INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
ORIGINAL RESEARCH ARTICLE
OPEN ACCESS

Combined effect of *Bacillus cereus* CPOU13 and *B. subtilis* SPC14 on polycyclic aromatic hydrocarbons degradation *in vitro*

Poornachander Rao M.[1]*, Anitha Y.[2], Satyaprasad K.[3]

[1]Department of Botany, Government Degree College, Mahabubabad, Warangal (Dist.), Telangana State.
[2]CRIDA (ICAR, New Delhi), Hyderabad, Telangana State.
[3]Professor in Botany (Rtd.), Department of Botany, Osmania University, Hyderabad

Received for publication: March 10, 2016; **Accepted**: March 15, 2016

Abstract: Biodegradation of polycyclic aromatic hydrocarbons (PAHs) with suitable bacterial strains conveys much interest in recent years. We studied biodegradation of PAHs namely phenanthrene, anthracene and pyrene using two efficient PAHs degrading strains, *B. cereus* CPOU13 and *B. subtilis* SPC14 *in vitro* experiments. Standard HPLC chromatograms for phenanthrene, anthracene and pyrene were plotted separately based on HPLC peak area values and Retension time of known concentrations of the test PAHs and using software, 'Origin 6.0'. Biodegradation of PAHs mixture containing phenanthrene, anthracene and pyrene was studied *in* for 13 days. We found that the combination of bacterial strains, *B. cereus* CPOU13 and *B. subtilis* SPC14 degraded high amounts of phenanthrene, anthracene and pyrene in 13 days of incubation. The recorded degradation percentages of phenanthrene, anthracene and pyrene were 85.31, 92.82 and 85.70 respectively. Concentration of phenanthrene was degraded from 217µg/5ml to 31.9µg/5ml. Concentration of anthracene was degraded from 211µg/5ml to 16µg/5ml. Concentration of pyrene was degraded from 233µg/5ml to 33µg/5ml in 13 days of incubation. We also observed biodegradation of phenanthrene, anthracene and pyrene from 1st day to 13th day.

Key words: Biodegradation; PAHs; B. cereus CPOU13; B. subtilis SPC14; phenanthrene; anthracene; pyrene

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that formed by the incomplete combustion of organic materials, such as wood or fossil fuels. PAHs consist three or more benzene rings, at least two are fused with two neighboring rings and shares two adjacent carbon atoms (1). There are thousands of PAHs in the environment but in practice PAHs analysis is restricted to determination of 6 to 16 compounds. Individually all PAHs differ substantially in their physical and chemical properties (2). The ubiquitous occurrence of PAHs is largely due to their formation and release in all processes of incomplete combustion of organic materials and these organic contaminants are resistant to degradation, can remain in the environment for long periods (3, 4).

Presence of PAHs in the environment at high levels exerts negative impact on all life forms due to their carcinogenic, mutagenic, teratogenic and phytotoxic properties and this enforced the scientific world to concentrate on the immediate actions for remediation of PAHs (5-7). Biodegradation with suitable bacterial strains conveys much interest in recent years due to its low cost, effectiveness, restoration of medium where it operated and possesses several advantages. Few bacterial strains act as key participants in PAHs biodegradation as they possessing special enzymes. Strains suitable for PAHs biodegradation studies are frequently isolate from their native contaminated sites and this may be due to their long term exposure and tolerance towards PAHs (8). However, consortia or multi-bacterial strains of PAHs degrading strains are more effective in biodegradation as compared to single strains (9, 10). In the present investigation, we used two effective PAHs degrading bacteria strains namely *B. cereus* CPOU13 (11) and *B. subtilis* SPC14 (12) those isolated from two different PAHs contaminated sites to study degradation of PAHs phenanthrene, anthracene and pyrene *in vitro* experiments.

***Corresponding Author:**
Dr. M. Poorna Chander Rao
Asst. Prof. in Botany,
Govt. Degree College,
Mahabubabad, Warangal (Dist.),
Telangana State.

