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RESEARCH ARTICLE

RHIZODEGRADATION OF PHENANTHRENE, ANTHRACENE AND PYRENE BY AUGMENTING BACILLUS CEREUS AND BACILLUS SUBTILIS STRAINS

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Abstract

Rhizodegradation is one of the best methods for the effective removal of dangerous polycyclic aromatic hydrocarbons pollutants from soil. This is operative due to the high persistent, non-bioavailability nature of PAHs and combined, sequential reactions of bacteria present in rhizosphere of plants. We have conducted pot-culture method to study the degradation of three PAHs compounds namely phenanthrene, anthracene and pyrene in artificially contaminated soils of rhizosphere and non-rhizosphere soil treatments of blackgram(Vignamungo L.) that augmented by two potential PAHs degraders namely Bacillus cereus CPOU13 and Bacillus subtilis SPC14 isolated from naturally contaminated soils for 90days. HPLC studies revealed that degradation percentages of the three PAHs in treatments were more where selected strains augmented to the soil treatments over the non-augmented soils. The rhizosphere treatments that have augmented strains recorded more degradation percentages of phenanthrene, anthracene and pyrene over the rhizosphere treatments that were non-augmented. Pyrene, a high molecular weight PAHs degraded maximum to 96.24% in rhizosphere soil treatment that is augmented with the strains while moderate degradation of pyrene recorded in non-autoclaved soil treatments that contain natural microbial communities. The study of counting of bacterial populations during the experimental period revealed that the populations of the selected and other natural bacteria were gradually increased from the first day, reached maximum by 60days and became almost consistent in 90days in all the treatments. It was also observed that the populations of bacteria were high in rhizosphere treatments compared to the non-rhizosphere soil treatments. With these results it has been predicted that degradation of PAHs in rhizosphere soil treatments is closely associated with the increasing PAHs degrading bacterial populations of selected bacterial strains that may consume more quantity of PAHs for their metabolic activities in rhizosphere soils. Key words: Rhizodegradation, PAHs, HPLC, pot culture.

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Introduction:-

Rhizodegradation is one of the best methods for the effective removal of organic pollutants like Polycyclic Aromatic Hydrocarbons(PAHs) present in soil bodies. It is a product of complex interactions between plants and bacteria that took place in the rhizosphere(Dominguez et al., 2020). This highly relies on balanced association of plant and microbial communities present in the rhizosphere and its surrounding non-rhizosphere soils. Numerous bacterial strains of rhizosphere were reported to degrade a variety of PAHs and most of them are isolated from contaminated soil and few are isolated from non-contaminated soils. Understanding complex molecular communications that take place in rhizosphere zones and exploiting these communications help to achieve better results in the elimination of contaminants like PAHs and it is a fascinating area of research for present and future perspective of bioremediation methods(Bishtet al., 2015).

The relative contribution of rhizobacteria and plant species and other communicative factors highly influences the degradation mechanisms of PAHs in soils. These factors determine the effectiveness and suitability of a remedial strategy for PAH-contaminated sites(Afegbua and Batty 2018). Rhizodegradation systems get strengthened and become more effective when PAHs degrade bacteria from contaminated soils augmented to desired rhizosphere soils. The enhancement of PAHs degradation in soil by adding potent PAHs degrading bacteria like *Mycobacterium* spp., extended the degradation of phenanthrene, pyrene and fluoranthene(Johnsenet al., 2007). This type of studies with potent PAHs degrading strains having the more remediation abilities recorded significantly improved PAHs remediation from soil treatments(Nieet al., 2002; Glick et al., 2005; Farwell et al., 2007). Hence, the present investigation has taken to study the rhizodegradation of PAHs by augmenting a combination of two potent PAHs degrading strains(*Bacillus cereus* CPOU13 and *Bacillus subtilis* SPC14) that were isolated from PAHs contaminated soils and added to the rhizosphere and non-rhizosphere soil treatments of a pulse crop, blackgram. The pot-culture method was taken for this study and the soil samples used for the study were artificially contaminated(600ppm) with three PAHs compounds namely phenanthrene, anthracene and pyrene.

