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Biodegradation of Pyrene Using *Bacillus* sp.C7 Isolated from Coal Deposited Soil

M. A. Karale[1], T. A. Kadam[1*], H. J. Bhosale[1] and K. P. Maske[2]

[1]School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded-431606, Maharashtra, India.
[2]Department of Microbiology, Rajarshi Shahu Mahavidyalaya, Latur (M.S.), India.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MAK and TAK involved in conceptual designing of this study. Author HJB helped to draft this manuscript and author KPM helped in optimization study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the efficiency of bacterial isolates in pyrene degradation.
Place and Duration of Study: The study was carried out at School of Life Sciences, S.R.T.M. University, Nanded, (M.S.), India during June 2015 and March 2016.
Methodology: Coal deposited soil was collected and used for isolation of pyrene degrading bacteria. The isolation of bacteria was carried out following three successive enrichments of soil sample in Bushnell Haas broth supplemented with 200, 400 and 1000 mg/L pyrene respectively. The morphologically distinct 10 bacterial isolates obtained on Bushnell Haas agar medium supplemented with 1000 mg/L pyrene as sole carbon and energy source were screened for pyrene degradation ability by DCPIP assay. The efficient pyrene degrading *Bacillus* sp. C7 was selected and used for further studies. The parameters in terms of pyrene concentration, pH of the medium, temperature, incubation period and suitable surfactant, carbon and nitrogen source were optimized using OVaT approach.
Results: The efficient pyrene degrading strain C7 was selected and identified on the basis of morphological and biochemical characterization as *Bacillus* sp. C7. The strain was able to

**Corresponding author: E-mail: karale.mahesh@gmail.com;*

catabolize 81.53% of pyrene (1000 mg/L) within 20 days of incubation. The strain showed highest pyrene degradation at 1000 mg/L, 30℃, pH 7.0, after 20 days of incubation and in the presence of tween-80, glucose as co-metabolite and beef extract as nitrogen source.
Conclusion: The study identified a pyrene degrading soil bacterium *Bacillus* sp. C7. The optimization approach used in the study revealed tween-80, glucose and beef extract are principle components for pyrene degradation. Overall 5.17% rise over control in pyrene degradation was observed after optimization studies.

Keywords: Pyrene; B. cereus; degradation; optimization.

1. INTRODUCTION

Pyrene is a high molecular weight (HMW) polycyclic aromatic hydrocarbon (PAH) possessing four benzene rings arranged in clusters. Pyrene along with other PAHs is an ubiquitous pollutants as a result of both natural and anthropogenic activities. Long term persistence of these compounds in nature is due to their low bioavailability and higher sorption on soil and sediment [1]. Pyrene is one of the PAHs, which have been considered to be priority pollutants by the United States Environmental Protection Agency (US-EPA) [2]. PAHs constitute a major environmental concern on ecosystem because of their adverse health effects on human being, bind covalently with cellular DNA, carcinogenic [3] and endocrine disrupting activity [4]. Degradation of PAHs from contaminated sites occurs through physical, chemical and biodegradation methods. Bioremediation is an economic, safe, ecofriendly and efficient method [5]. Pyrene as an indicator of the PAH group to study the biological degradation [6,7]. Diverse microorganisms, including bacteria, fungi, cyanobacteria and algae have ability to utilize PAHs as a sole source of carbon and energy. In addition, various factors affect the rate of biodegradation; solubility is one of them [8]. The low water solubility, production of toxic or dead end metabolites, metabolite repression, and presence of preferred substrates, lack of cometabolic or inducer and physical condition (temperature, oxygen, pH value and water content in soil) are often found to affect biodegradation [9,10]. Another factor that may improve PAH degradation is the addition of readily assimilated carbon sources [11]. Moreover, pH is an important factor that affects the solubility as well as metabolism of PAHs. The optimal range of bacterial degradation in between 5.5 and 7.8 [12,13].

