacillus cereus* CPOU13 that isolated from oil contaminated site in Hyderabad [11]. In the present study we investigated the effects of different factors on biodegradation of PAHs and quantification of degrading PAHs *in vitro*.

MATERIALS AND METHODS

Effect of phenanthrene, anthracene and pyrene concentrations on the growth of *Bacillus cereus* CPOU13

The method to determine the effect phenanthrene, anthracene and pyrene concentrations on the strain growth was adopted from John et al. (2012) [12]. 75 ml of MSM was dispensed into 250 ml flasks and sterilized by autoclaving. The flasks were then divided into six sets of six flasks. Further, 10, 50, 100, 150, 200 and 250 ppm levels of phenanthrene, anthracene and pyrene were dissolved separately and the strain was inoculated to each flask. Inoculated flasks were then incubated at 28ºC and 130 rpm speed for 3 days. 5 ml of aliquot was aseptically collected from each flask and assayed for the level of microbial growth. Growth was recorded in terms of optical density (OD) readings at 600 nm using a UV spectrophotometer.

Effect of pH on growth of *Bacillus cereus* CPOU13

Effect of pH (negative logarithm of hydrogen ion) on growth of the strain was tested at a wide range of pH from 5 to 10 on MSM broth. pH of the media was adjusted by NaOH/HCl and incubated under standard growth conditions. Growth of the strain was recorded in terms of OD at 600 nm after 3 days of incubation.

Construction of standard chromatograms for HPLC studies

Method for the preparation of standard chromatograms for phenanthrene, anthracene and pyrene using high performance liquid chromatography (HPLC) was adopted from Boonchan et al. (2000) [13]. Phenanthrene, anthracene and pyrene chemicals with 99% purity purchased from Sigma-Aldrich, USA and

used throughout the experiments. Accurately weighed 100 mg of phenanthrene, anthracene and pyrene was transferred separately to 100 ml volumetric flasks and 2-3 ml of acetonitrile was added to dissolve. Solution was diluted with acetonitrile (HPLC grade) to obtain volume 1000 mg/L stock solution and ran HPLC. Standard HPLC chromatograms were prepared using known concentrations of phenanthrene, anthracene and pyrene *viz.,* 25, 50, 100, 200 and 250 ppm and software, 'Origin 6.0' by retrieving peak area values of PAHs at respective retention times.

HPLC analysis

High performance liquid chromatography studies were conducted using a reverse phase HPLC (SHIMADZU, model RF-10AXL). The instrument consists of dual pump system and connected with UV detector (SPD-20A). Instrument was equipped with column C 18 (250 mm x 4.6 mm, 5 A° particle size) of Phenomenex Co. Mobile phase was consisted of 75% acetonitrile and 25% of de-ionized water. Detector was set at 250 nm and mobile phase was maintained at flow rate of 0.8 ml/min in isocratic mode. 20 µl of sample was injected into HPLC with a HPLC injector (Rheodine injector) that prior filtered with 0.22 µm syringe filters. Data of each peak on HPLC chromatogram was analyzed using chromatography software 'LC Solutions'.

Degradation of polycyclic aromatic hydrocarbons (PAHs) *in vitro*

Degradation of phenanthrene, anthracene and pyrene in a mixture was studied according to the method of Moody et al. (2001) [14]. 250 ml of Minimal salt medium (MSM) was prepared in 1000 ml conical flasks and enriched with 150 ppm of PAHs (each PAH 50 ppm). One ml culture of the strain was added separately to MSM flasks and in control flasks no culture was added. The cultured flasks were incubated under standard growth conditions for 13 days in the dark. A 5 ml of aqueous portion was withdrawn from each flask at regular time intervals of 0, 1, 2, 3, 7, 8 and 13 days and extracted with three equal volumes of ethyl acetate after adjusting pH to 2.5 using 1N HCl and the step of extraction was repeated thrice. Each time a pinch of anhydrous disodium sulphate (Na_2SO_4) was added to remove residual water content from the samples. Extract was concentrated using rotary evaporator under reduced pressure at 34ºC under vaccum conditions. Finally, the samples were dissolved in 3 ml of acetonitrile (ACN) and preserved at 4ºC for HPLC studies as described earlier.