Materials and Methods

Construction of standard chromatogram for HPLC studies:

Method for the construction of standard chromatograms for PAHs using high performance liquid chromatography (HPLC) was adopted from Boonchan (13). Phenanthrene, anthracene and pyrene with 99% purity purchased from Sigma-Aldrich, USA and used throughout the experimental work as test PAHs. Accurately weighed 100 mg of phenanthrene, anthracene and pyrene transferred to a 100 ml volumetric flask and 2-3 ml of acetonitrile was added to dissolve. Solution was diluted with acetonitrile to obtain 1000 mg/L stock solution. The stock solution further diluted to obtain the concentrations of 25, 50, 100, 200 and 250 ppm and the samples run in HPLC. Standard chromatograms for phenanthrene, anthracene and pyrene were plotted separately using software, 'Origin 6.0' by retrieving the peak area values at respective retention times of each PAH.

HPLC analysis:

High performance liquid chromatography (HPLC) studies were conducted with 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 PAHs mixture containing phenanthrene, anthracene and pyrene was studied by the method of Moody (14). 250 ml of Minimal salt medium (MSM) was prepared in 1000 ml conical flasks and enriched with phenanthrene, anthracene and pyrene (each 50 ppm) and made 150 ppm. One ml of exponential growth phase culture (approximately 10^7 colony forming units) of strains (*B. cereus* CPOU13 and *B. subtilis* SPC14) were added separately to MSM flasks and in control flasks no culture was added. The cultures were incubated under standard growth conditions (at 24°C on a rotary shaker at 150 rpm speed) 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 34°C under vaccum conditions. Finally, the samples were dissolved in 3 ml of acetonitrile (ACN) and preserved in a refrigerator at 4°C for HPLC studies as described earlier.

Results

Preparation of standard chromatograms for PAHs:

Standard chromatograms for phenanthrene, anthracene and pyrene were plotted by running HPLC with the known concentrations 25, 50, 100, 200 and 250 ppm separately. Each PAH formed a peak at constant retention time and peak areas were increased with the increase in concentration. Based on the values of peak areas against known concentrations (25, 50, 100, 200 and 250 ppm) standard chromatograms for each PAH were plotted separately using the software 'Origin 6.0'. The peak area values against known concentrations of PAHs are presented in Table 1.

Table 1: HPLC peak area values of phenanthrene, anthracene and pyrene at known concentrations

Sl. No.	Concentration (ppm)	Peak Area Values		
		Phenanthrene	Anthracene	Pyrene
1	25	25.880	20.688	10.233
2	50	221.241	80.641	40.498
3	100	625.788	260.992	120.054
4	200	855.055	640.167	280.330
5	250	1017.880	918.880	420.777

Phenanthrene peak was appeared at 13 min retention time on HPLC chromatograms. Anthracene peak was recorded at retention time of 13.5 min. Pyrene was formed a peak at the retention time of 16.5 min. The standard chromatograms were constructed separately for each PAH to study PAHs concentrations in the standard solution based on their retention time and peak area (Fig 1, 2, 3). A linear standard graph obtained for each PAH and their correlation coefficient values are 0.9935 for phenanthrene, 0.9916 for anthracene and 0.9865 for pyrene. The value of the correlation coefficient (R^2) obtained for each calibration chromatogram shows that the correlation between relative peak area and concentration was linear and reproducible within selected concentration range. The 'y'- value representing linear correlation equation for phenanthrene, anthracene and pyrene were 3.921x, 3.9528x and 1.7749x respectively. Thus, the data obtained from standard chromatogram was reliable and accurate (Fig 1, 2, 3).