In general, the optimization of pyrene bioremediation showing the simultaneous effect of different environmental factors have been less studied. Hence, in present study the effects of seven factors viz. concentration of pyrene, pH, temperature, incubation period, surfactants, carbon source and nitrogen source on the growth and biodegradation of pyrene of *Bacillus* sp.C7 isolated from coal deposited soil sample were studied.

2. MATERIALS AND METHODS

2.1 Chemicals and Media

Pyrene (purity 98.9%) was procured from Sigma Chemical Co. (Germany). The solvents used, ethyl acetate, acetonitrile, acetone was procured from SD-fine chemicals Mumbai. Bushnell Haas Broth (BHB) and Luria Britani Broth (LB Broth) were from Hi-Media Pvt.Ltd. Mumbai.

2.2 Soil Sample

Soil sample was collected from the coal deposited site of thermal power station Parali Vaijanath (M.S.). Soil sample was collected in a sterile polythene bag and stored in the lab at 4^{o}C until the isolation complete.

2.3 Isolation of Pyrene Utilizing Bacteria

Pyrene degrading bacteria were isolated from coal deposited thermal power station soil sample by three successive enrichments in BHB [14] supplemented with different concentration of pyrene (200 mg/L, 500 mg/L, 1000 mg/L) as a sole source of carbon and energy. Pyrene was dissolved in acetone and added in 250 mL Erlenmeyer flasks, evaporated and wrapped with aluminium foil to prevent photolysis. BHB medium (50 mL, pH-7.0±0.2) was added to the culture flask (200 mg/L). One gm of soil sample was inoculated in BHB medium flask and kept in the dark on rotary shaker incubator at 32^{o}C for 15 days at 120 rpm.

After 15 days of incubation, 5 mL of enriched broths were transferred to fresh BHB with 500 and 1000 mg/L of pyrene and incubated as stated earlier. The broth was serially diluted from 10^{-1} to 10^{-7} and 100µl were spread on BH agar containing 1000 mg/L pyrene. The plates were incubated in the dark for 2-3 days. The well isolated colonies were maintained on LB agar slant.

2.4 Screening of Pyrene Utilizing Bacteria

In order to screen the best isolates for pyrene utilization, purified isolates were grown in LB broth for 48 hrs. Centrifuged and collected cell pellet was washed with phosphate buffer (pH 7.0) and used for 2, 6-DCPIP assay.

2.4.1 Dicholorophenol indophenol (2, 6-DCPIP) assay

The assay mixture containing 2.25 mL of Fe-free W medium [15], 150 µl of $FeCl_3.6H_2O$ solution (150 µg/mL) and 150 µl of 2, 6-DCPIP solution (50 µg/mL) was mixed with 240 µl of bacterial cell suspension (O.D. 1.0 at 600 nm) and 25 µl of pyrene (1000 mg/L in acetone). The reaction mixture was incubated at 32℃ under shaking conditions (120 rpm) for 3 days. Pyrene degradation ability of the isolates was observed by recording a change in the color of the medium from blue to colorless [16].

2.5 Identification of Efficient Pyrene Degrading Bacterial Isolates

Morphological and biochemical studies were carried out to identify the efficient pyrene degrading bacteria [17,18]. The tests carried out includes, Gram staining, morphology, catalase, oxidase, methyl red, VP test, indole, and nitrate reduction, hydrolysis of starch, gelatin, lipid, casein, and citrate and sugar utilization.

2.6 Biodegradation Study

2.6.1 Preparation of inoculum of strain C7

Efficient pyrene degraded strain of bacterial isolate was inoculated into sterilized 10 mL LB broth and incubated for 48 hrs at 32°C. Centrifuged at 4000 rpm for 5 min and collected cell pellet, the pellet was washed twice by using phosphate buffer (pH 7.0) and suspended in 5 mL of phosphate buffer (pH 7.0) and used as inoculum for biodegradation study.