RESULTS

Effect of Polycyclic aromatic hydrocarbons concentrations on *Bacillus cereus* CPOU13 growth

Effect of three test PAHs concentrations (from 10 to 250 ppm) on the strain's growth was studied on MSM and the results are presented in Table 1. The strain showed gradual decrease in growth with increasing concentrations of PAHs from 10 ppm to 250 ppm and incase of control sample no bacterial growth was recorded (Fig. 1).

Table 1: Growth of *B. cereus* CPOU13 at different concentrations of PAHs

PAHs enrichment	OD at different concentrations of PAHs (ppm)						
	0	**10**	**50**	**100**	**150**	**200**	**250**
Phenanthrene	0	0.29	0.26	0.23	0.23	0.21	0.19
Anthracene	0	0.28	0.25	0.22	0.21	0.17	0.12
Pyrene	0	0.24	0.22	0.21	0.19	0.16	0.11
Control (Without PAHs)	0	0	0	0	0	0	0

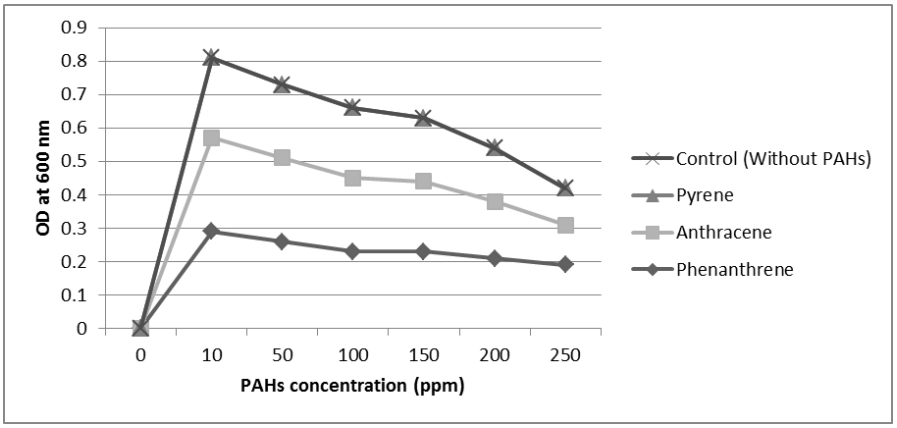

Fig. 1: Growth of *B. cereus* CPOU13 on MSM enriched separately with different concentrations of PAHs

Effect of incubation time on *B. cereus* CPOU13 growth

Growth of the strain was assayed for 13 days on MSM enriched separately with phenanthrene, anthracene or pyrene. The results are presented in Table 2 and Fig. 2. The strain recorded gradual increase in growth from 0th day to 3rd day and became consistent between 3rd and 7th days of incubation. Growth of the strain was dropped after 7th day and recorded minimum on 13th day. Maximum growth of the strain was observed on phenanthrene enrichment over the anthracene and pyrene enrichments and minimum growth was recorded on pyrene enrichment.

Table 2: Growth of *B. cereus* CPOU13 on MSM enriched separately with phenanthrene, anthracene or pyrene for 13 days

PAHs enrichment	OD at different time intervals (days)						
	0	**1**	**2**	**3**	**7**	**10**	**13**
Phenanthrene	0	0.13	0.28	0.41	0.43	0.39	0.28
Anthracene	0	0.12	0.26	0.37	0.38	0.31	0.16
Pyrene	0	0.09	0.18	0.27	0.29	0.23	0.14
Control (Without PAHs)	0	0	0	0	0	0	0