Figure 1: Standard graph for the HPLC chromatograms of phenanthrene

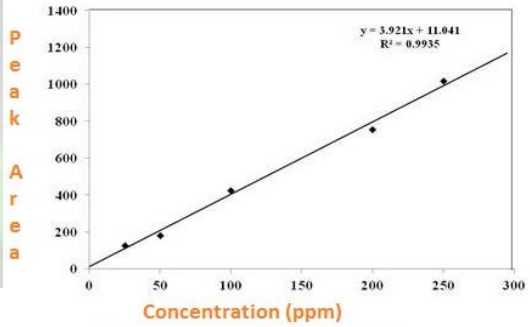


Figure 2: Standard graph for the HPLC chromatograms of anthracene

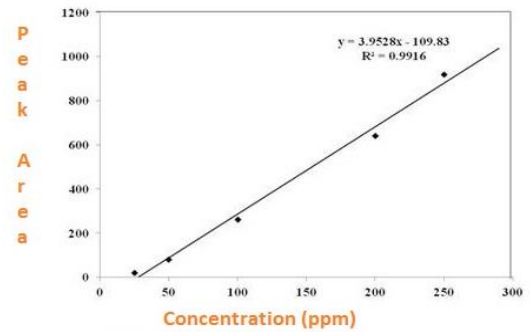
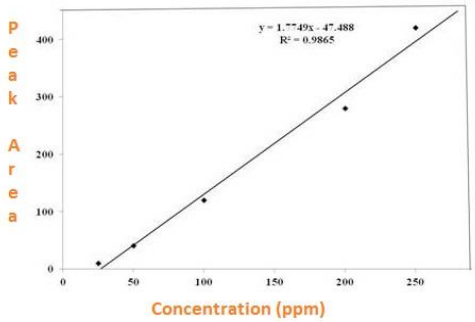


Fig. 3: Standard graph for the HPLC chromatograms of pyrene



Degradation of PAHs by *B. cereus* CPOU13 and *B. subtilis* SPC14 *in vitro*:

Combined biodegradation capability of *B. cereus* CPOU13 and *B. subtilis* SPC14 was assayed *in vitro* conditions on MSM enriched with a mixture of phenanthrene, anthracene and pyrene each at 50 ppm concentration. This was conducted by high performance liquid chromatography (HPLC) with C 18 column. Degradation of PAHs recorded at regular time intervals of 0th, 1st, 2nd, 3rd, 7th, 8th and 13th days of incubation. The observed retention peak areas of phenanthrene, anthracene and pyrene were analyzed by software 'LC-solutions'. Based on retention times and respective peak area values, concentrations of PAHs in the samples were determined with the help of standard chromatograms plotted for known concentrations of PAHs. Further, based on the concentration of each PAH remained in respective samples degradation percentages were calculated.

The combination of bacterial strains, *B. cereus* CPOU13 and *B. subtilis* SPC14 resulted in high degradation performance for phenanthrene, anthracene and pyrene in 13 days of incubation (Fig. 4, 5). Degradation percentages and concentration depletions of phenanthrene, anthracene and pyrene by the strains showed a gradual increase from the 1st day to 13th day. The results are presented in Table 2.

Degradation of phenanthrene was recorded up to 85.31% in 13 days as its initial concentration degraded from 217µg/5ml to 31.9µg/5ml. Degradation of anthracene was recorded up to 92.25% as its concentration declined from 211µg/5ml to 16µg/5ml in 13 days. Degradation of pyrene was reached to 85.70% as its initial concentration depleted from 233.14µg/5ml to 33µg/5ml in 13 days of incubation.

Figure 4: Degradation of phenanthrene, anthracene and pyrene by *B. cereus* CPOU13 and *B. subtilis* SPC14 in 13 days (Error bars represent standard deviation of three replacates)