2.7 Growth Pattern of Isolate in Pyrene Containing Medium

The 50 mL of BHB was added in different 250 mL Erlenmeyer flask containing a final concentration of pyrene 1000 mg/L. The media were sterilized and inoculated with inoculum by adjusting 0.1 O.D. at 600 nm. The flasks were wrapped with aluminium foil and incubated aerobically in the dark for 25 days at 32°C and 120 rpm. The blank was prepared without adding test inoculum in BHB medium. The experiment was set up in triplicate. The samples were withdrawn at an interval of 5 days and growth was measured in terms of O.D. at 600 nm.

2.8 Biodegradation of Pyrene by C7 Strain

For the determination of residual pyrene concentration, the C7 culture samples were extracted thricely with ethyl acetate (1:1 v/v) after acidification to pH-2 with 1 N HCl. The ethyl acetate phase was further extracted three times with an equal volume of NaOH (60 mL, 10 mM). The organic phase (neutral fraction) was dried over anhydrous sodium sulfate (Na_2SO_4) and solvent was removed in vacuo. The residue was dissolved in 20 mL methanol and used for quantitative analysis of residual pyrene at 335 nm. The % degradation of pyrene was calculated by using the formula.

$$\%\ degradation = \frac{A - B}{A} \times 100$$

A = Initial concentration of pyrene.
B = Final concentration of pyrene.

2.9 Optimization of Growth and Pyrene Degradation by C7 Strain Using one Variable at a Time Approach (OVaT)

The factors evaluated for optimal growth and pyrene degradation included a concentration of pyrene, pH, temperature, incubation period, surfactant and nutrients, carbon and nitrogen sources. The levels of each factor studied were selected as described below. The *Bacillus* sp. C7 were inoculated into a 250 mL Erlenmeyer flask containing 50 mL BHB medium, the growth and % degradation were determined by taking O.D. at 600 nm and 335 nm, respectively.

2.9.1 Effect of concentration of pyrene on pyrene degradation by *Bacillus* sp. C7

Optimization of different concentration of pyrene for pyrene degradation by *Bacillus* sp. C7 was

studied at 400 mg/L, 600 mg/L, 800 mg/L, 1000 mg/L, 1200 mg/L and 1400 mg/L. Flasks containing 50 mL BHB medium having different concentrations of pyrene. Flasks were inoculated with 1 mL inoculums of *Bacillus* sp. C7 from exponential growth phase having 0.1 O.D. at 600 nm. Flasks were incubated on a rotary shaker at 120 rpm and 32^{o}C for 20 days. Growth was monitored at 20th days of incubation in the form of O.D. at 600 nm and contents from all flasks were subjected to liquid-liquid extraction by using ethyl acetate as stated earlier. The residual concentration of pyrene was determined by UV-Vis-spectrophotometer at 335 nm and % degradation was calculated as mentioned earlier. Once a factor was optimized, its optimum value was used for the subsequent factor optimization study.

2.9.2 Effect of pH on pyrene degradation by *Bacillus* sp. C7

To determine the optimal pH for pyrene degradation, flasks containing 50 mL of BHB medium having 1000 mg/L of pyrene as a sole source of carbon and energy were adjusted to pH 5.0, 6.0, 7.0, 8.0 and 9.0. Flasks were inoculated with 1 mL inoculum of *Bacillus* sp. C7 and flasks were incubated on a rotary shaker at 120 rpm and 32^{o}C for 20 days. After 20th day of incubation contents from all flasks were subjected to liquid-liquid extraction and determined the residual concentrations of pyrene and percent degradation.

2.9.3 Effect of temperature on pyrene degradation by *Bacillus* sp. C7

The active culture of *Bacillus* sp. C7 were inoculated in BHB medium containing 1000 mg/L pyrene and adjusted to pH 7.0 and the flasks were incubated at different temperatures ranging from 20^{o}C, 30^{o}C, 40^{o}C, 50^{o}C and 60^{o}C on shaking incubator at 120 rpm for 20 days. After the 20th day of incubation, growth, the residual concentration of pyrene and pyrene degradation were recorded as stated previously.