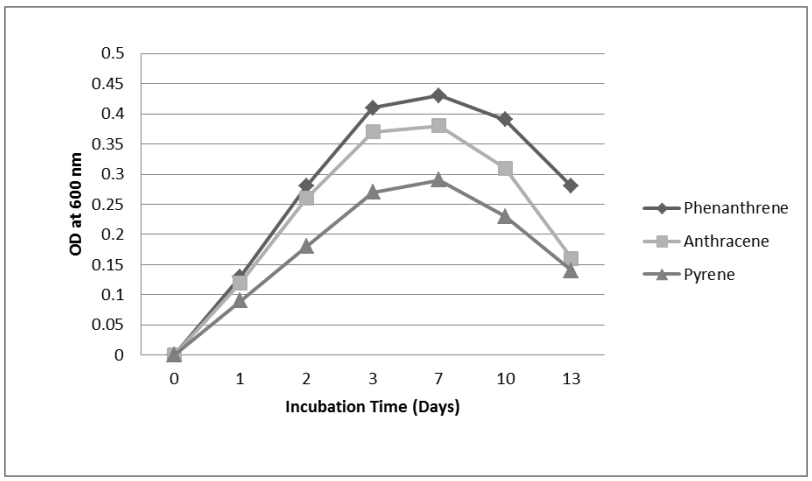

Fig. 2: Growth of *B. cereus* CPOU13 on MSM enriched separately with phenanthrene, anthracene or pyrene for 13 days

Effect of pH on *B. cereus* CPOU13 growth

pH of the medium influences bacterial growth and thereby degradation of PAHs. In view of this, growth of the strain was assessed at different pH, acidic to basic on MSM. The strain recorded better growth from pH 6 to pH 8 and it was declined as the acidity and alkalinity increased in the medium. The growth was relatively less but moderate at pH 9 (Table 3 and Fig. 3)

Table 3: Growth of *B. cereus* CPOU13 at different pH on MSM

pH	4	5	6	7	8	9	10
OD at 600 nm	0.23	0.85	1.78	1.82	1.83	1.08	0.56
Control (Without PAHs)	0	0	0	0	0	0	0

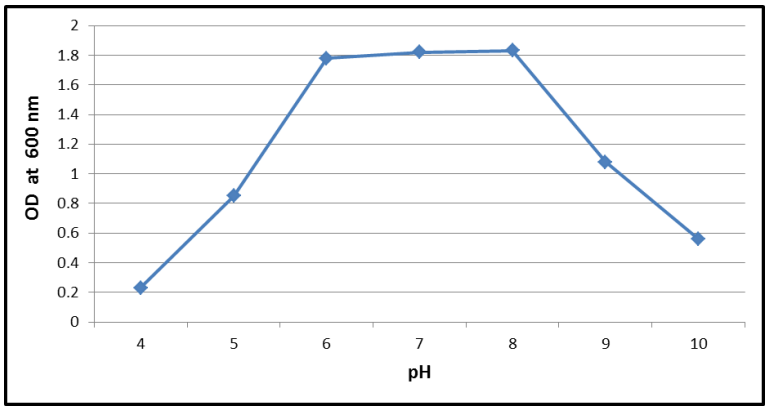

Fig. 3: Growth of *B. cereus* CPOU13 at different pH

Degradation of phenanthrene, anthracene and pyrene by *B. cereus* CPOU13 *in vitro*

The standard chromatograms were constructed separately phenanthrene, anthracene and pyrene based on their retention time and peak area. A linear standard graph was drawn for each PAH and their obtained correlation coefficient (R^2) values are 0.9935 for phenanthrene, 0.9916 for anthracene and 0.9865 for pyrene (Results are not shown). Biodegradation of phenanthrene, anthracene and pyrene by *B. cereus* CPOU13 was assayed *in vitro* conditions on MSM. Degradation of PAHs was studied at regular time intervals of 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 7^{th}, 8^{th} and 13^{th} days. The observed retention peak areas of phenanthrene, anthracene and pyrene were analyzed by software 'LC-solutions'. Based on these data concentrations of phenanthrene, anthracene and pyrene in test samples were determined using standard chromatograms that plotted early. The strain degraded three test PAHs to a greater extent in 13 days of incubation and the results are presented in Table 4 and Fig. 4 and 5.

The strain, *B. cereus* CPOU13 degraded phenanthrene up to 73.46% and initial concentration of phenanthrene (216.32 µg) was declined to 56.57 µg. Degradation of anthracene reached up to 85.76% as 209.20 µg of initial concentration was reduced to 32.63 µg. Degradation of pyrene was recorded up to 47.88% and 230.14 µg of initial concentration was degraded to 119.95 µg. Degradation of phenanthrene, anthracene and pyrene was observed from first day and continued up to 13^{th} day. In the mixture of PAHs, anthracene

degraded at maximum percentage (85.76) while pyrene degraded at minimum percentage (47.88) (Fig. 4, 5).