Materials and methods:-

Pot culture studies:

Soil used for pot culture was collected from Botanical Garden, Osmania University campus, Hyderabad, Telangana State. The soil had no previous history of PAH contamination. The soil was air-dried at room temperature(28-31°C) for 24h to constant weight before use. Concentrations of nitrogen, phosphorus and potassium were estimated by the methods of Subbaiah and Asija (1956), Olsen et al., (1954) and Muhret al., (1965) respectively.

Phenanthrene, anthracene and pyrene(Sigma 99% purity) were accurately weighed and dissolved in acetone separately. Each PAH solution was transferred to a glass sprayer and spiked onto the experimental soil and made the final concentration of 600mg/kg soil(600ppm). The soil was mixed thoroughly and equally distributed. Spiked soil was air-dried at room temperature(28-30°C) for more than 24h or until the smell of acetone disappeared. Soil used for autoclaved treatments were autoclaved at 121°C for 15min before PAHs spiking and the soil used for non-autoclaved treatment soil was directly used without autoclaving.

One milliliter 7-day cultures of *B. cereus* CPOU13 and *B. subtilis* SPC14 grown in LB broth were transferred separately to 50ml nutrient broth and incubated for 24h at 30°C on a rotary shaker at 150rpm speed. One ml of the bacterial culture was suspended in 9ml of nutrient broth and mixed with soil to a final concentration of 3.3×10^4 CFU of the strain *B. subtilis* SPC14 per gram dried soil. Bacterial enumeration was done by viable plate counting after serial dilution(Chouychaiet al., 2009).

Experimental design and analytical method:

The method for evaluating rhizodegradation of PAHs by the strains, *B. cereus* CPOU13 and *B. subtilis* SPC14 was adopted from Chouychaiet al., (2009). In the present study, the treatments were categorized and set to following seven types:

Non-rhizosphere soils

(a) Autoclaved soil(ACS) - control
(b) Non-autoclaved soil(NACS)
(c) *B. cereus* CPOU13 and *B. subtilis* SPC14 in autoclaved soil
(d) *B. subtilis* SPC14 in non-autoclaved soil

Rhizosphere soils

(e) Plant in non-autoclaved soil
(f) Plant with *B. cereus* CPOU13 and *B. subtilis* SPC14 in non-autoclaved soil
(g) Plant with *B. cereus* CPOU13 and *B. subtilis* SPC14 in autoclaved soil

Three replicates were maintained per treatment. The soil used for autoclaved treatments was autoclaved at 121°C for 15min for three times in three days.

Extraction of PAHs from pot culture soils:

The method for PAHs extraction from soil was adopted from Yuan et al., (2000). 2grams of soil from each pot was collected after the 90days and placed separately in 50ml test tubes. 5ml of n-hexane was added to each test tube prior to being shaken with a rotary shaker for 24hrs at 160rpm. Then a layer of n-hexane was collected and aqueous layer was further extracted with additional n-hexane and adding anhydrous Na_2SO_4 for complete moisture removal. The step was repeated for 3-4 times. Extracts were centrifuged at 12,000g for 10min and filtered through 0.2µm filters. Finally, extracts were concentrated using a vacuum evaporator under low pressure conditions. The remnants of the sample were dissolved in 3ml of HPLC grade acetonitrile and stored at 4°C until the HPLC analysis. HPLC studies were conducted as described in previous sections. Unknown concentrations of phenanthrene, anthracene and pyrene in the soil samples were determined using standard chromatograms.

Statistical analysis:

All the experiments in the study were performed in triplicates. Mean and standard deviation of triplicate in independent experiments were calculated. Mean values were compared with the values of LSD(least significant difference) to find significance at 0.01 and 0.05 probabilities. LSD values were calculated using a software STAR(Statistical Tools for Agricultural Research) made by CRIDA(Central Research Institute for Dryland Agriculture, Hyderabad, Telangana State).