2.9.4 Effect of incubation period on pyrene degradation by *Bacillus* sp. C7

To study the influence of incubation period, the active culture of *Bacillus* sp. C7 was inoculated in BHB medium. The broth was sequentially tested for the growth and % degradation of pyrene at the interval of 5 days (5, 10, 15, 20 and 25 day) up to 25 days. The percentage degradation was measured and the optimum period was used for further experimentation.

2.9.5 Effect of surfactants on pyrene degradation by *Bacillus* sp. C7

The five surfactants (Tween 20, Tween 80, Triton X-100, Brij 35 and SDS (sodium dodecyl sulphate)) were used at 0.05% level in the biodegradation studies. Flasks containing 50 mL BHB medium (pH 7.0) having a pyrene 1000 mg/L were amended with different surfactants and active culture of *Bacillus* sp. C7 was inoculated in respective broths. The flasks were incubated for 20 days at 30^{o}C and after incubation, the surfactant supporting higher degradation of pyrene was identified. The studied parameters includes growth at 600 nm, residual concentration of pyrene and % degradation of pyrene.

2.9.6 Effect of carbon sources on pyrene degradation by *Bacillus* sp. C7

The five different carbon sources (glucose, sucrose, lactose, galactose and ribose) as co-metabolite at the 250 mg/L concentration were added separately to the flasks containing 50 mL BHB medium (pH 7.0) containing 1000 mg/L pyrene and biodegradation study was carried out as mentioned earlier.

2.9.7 Effect of nitrogen sources on pyrene degradation by *Bacillus* sp. C7

The five different nitrogen sources (meat extract, beef extract, yeast extract, peptone and ammonium sulphate) at the 250 mg/L concentration were added separately to the flasks containing 50 mL of BHB medium (pH 7.0) containing 1000 mg/L pyrene and used for biodegradation studies.

2.10 Statistical Analysis

All the experiments were performed in triplicate and values are mentioned as average.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Bacterial Isolates

After isolation by the enrichment culture technique the 10 bacterial isolates were screened for evaluation of pyrene degradation

efficiency in the presence of 2,6-DCPIP (Fig. 1). The fast dye reducing and efficient in degradation of pyrene bacterial isolate C7 was identified as *Bacillus* sp. C7 on the basis of tests given in *Bergey's Manual of Systematic Bacteriology* (9th edition) (Table 1). The *Bacillus* sp. C7 was examined based on its morphological, physiological and biochemical characteristics as the genus *Bacillus*. In the present study the selected isolate showed reduction of DCPIP dye within 24 hrs of incubation. Similarly Karale et al. [16] reported three anthracene degrading bacterial isolates *Aeromonas hydrophila, Bacillus polymyxa* and *Streptococcus mutans* completely reduces DCPIP dye within 48 hrs of incubation. In similar studies, Bidoia et al. [19] reported that *B. subtilis* can completely reduce DCPIP in 138 hrs, 125 hrs, 75 hrs and 87 hrs for synthetic, semi-synthetic, mineral and used oil respectively.

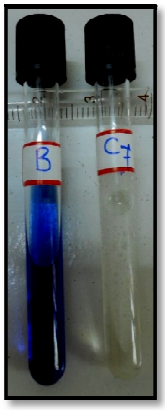

Fig. 1. DCPIP (2,6-Dichlorophenol indophenol) assay of pyrene utilizing bacterial isolate C7