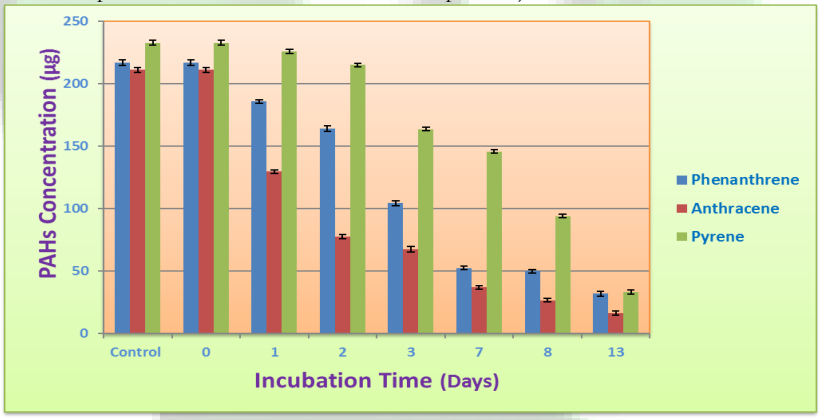
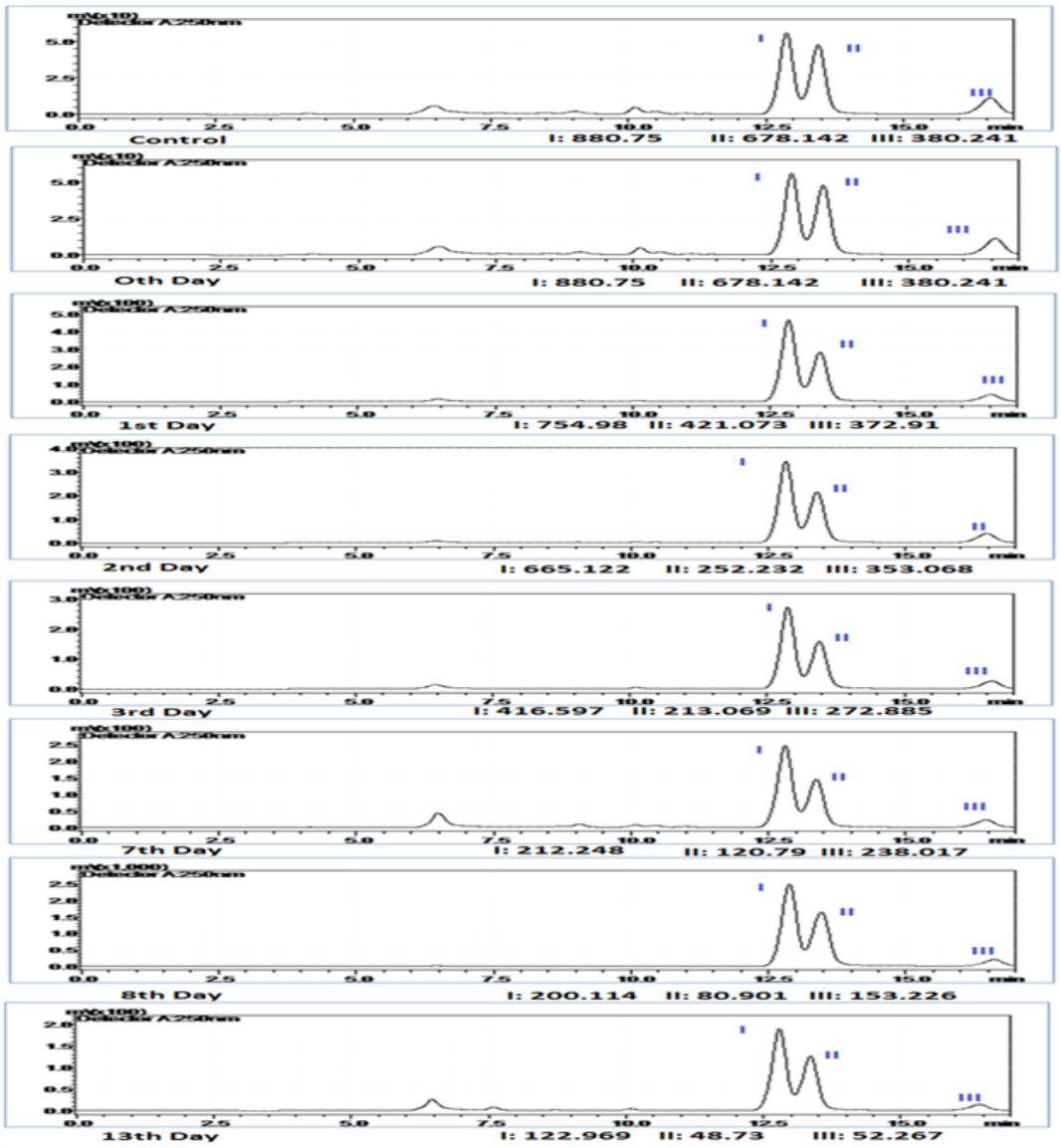