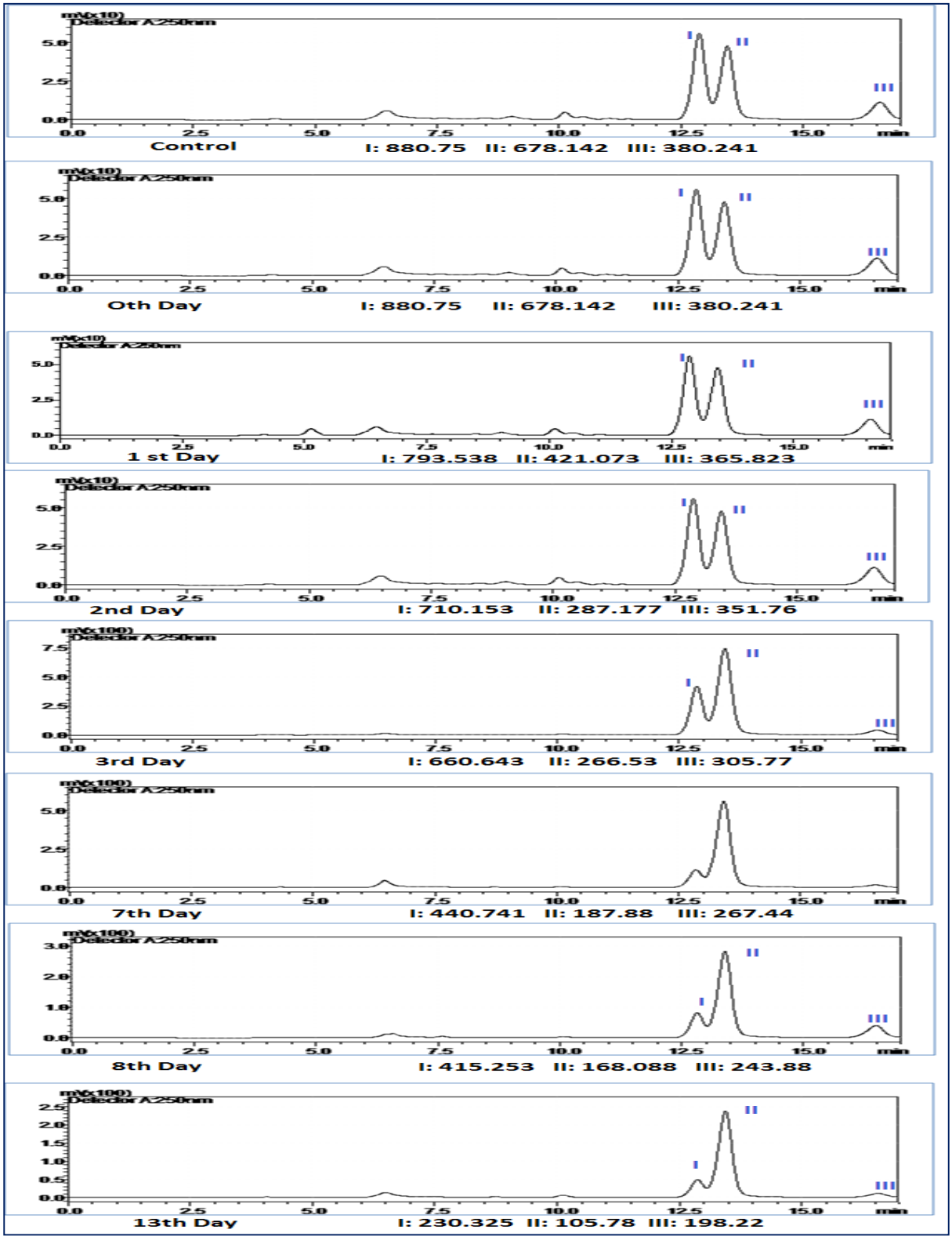

Fig. 4: HPLC chromatograms of PAHs during their degradation by *B. cereus* CPOU13 *in vitro*
(I= Phenanthrene; II= Anthracene; III= Pyrene)