B= blank, C7= bacterial isolate C7

3.2 Biodegradation Studies

The growth (O.D. at 600 nm) of *Bacillus* sp. C7 in pyrene containing medium (1000 mg/ L) was increased in the BHB medium from 0.594 to 0.999 from 5 days to 25 days of incubation respectively. However, the increase in growth after 20th day was not constantly increasing (Fig. 2.). Similarly, degradation of pyrene (%) during the study was increased sharply from 5 days (23.43%) to 20 days (81.53%), and the degradation in control flask was 3.12% (Fig. 3). The plot of growth and % degradation against incubation time demonstrated that *Bacillus* sp. C7 posse's ability to degrade pyrene in a linear fashion with time. The *Mycobacterium* sp. strains PYR-1 [20] and RJGII 135 [21] mineralized approximately 50% of added pyrene, while *Rhodococcus* sp. strain UW1 [22] mineralize 72% of added pyrene after 14 days of incubation *Achromobacter xylooxidans* mineralize 80% of pyrene [23] while isolated *Bacillus* sp. C7 degraded pyrene up to 81.96%.

3.3 Optimization of Pyrene Degradation

The growth of *Bacillus* sp. C7 and degradation of pyrene increased continually as the concentration of pyrene increases from 400 mg/L to 1000 mg/L. Above 1000 mg/L pyrene the growth and degradation was decreased which might be due to toxic effect of high concentrations on bacteria. The maximum growth (0.998) and degradation of pyrene (80.53%) were obtained at 1000 mg/L after 20 days incubation period. The 1000 mg/L pyrene concentration was appeared as the optimal concentration of pyrene for degradation studies (Fig. 4).

Table 1. Biochemical characteristics of C7

Sr. no.	Biochemical tests	Result
1	Indole production	-
2	Methyl red	+
3	VP	+
4	Citrate utilization	+
5	Catalase	+
6	Oxidase	-
7	Nitrate reduction	+
8	Growth in NaCl (2%)	-
9	Motility	Motile
10	Grams nature	+
11	Sugar fermentation.	
	1) Glucose	+
	2) Xylose	-
	3) Mannitol	-
	4) Arabinose	-
12	Hydrolysis of	
	1) Casein	+
	2) Gelatin	+
	3) Starch	+
	4) Lipid	+

The effect of initial pH of medium on pyrene degradation by *Bacillus* sp. C7 is shown in (Fig. 5) it was observed that the neutral pH of the medium favors the growth and pyrene degradation of *Bacillus* sp. C7. The growth (1.059) and % degradation (80.91) was highest at pH 7.0. Therefore, given that the highest values of both parameters (Growth and %

degradation) were observed at pH 7.0, this value will be considered as the most efficient in the pyrene biodegradation process. The pH favored for bacterial growth is neutral, whereas, for fungi growth is 3-4 [24].

At 30°C, the growth of *Bacillus* sp. C7 (1.003) and pyrene degradation (81.30%) was highest as shown in (Fig. 6). High temperature not only affects growth of organism and degradation rate, but also lost the enzyme activity needed during degradation. That might be the reason for observed less growth and pyrene degradation beyond 30°C. Similar results were observed with degradation of anthracene and phenanthrene by using white rot fungi *Phanerochaete chrysosporium* [25] Biodegradation efficiency decreased as temperature increased or decreased. Solubility of PAHs increased with an increase in temperature [26], which increased bioavailability of PAH molecules. Moderate fluctuation of temperature beyond the optimal value will cause a reduction in microbial respiration. The moderate fluctuation in temperature also affects microbial growth rate but not degradation rates because degrading population is able to degrade PAH efficiently in temperature range 20 and 30°C [27].

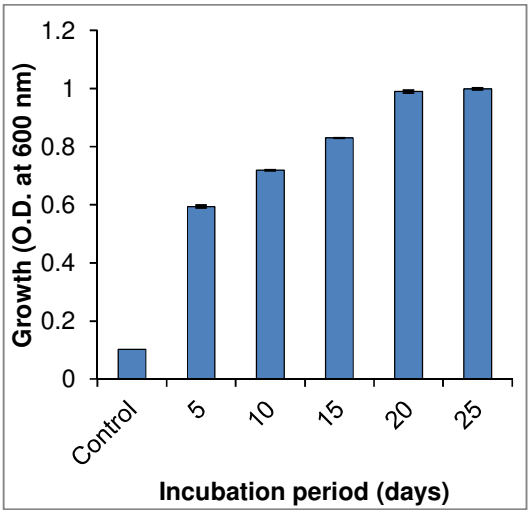

Fig. 2. Growth pattern of *Bacillus* sp. C7 in pyrene containing medium. Values are mean ± S.D. n=3