Table 2: Degradation of phenanthrene, anthracene and pyrene by combined treatment of *B. cereus* CPOU13 and *B. subtilis* SPC14

Sl. No.	Day	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 Day	880.750	217±2.07	0	678.142	211±2.03	0	380.241	233±2.08	0
3	1st Day	754.98	186±1.54	14.39	421.073	130±1.54	38.63	372.91	226±1.63	3.00
4	2nd Day	665.122	164±2.08	24.41	252.232	77±1.56	63.29	353.068	215±1.39	7.65
5	3rd Day	416.597	104±2.00	51.92	213.069	67±2.07	68.07	272.885	164±1.58	29.64
6	7th Day	212.248	52±1.54	75.82	120.79	37±1.61	82.51	238.017	146±1.64	37.42
7	8th Day	200.114	45±1.51	77.04	80.901	27±1.48	87.39	153.226	94±1.39	59.60
8	13th Day	122.969	31.9±2.09	85.31	48.73	16±1.62	92.25	52.267	33±1.59	85.70

(RT= Retention; ± represents standard deviation of three replicates)

Figure 5: HPLC chromatograms showing the degradation of PAHs *in vitro* by *B. cereus* CPOU13 and *B. subtilis* SPC14



(I= Phenanthrene; II= Anthracene; III= Pyrene)

Discussion

Bacterial strains generally evolve to utilize more carbon molecules than one type of carbon molecules for their growth and metabolism. This flexibility for nutritional resources helps the bacteria to strive many nutritional constraint conditions. As several researchers suggested many bacterial strains utilize more than one type of PAHs as nutritional source in addition to commonly utilized carbohydrates. Bacterial strains acquire these qualities by secreting few specific enzymes that can catalyze many PAHs (15). These abilities of bacteria result in degradation or mineralization of PAHs on media components. However utilization of PAHs as nutritional sources *in vitro* experiments believed to be limited because of their hydrophobicity, crystalline and other related properties which decrease their availability to degrading bacterial strains (16, 17).

Different analytical methods are adoptable to determine PAHs quantities in liquid samples during biodegradation. Reverse phase high performance liquid chromatography (RP-HPLC) is one of the prime methods used because it grants several advantages including sensitive and selective detectors, easy sample preparation, and less risk of solute degradation.

In our study phenanthrene, anthracene and pyrene formed different peaks in HPLC chromatograms at respective retention times (RT) which based on their respective number of rings and molecular weights. Sequential appearance of test PAHs on

chromatogram was phenanthrene, anthracene and pyrene. These results are in agreement with the results of Haritash and Kaushik (18). Anthracene formed strong signals and high peaks in respective HPLC chromatograms due to their linear structure and high UV detection (19). The retention times observed for phenanthrene, anthracene and pyrene in HPLC chromatograms are 13, 13.5 and 16.5 minutes respectively. We also observed that peak areas of test PAHs increased with their concentration. Plotted standard chromatograms for each PAH had above the 0.9 correlation coefficient value. Hence, these results are reliable and amenable for determination of unknown concentrations of phenanthrene, anthracene and pyrene.

Strains suitable for biodegradation studies are generally isolate from their native contaminated sites. In more extent, consortia of PAHs degrading bacteria are more effective in degradation as compared to selected single strains (20). Yu (21) investigated biodegradation of mixture PAHs consisting of fluorene, phenanthrene and pyrene using bacterial consortium made up of three strains of *Rhodococcus* sp., *Acinetobacter* sp. and *Pseudomonas* sp. and reported that addition of the bacterial consortium significantly enhanced the efficiency of fluorene and phenanthrene biodegradation. In the present study we used two potent PAHs degrading bacterial strains that isolated from two PAHs contaminated sites.