Table 4: Degradation of phenanthrene, anthracene and pyrene by *B. cereus* CPOU13 on MSM enriched with a mixture of PAHs *in vitro*

Sl. No.	Days	Phenanthrene		Degradation (%)	Anthracene		Degradation (%)	Pyrene		Degradation (%)
		Rt. Peak	Conc. (µg/5ml)		Rt. Peak	Conc. (µg/5ml)		Rt. Peak	Conc. (µg/5ml)	
1	Control	880.750	217±2.07	0	678.142	211±2.03	0	380.241	233±2.08	0
2	0th	880.750	217±2.07	0	678.142	211±2.03	0	380.241	233±2.08	0
3	1st Day	793.538	196±2.06	9.867	421.073	135±1.53	36.22	365.823	223±1.99	4.05
4	2nd Day	710.153	175±2.07	19.31	287.177	88±1.53	58.35	351.760	213±1.53	8.42
5	3rd Day	660.643	163±1.15	25.07	266.530	83±1.52	60.73	305.770	186±2.08	20.23
6	7th Day	440.741	109±2.08	49.80	187.880	57±1.52	72.86	267.440	162±1.53	30.32
7	8th Day	415.253	104±1.12	52.21	168.088	52±1.15	75.59	243.880	147±5817	36.61
8	13th Day	230.325	58±1.72	73.46	105.780	33±2.08	84.23	198.220	119±1.16	48.76

(Rt. Peak = Retention Peak; ± represents standard deviation of three replicates)

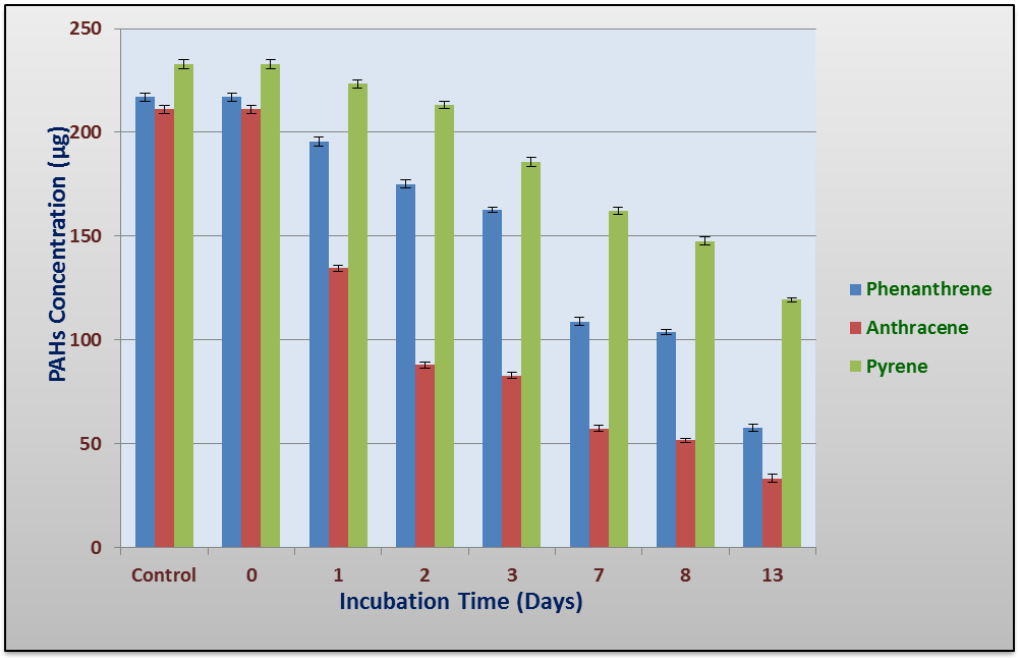

Fig. 5: Biodegradation of phenanthrene, anthracene and pyrene by *B. cereus* CPOU13

DISCUSSION

Polycyclic aromatic hydrocarbons contamination is of environmental concern as they exhibit carcinogenic and mutagenic properties. Utilization of bacterial strains that possesses abilities to degrade PAHs in the studies of biodegradation has prime importance and takes high priority. The present study is conducted to determine the biodegradation abilities of a novel bacterial strain, *B. cereus* CPOU13 that isolated from a PAHs contaminated site.