HPLC analysis:

Determination of PAHs compounds degradation was studied with a reverse phase High Performance Liquid Chromatography(HPLC). The instrument consists of a dual pump system and connected with a UV detector(SPD-20A). Instrument was equipped with column C18(250mm × 4.6mm, 5A° particle size) of Phenomenex Co. Mobile phase consisted of 75% acetonitrile and 25% deionized water. Detector was set at 250nm and the mobile phase was maintained at a flow rate of 0.8ml/min in isocratic mode. 20µl of sample was injected into HPLC with a HPLC injector(Rheodine injector) that prior filtered with 0.22µm syringe filters. Data of each peak on the HPLC chromatogram was analyzed using chromatography software 'LC Solutions'.

Total bacterial population in pot culture soils:

The plate count method was followed to enumerate total bacterial population for pot culture soil treatments and this was done as described by Kim et al., (2007). One gram of soil from each treatment was added to 9ml of sterilized water and mixed vigorously. After settling, 1ml of supernatant was transferred to another test tube containing 9ml of sterilized water to achieve a dilution of 10^{-2}. Other samples were treated in the same manner. A serial dilution technique subsequently yielded five additional test tubes with the dilutions from 10^{-3} to 10^{-7}, each with three replicate samples. For dilutions of 10^{-3} to 10^{-7} spread on Tryptic soy agar plates and incubated at room temperature(25°C) for 10days. Total bacterial colony forming units(CFUs) were counted and means were taken for the studies.

Results:-

Extraction efficiency of phenanthrene, anthracene and pyrene from pot culture soil samples:

Extraction efficiency of PAHs from soils is important in PAHs degradation studies. In view of this, the soil samples from pot culture were extracted with acetonitrile and assayed for determining extraction efficiency using HPLC. The PAHsPhenanthrene, Anthracene and Pyrene recorded their Extraction efficiencies percentages 59.65, 70.59 and 25.69 respectively.

Soil analysis:

Composition of soil and the nutrients play an important role in supporting microbial activity and plant growth. In a view of this, concentrations of macronutrients such as nitrogen(N), phosphorus(P) and potassium(K) estimated from the soil samples and found concentrations of macronutrient are 112-120, 120-150, and 35-40 respectively.

Effect of *B. cereus* CPOU13 and *B. subtilis* SPC14 combination on rhizodegradation of phenanthrene, anthracene and pyrene in pot culture:

Combination of selected bacterial strains, *B. cereus* CPOU13 and *B. subtilis* SPC14 resulted in an increased effect on degradation of phenanthrene, anthracene and pyrene in pot culture studies(**Fig. 1** and **2**). Degradation of test PAHs was more in rhizosphere soils than non-rhizosphere soils. Results are presented in **Table 1**.

Decrease in the concentration of phenanthrene by the strains was more in autoclaved rhizosphere soil(96.24%) while in non-autoclaved rhizosphere soil it was slightly less(93.45%). The strains combination resulted in moderate degradation(30-40%) in autoclaved and non-autoclaved non-rhizosphere soils. Non-autoclaved rhizosphere soil recorded very low degradation of phenanthrene(0-15%).

Autoclaved rhizosphere soil treatment consisted of the strains recorded maximum degradation of anthracene up to 82.30%. The same pair of bacterial strains reported slightly reduced degradation of anthracene in non-autoclaved rhizosphere soil(76.80%). Very low degradation of anthracene(0-15%) was observed in non-autoclaved rhizosphere soil.

The maximum degradation of pyrene by the strains up to 96.24% noticed in autoclaved rhizosphere soil and moderate degradationof this PAHwas observed in autoclaved and non-autoclaved non-rhizosphere soil treatments.
The selected strains, *B. cereus* CPOU13 and *B. subtilis* SPC14 have good degradation abilities in the rhizosphere of blackgram. This combination reported higher degradation of phenanthrene, anthracene and pyrene up to 96.59%, 82.30% and 96.24% respectively.