Nasseri (22) reported that consortium of two bacterial isolates of *Pseudomonas* species showed maximum biodegradation of phenanthrene than the individual treatments. Leblond (23) also reported consortium of four different bacterial strains was more effective than single strain in PAHs biodegradation. Churchill (24) suggested that use of single bacteria for biodegradation took much more time than the time taken by a mixture of culture. This effect may be due to the ability of certain strains in removal of intermediates produced by other members of consortium that facilitates additional PAHs removal **(**25**).** In the present investigation, degradation of PAHs mixture (phenanthrene, anthracene and pyrene) by *B. cereus* CPOU13 and *B. subtilis* SPC14 was studied *in vitro* for 13 days. The combined treatment recorded high degradation rates of phenanthrene, anthracene and pyrene. Degradation percentages of phenanthrene, anthracene and pyrene in 13 days of incubation were 85.31, 92.82 and 85.70 respectively. These results indicating that combination of these strains is compatible and results in high degradation percentages.

Guo (26) suggested that certain defined bacterial consortia are very effective in biodegradation than the other consortia partners and isolated strains. They studied degradation of multi-PAHs by bacterial consortium and reported that among three bacterial strains, SPNT (*Paracoccus versutus*) degraded phenanthrene, fluoranthene and pyrene completely within 10 days. Strains, HCSS (*Rhodococcus opacus*) and MWFG (*Sphingomona* sp.) recorded lower degradation percentages for all three PAHs, especially pyrene, which had only 20% degradation. Similarly, in the present study combination of two PAHs degrading strains recorded high degradation of phenanthrene and anthracene in 13 days of incubation. Pyrene, a high molecular weight PAH also degraded to high percentage. The present results indicating that efficacy of PAHs degradation is high and can be improved by adding more bacterial strains. More efforts have to pay for the construction of highly PAHs degrading consortium for 100 percent PAHs degradation in short periods.

References

1. Wilson SC and Jones KC. "Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review." Environmental Pollution. 81.3 (1993): 229-249. Print.

2. Masclet P and Mouvier G. "La chimie atmosphrique des hydrocarbures aromatiques polycycliques." Pollution Atmospherique, (1988): 25. Print.

3. Sims RC and Overcash MR. "Fate of polynuclear aromatic compounds (PNAs) in soil-plant systems." Residue Reviews 88 (1983): 1-68. Print.

4. Kordybach BM. "Sources, Concentrations, Fate and Effects of Polycyclic Aromatic Hydrocarbons (PAHs) in the Environment. Part A: PAHs in Air- Review". Polish Journal of Environmental Studies. 8. 3 (1999): 131-136. Print.

5. Chaudhry Q, Blom-Zandstra M, Gupta S and Joner EJ. "Utilizing the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment." Environmental Science and Polluton Research. 12 (2005): 34-48. Print.

6. Vaajasaari K and Joutti A. 'Field-scale assessment of phytotreatment of soil contaminated with weathered hydrocarbons and heavy metals." Journal of Soils and Sediments. 6. 3 (2006): 128-136. Online.

7. Euliss K, Ho CH, Schwab AP, Rock S and Banks MK. "Greenhouse and field assessment of phytoremediation for petroleum contaminants in a riparian zone." Bioresource. Technology. 99 (2008): 1961-1971. Print.

8. Cordova-Rosa SM, Dams RI, Corova-Rosa EV, Radetski MR, Correa AXR and Radetski CM. "Remediation of phenol contaminated soil by a bacterial consortium and Acinetobacter calcoaceticus isolated from an industrial waste water treatment plant." J. Hazard Mater 164 (2009): 61-6. Online.

9. Ghazali FM, Rahman RNZA, Salleh AB and Basri M. "Biodegradation of hydrocarbons in soil by microbial consortium." International Biodeterioration and Biodegradation. 54 (2004): 61-7. Print.

10. Goux S, Shapir NE, Fantroussi S, Lelong S, Agathos SN and Pussemier L. "Longterm maintenance of rapid atrazine degradation in soils inoculated with atrazine degraders." Water Air and Soil Pollution: Focus 3 (2003): 131-42. Print.

11. Rao MP, Rajithasri AB, Sagar K, Satyaprasad K. "Isolation of polycyclic aromatic hydrocarbons degrading PGPR from a oil products contaminated site in Hyderabad, Telangana State, India." International Journal of Pharma and Biosciences. 6. 4 (2015): 305-317. Print.