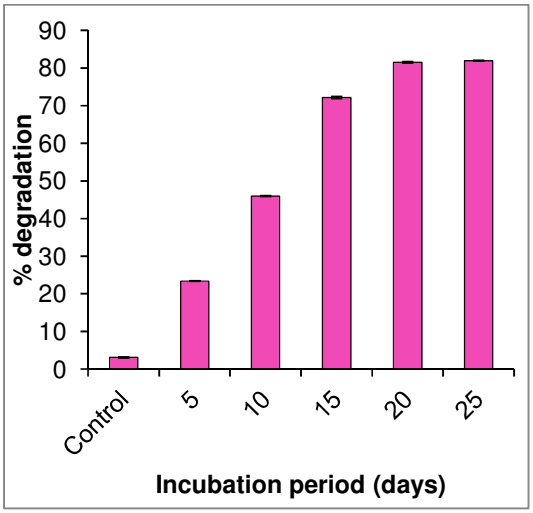

Fig. 3. Percentage degradation of pyrene by *Bacillus* sp. C7. Values are mean ± S.D. n=3

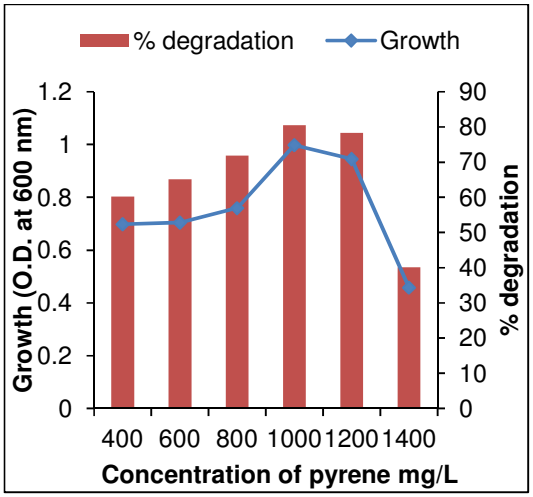

Fig. 4. Effect of concentration of pyrene on pyrene degradation by *Bacillus* sp. C7

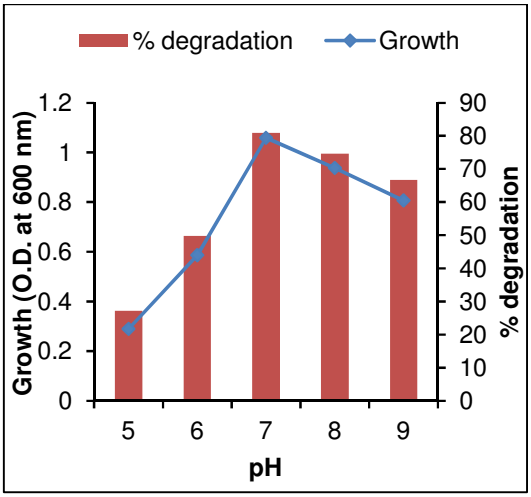

Fig. 5. Effect of pH on pyrene degradation by *Bacillus* sp. C7

The growth and pyrene degradation rate of *Bacillus* sp. C7 was increased with increase in incubation period upto 20 days. The growth and pyrene catabolism rate was more in log phase of growth (5-20 days). The growth and degradation of pyrene was constant beyond which the parameters remain unaffected (Fig. 7).