Total bacterial population in *B. cereus* CPOU13and *B. subtilis* SPC14 augmented soils during rhizodegradation:

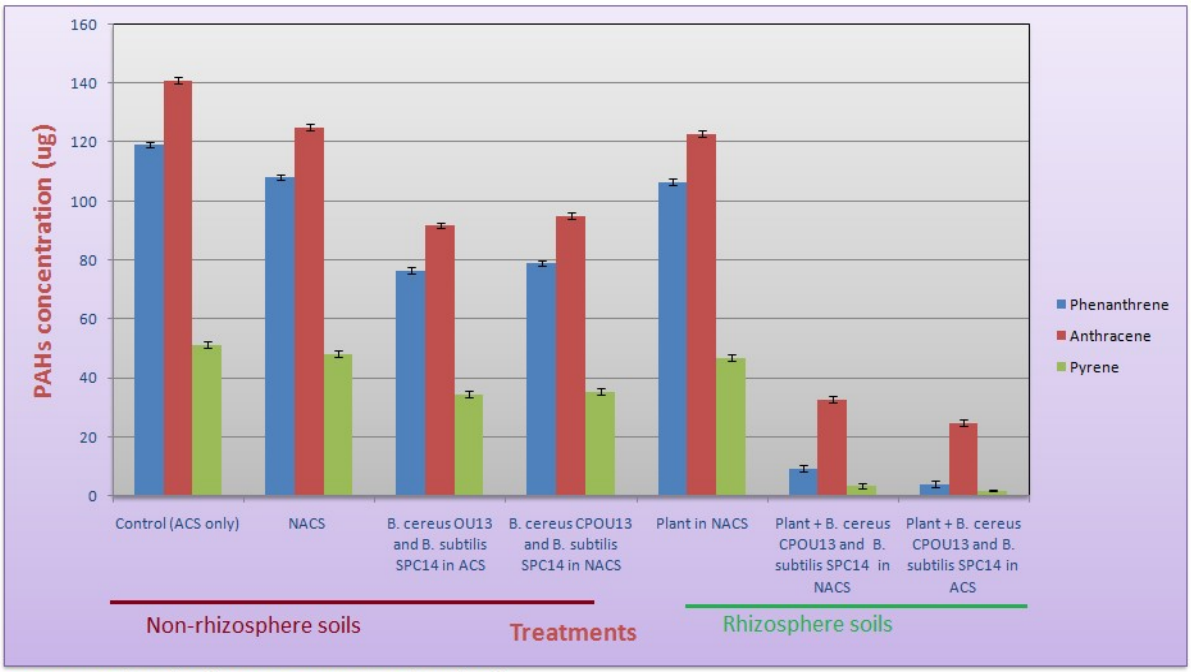
Augmentation of *B. cereus* CPOU13and *B. subtilis* SPC14 produced a prominent effect on bacterial population during rhizodegradation of PAHs. The results are presented in **Fig. 1** and **Table 2**. Bacterial populations were gradually increased from the beginning of the study *i.e.* 0th day, reached maximum by 60days and became almost consistent between 60 to 90days in all soil treatments. Populations of bacteria were high in rhizosphere treatments over the non-rhizosphere soil treatments. Non-autoclaved soils recorded more bacterial populations when compared to autoclaved soils. However, bacterial populationswerehighly increased in autoclaved rhizosphere soils. Statistically, the mean differences between treatments and time intervals are significant at 5% level.

Table 1:- Effect of *B. cereus* CPOU13 and *B. subtilis* SPC14 on rhizodegradation of phenanthrene, anthracene and pyrene in pot culture(± represents standard deviation of three replicates).

Sl. No.	Treatment		Phenanthrene		Anthracene		Pyrene	
			Quantity (µg/g soil)	Degradation (%)	Quantity (µg/g soil)	Degradation (%)	Quantity (µg/g soil)	Degradation (%)
1	CONTROL(AUTOCLAVED SOIL)	NON-RHIZOSPHERE SOILS	119.31± 1.00	0	141.19± 0.91	0	51.39± 1.01	0
2	NON-AUTOCLAVED SOIL		108.21± 0.99	I. 9. 3	125.10± 1.02	11.4	48.24± 0.89	6.11
3	*B. cereus* OU13 and *B. subtilis* SPC14 in Autoclaved soil		76.60±2.00	35.8	91.90±1.00	34.91	34.68±1.00	32.52
4	*B. cereus* OU13 and *B. subtilis* SPC14 in non-autoclaved soil		79.11± 0.98	33.71	95.09±0.98	32.65	35.47± 0.99	30.98
5	PLANT IN NON-AUTOCLAVED SOIL	RHIZOSPHERE SOILS	106.64± 1.00	10.62	122.91± 1.00	12.95	47.91± 1.05	8.72
6	Plant + *B. cereus*		9.49±0.91	92.05	32.75±0.9	76.8	3.365±0.9	93.45