Bisht et al. (2010) [15] investigated the growth of bacterial strains namely, *Kurthia* sp. SBA4, *Micrococcus variance* SBA8, *Deinococcus radiodurans* SBA6, *B. circulans* SBA12 on MSM enriched with different concentrations anthracene and naphthalene and they reported that growth of the bacteria was decreased with

increasing PAHs concentration. As they suggested, higher concentrations of PAHs declines bacterial growth rapidly. Similarly, growth of *B. cereus* CPOU13 was decreased with increasing concentrations of phenanthrene, anthracene and pyrene. Decrease in bacterial growth and multiplication is may be due to the toxic effects of PAHs on bacterial membrane [16].

Incubation time is well noted to effect growth and multiplication of bacteria in different media. In the present study growth of the strain, *B. cereus* CPOU13 was increased from first day of the experiment up to 3rd day, became consistent between 3rd to 7th days and declined afterwards. The results indicating that the strain can grow better on MSM enriched with phenanthrene, anthracene and pyrene enrichments for 7 days and more degradation of PAHs can be observed until 7th day. The present results are coinciding with the findings of Hunter et al. (2005) and Lily et al. (2009) [17-18].

pH is one of the important factor that influences bacterial growth in media. As several studies suggested bacteria grow better within pH range 7 to 9 and also support the degradation of PAHs [19-20]. In the present study, better growth of *B. cereus* CPOU13 was observed between pH 6 to 8 and this data helps in maintaining pH of MSM for optimum PAHs degradation. Our findings are similar with the results of Hou et al. (2007), Lin et al. (2010) [21-22].

In the present investigation degradation of PAHs (phenanthrene, anthracene and pyrene) in mixture by *B. cereus* CPOU13 was studied *in vitro* conditions for 13 days. Different analytical methods are adapted to determine PAHs concentrations in liquid samples and Reverse phase- HPLC is one of the prime methods used. This grants advantages like sensitive and selective detectors (fluorimetric), easy sample preparation, and less risk of solute degradation. Polymeric C 18 column is preferred for successful separation of PAHs in HPLC.

In the present study degradation of phenanthrene, anthracene and pyrene was observed throughout the incubation period (13 days). However, rapid degradation was observed until 7th day and after this time period we noticed low rate of biodegradation. Lily et al. (2009) obtained similar results with the degradation of benzo(a)pyrene by *B. subtilis* BMT4i. It is clear that the strain showed high degradation due to high growth and multiplication in selected media under applied growth conditions [23]. *B. cereus* CPOU13 degraded phenanthrene, anthracene and pyrene up to 73.46, 84.23 and 48.7 percentages respectively in 13 days of incubation. These results indicating that the strain is very effective in biodegradation of selected PAHs. Similar reports were published by Daane et al. (2001); Zuang et al. (2004) [24, 25]. Efficiency of *Bacillus* strains to degrade PAHs in mixture is well reported in earlier researches also [17, 18].

Provision of adequate time i.e. incubation time course is necessary for effective biodegradation of PAHs. As researchers suggested inadequate incubation time may limit degradation of high molecular weight PAHs [26]. In the present investigation we found high level of PAHs degradation in 13 days of experiment and it became possible by abilities of the strain. Hunter et al. (2005) and Lily et al. (2009) [17-18] also published similar results. Biodegradation of PAHs is depends on their chemical structure and corresponding physiochemical properties [27] and low molecular weight PAHs degrade rapidly than high molecular weight PAHs [24, 28]. Similar results are obtained in the present study. Pyrene, a high molecular weight PAH degraded to a low percentage (48.1%) by *B. cereus* CPOU13 while low molecular weight PAHs such as phenanthrene and anthracene were degraded up to 73.46% and 84.23% respectively. This may be due to high molecular weight PAHs are more recalcitrant and hard to microbial attack [29-31].

CONCLUSION

The results of the present study indicating that the strain, *B. cereus* CPOU13 is very effective in degradation of phenanthrene, anthracene and pyrene *in vitro* conditions when favourable growth conditions applied. Yet more research has to take place in this piece of work for utilizing this strain in all conditions.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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