Fig. 8. shows that the addition of surfactants in degradation medium significantly affects on the growth and degradation of pyrene. The highest growth (1.629) and degradation (85.26%) was observed by the addition of Tween-80 which significantly improves the availability of carbon source for the growth of *Bacillus* sp. C7. The other surfactants also improve both the factor, but the rate was lower. The lowest growth (0.905) and degradation (77.22%) was observed in the presence of Brij-35. According to these results, Tween-80 was considered as best to improve the pyrene biodegradation. The similar result was observed by [28] during the optimization of surfactants. The use of surfactants to the culture media at a concentration above the CMC is essential to increase PAH degradation rate [29]. However, [30] reported a negative effect when the surfactants were added above the CMC reduces the bioavailability of PAHs [31]. In addition, it is important to consider the possible use of surfactant as a carbon source, preferentially to PAHs, by the strains which would reduce biodegradation rates [32].

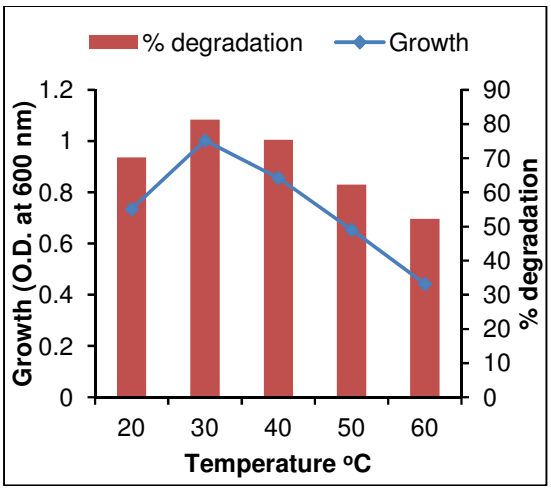

Fig. 6. Effect of temperature on pyrene degradation by *Bacillus* sp. C7

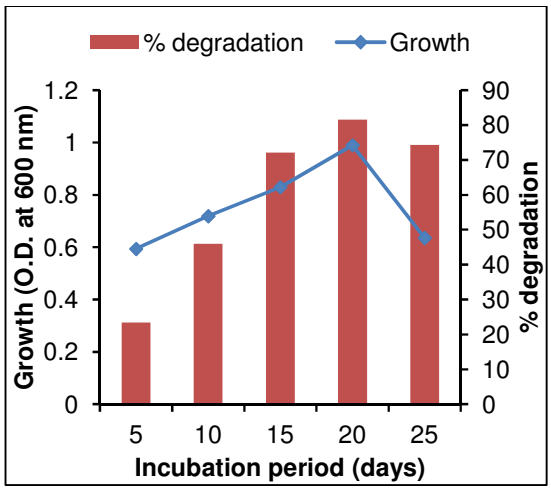

Fig. 7. Effect of incubation period on pyrene degradation by *Bacillus* sp. C7

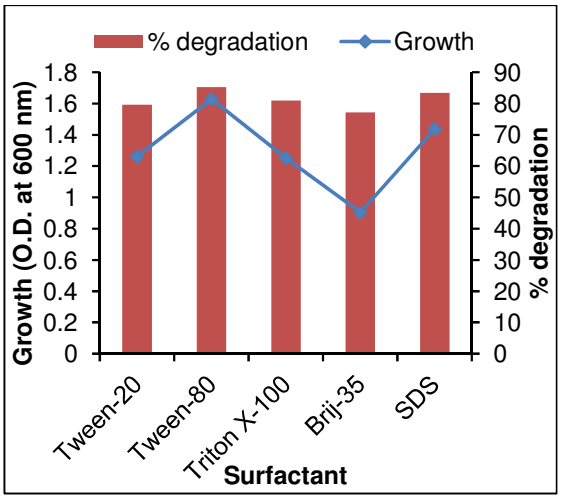

Fig. 8. Effect of surfactants on pyrene degradation by *Bacillus* sp. C7

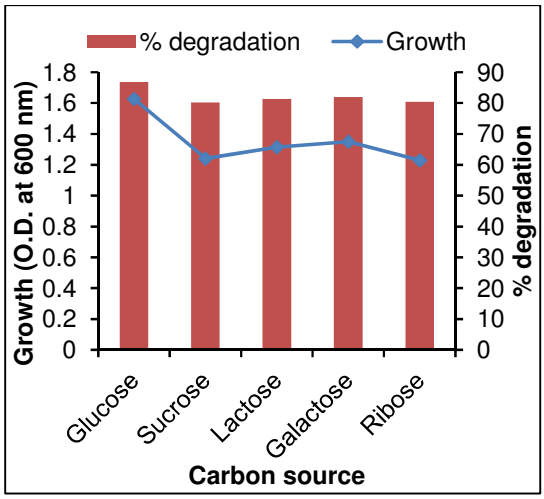

Fig. 9. Effect of carbon sources on pyrene degradation by *Bacillus* sp. C7

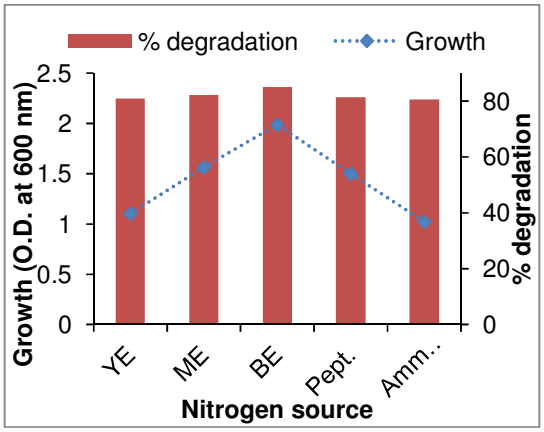

Fig. 10. Effect of nitrogen sources on pyrene degradation by *Bacillus* sp. C7

YE= Yeast extract, ME= Meat extract, BE= Beef extract, Pept. = Peptone, Ammo. Sulp. = Ammonium sulphate, SDS= sodium dodecyl sulphate