	OU13 and *B. subtilis* SPC14 in non-autoclaved soil				2		0	
7	Plant + *B. subtilis* SPC14 in autoclaved soil		4.07±1.00	96.59	24.99±1.02	82.3	1.93±0.09	96.24

Fig 1:- Effect of *B. cereus* CPOU13 and *B. subtilis* SPC14 on rhizodegradation of phenanthrene, anthracene and pyrene in pot culture after 90days(Error bars represent standard deviation of three replicates).



ACS = Autoclaved soil; NACS = Non-autoclaved soil ;

Table 4:- Total bacterial population in *B. cereus* CPOU13 and *B. subtilis* SPC14 augmented soils in pot culture(± represents standard deviation of three replicates).

S. No.	Bacterial population(×10^4 CFU/g soil)						
	Treatment		0th day	15days	30days	60days	90days
1	Control(Autoclaved soil only)	Non-rhizosphere soils	0	0	0	0	0
2	Non-autoclaved soil only		73	76	76	79	76
3	*B. cereus* CPOU13 and *B. subtilis* SPC14 in autoclaved soil		3.3	6	7.6	7.6	7.3
4	*B. cereus* CPOU13 and *B. subtilis* SPC14 in non-autoclaved soil		160	230	230	260	200
5	Plant in non-autoclaved soil	Rhizosphere soils	73	86	93	130	100
6	Plant + *B. cereus* CPOU13 and *B. subtilis* SPC14 in non-autoclaved soil		160	260	330	400	400
7	Plant + *B. cereus* CPOU13 and *B. subtilis* SPC14 in autoclaved soil		3.3	83	130	330	330

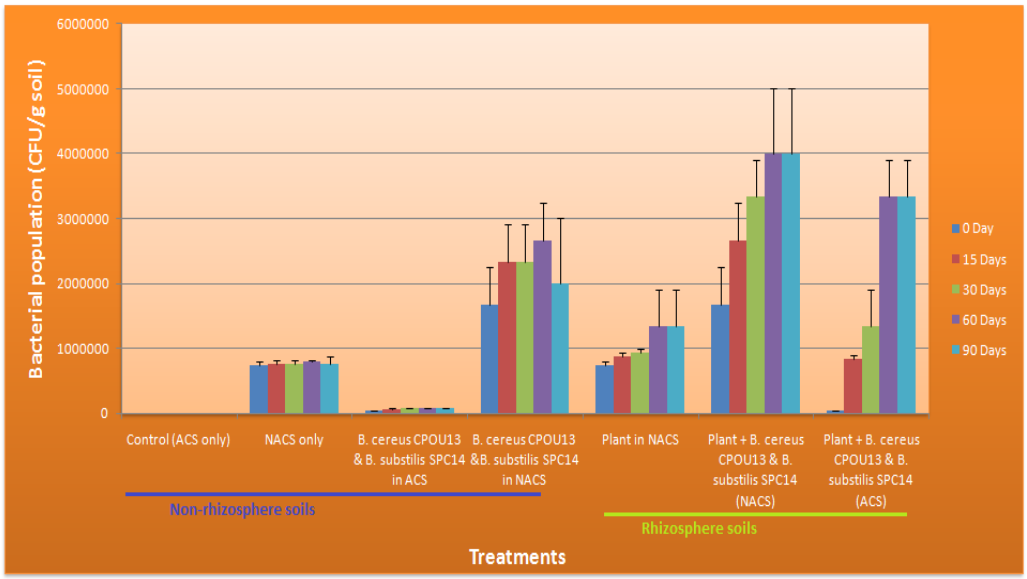


Fig. 2:-Total bacterial population in *B. cereus* CPOU13 and *B. subtilis* SPC14 augmented together in rhizosphere and non-rhizosphere soils(Error bars represent standard deviation of three replicates).