12. Rao MP, Rajithasri AB, Ashokvardhan T, Satyaprasad K. "Effective polycyclic aromatic hydrocarbons degrading rhizobacteria with PGP traits from plants growing on fly ash in the premises of thermal power station-Kothagudem, Telangana State, India." International Journal of Science and Research. (2013): 2319-7064. Paper ID: SUB154563. Online.

13. Boonchan S, Britz ML and Stanely GA. "Biodegradation of and mineralization of high molecular weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures." Appl. and Environ. Microbiol. 66. 3 (2000): 1007-1019. Online.

14. Moody JD, Freeman JP, Doerge DR and Cerniglia CE. "Degradation of phenanthrene and anthracene by cell suspensions of Mycobacterium sp. Strain PYR-1." Applied and Environmental Microbiology 67.4 (2001): 1476-1483. Print.

15. Egli T. "Microbial degradation of pollutants at low concentrations and in the presence of alternative carbon substrates: emerging patterns'. In: Agathos, S.N., Reineke, W. (Eds.), Focus on Biotechnology. 'Biotechnology for the Environment: Strategy and Fundamentals', Kluwer Academic Publishers, Dordrecht, 3. A (2002): 131-139. Print.

16. Ogram AV, Jessup RE, Ou L. and Rao PSC. "Effects of sorption on biological degradation rates of (2, 4-Dichlorophenoxy) acetic acid in soils." Applied and Environmental Microbiology. 49 (1985): 582-587. Print.

17. Harms H and Bosma TNP. "Mass transfer limitation of microbial growth and pollutant degradation." Journal of Industrial Microbiology and Biotechonlogy. 18 (1997): 97-105. Print.

18. Haritash AK and Kaushik CP. "Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review." J. Hazard. Mater. 169. 1-3 (2009): 1-15. Online.

19. Irwin RJ, Vanmouwerik M, Stevens M, Seese L and Basham ND. "Environmental contaminants encyclopedia." National Park Service, Water Resources Division, Fort Collins, Colorada. (1997). Print.

20. Jacques RJS, Okeke BC, Bento FM, Teixeira AS, Peralba MCR and Comargo FAO. "Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil." Bioresource Technology. 99.7 (2008): 2637-43. Print.

21. Yu SH, Ke L, Wong YS and Tam NFY. "Degradation of polycyclic aromatic (PAHs) by a bacterial consortium enriched from mangrove sediments." Environment International 31. 2 (2005): 149-54. Print.

22. Nasseri S, Kalantary R, Nourieh N, Naddafi K, Mahvi AH and Baradaran N. "Influence of bioaugmentation in biodegradation of PAHs contaminated soil in bio-slurry phase reactor Iran." Iranian Journal of Environmental Health, Science and Engineering. 7. 3 (2010): 199-208. Online.

23. Leblond J, Schultz WT and Sayler G. "Observations on the preferential biodegradation of selected components of polyaromatic hydrocarbon mixtures." Chemosphere. 42. 4 (2001): 333-343. Print.

24. Churchill S, Harper J and Churchill P. "Isolation and characterization of a Mycobacterium species capable of degrading three and four-ring aromatic and aliphatic hydrocarbons." Applied and Environmental Microbiology. 65. 2 (1999): 549-552. Print.

25. Heinaru E, Merimaa M, Viggor S, Lehiste M, Leito I and Truu J. "Biodegradation efficiency

of functionally important populations selected for bioaugmentation in phenol and oil-polluted area." FEMS Microbiology. Ecol. 51.3 (2005): 363-73. Print.

26. Guo CL, Zhou HW, Wong YS and Tam NFY. "Isolation of PAH-degrading bacteria from mangrove sediments and their biodegradation potential." Marine Pollution Bulletin. 51. 8-2 (2005): 1054-1061. Online.

Cite this article as:
Poornachander Rao M, Anitha Y, Satyaprasad K. Combined Effect of Bacillus cereus CPOU13 and B. subtilis SPC14 on Polycyclic Aromatic Hydrocarbons Degradation in vitro. *International Journal of Bioassays* 5.4 (2016): 4505-4511.

Source of support: Nil
Conflict of interest: None Declared