The effect of the addition of a carbon source as co-metabolite on the growth and pyrene degradation of *Bacillus* sp. C7 is shown in (Fig. 9.). The highest growth (1.628) and degradation (86.87%) was observed in the presence of glucose. The growth was decreased in the presence of sucrose (1.241) and ribose (1.229). The addition of glucose as the additional carbon source significantly improved the growth and pyrene degradation rate of *Bacillus* sp. C7. Pyruvate is carbon source that favors the growth of certain degrading strains such as *Pseudomonas putida* [33], whereas salicylate induces the synthesis and cultivation of degradative enzymes [34]. The change in the type of carbon source supplied to PAH degrading microorganisms an adaptation period for the enzymatic system was required, reducing the mineralization rate of pollutants [35,14,36].

The analysis by the addition of a nitrogen source (Fig. 10), revealed that the different nitrogen sources added had significant effects on growth and degradation of pyrene. The addition of beef extract significantly improved the growth and degradation of pyrene. The beef extract was observed best form to supply the nitrogen source for both degradation and the growth of the *Bacillus* sp. C7.

4. CONCLUSION

This work described pyrene degradation by using *Bacillus* sp. C7, which was isolated from coal deposited site soil sample. This bacterium reduces 2,6-DCPIP more quickly as compared with other bacterial isolates. The bacterial isolates identified on the basis of its morphological, physiological and biochemical characteristics. The optimization study for degradation of pyrene with concentration of pyrene, pH, temperature, incubation period, surfactants, co-metabolite as carbon and nitrogen source revealed that 1000 mg/L concentration of pyrene, 7.0 pH, 30^{o}C temperature, 20 days of incubation period, tween-80 as a surfactant, glucose as co-metabolite and nitrogen source beef extract were optimum. Therefore, the *Bacillus* sp. C7 isolated in this study can be applied at pyrene polluted sites in presence of optimum parameters for maximum bioremediation of pyrene from polluted sites.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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