Discussion:-

Rhizodegradation of PAHs compounds by augmenting bacterial strains isolated from contaminated sites to the plant rhizosphere implicate greater involvement of bacterial populations and nutritious root exudates. Plant-bacterial interactions in rhizosphere with coupled metabolic capabilities completely degrade PAHs and avoid the formation of dangerous intermediates(Turkovskaya and Muratova 2019). Rhizodegradation may be very effective in PAHs removal with well-established and potential PAHs degrading bacteria through growth linked and co-metabolic reactions(Kanaly and Haryama, 2010). Previous researchers had found well defined microbial combinations were more effective than pure cultures or single bacterial strains in PAHs degradation(Lu et al., 2019, Silva et al., 2009). The enhanced PAHs degradation with combination of bacterial strains is possible due to broader enzymatic capacity of bacterial populations and other vital relations among the microbial communities(Kuiper et al., 2004). Furthermore enhanced pollutant removal efficiency might be due to enhanced microbial activity, increased nutrient supply and pollutant availability to the microorganisms by secreting specific enzymes and chemicals(Forjanet al., 2020; Mohan et al., 2008).

In the present study, the combination of *B. cereus* CPOU13 and *B. subtilis* SPC14 performed high rhizodegradation of PAHs in the treatments and these results are par with the findings of Nasseriet al., (2010) who reported more degradation of phenanthrene when they used bacterial consortium instead of single bacterium strains. Jacques et al., (2008) also reported microbial consortium of *Mycobacterium fortuitum, B. cereus, Microbacterium*, *Gordoniapolyisoprenivorans*, *Microbacteriaceae* bacterium and *Fusariumoxysporum* degrade more phenanthrene, anthracene and pyrene up to 96% to 99% within 70days of incubation. Some defined bacterial consortia degrading more hydrocarbons than other defined consortia due to their compatible co-metabolic activities(Ghazaliet al., 2004).

In the present investigation, the two strains of *Bacillus* reported the maximumdegradation of phenanthrene upto 96.59%, anthracene up to 82.30% and pyrene up to 96.24% in autoclaved rhizosphere soil while moderate and low degradationof the PAHs was observed in autoclaved and non-autoclaved non-rhizosphere soil treatments. These results disclosed the effect of PAHs degraders in the rhizodegradation system as their association with rhizosphere is important. In this mutual relation root exudation stimulates survival, multiplication and action of bacteria and inturn bacteria decrease pollutant concentration and their toxic effects on plants(Song et al., 2012). In addition, the plants and bacteria might have acquired the ability to withstand the toxicity of the PAHs and develop the potential to degrade them(Reichenauer and Germida, 2008; Song et al.,. 2012).

In this study, augmentation of the strains produced a significant effect on bacterial population during rhizodegradation. Bacterial community gradually increased from the beginning of the study, reached maximum by 60days and became almost consistent between 60 to 90days in the treatments. This increase was more in case of

rhizosphere soil treatments over the non-rhizosphere soil treatments and in non-autoclaved soils bacterial populations increased more when compared to autoclaved soils. In the study, the PAHs degrading bacteria augmentation in rhizosphere soils recorded accelerated degradation. Similar results were found with the findings of Yu et al., (2010) when they used ryegrass and PAHs degrading bacteria.

Higher densities and greater activities of microorganisms in the rhizosphere over the non-rhizosphere are considered to be responsible for enhanced degradation PAHs(Praeget al., 2019). Nutrients secreted from roots such as amino acids, organic acids, enzymes, carbohydrates may help the bacteria to spread over the soil and may become responsible for the present enhanced rhizodegradation of PAHs(Xuet al.,2009; Jensen et al., 2012). Hence, this investigation revoked the importance of bacterial population and microbial potential towards PAHs degradation achieved through compatible relation between the strains and host plant(Gentry et al., 2004; Balcom and Crowley, 2009; Zhang et al., 2012). Yet more research has to take place in this segment for more clarifications at molecular level and better understanding the mechanism of rhizodegradation.

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