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RHIZODEGRADATION OF PAHS BY AUGMENTING PGPR STRAIN *BACILLUS SUBTILIS* SPC14

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ABSTRACT

Utilization of PGPR strains for organic pollutants bioremediation systems has been become one of the common trends in recent years as it offers various advantages over the physical and chemical remediation methods. The present investigation was taken up to study the rhizodegradation of three PAHs compounds namely phenanthrene, anthracene and pyrene contaminated soils with a PAHs degrading PGPR strain, *Bacillus subtilis* SPC14 in pot culture experiments for 90days. Soil treatments of the study divided into rhizosphere and non-rhizosphere treatments then they were further divided broadly into autoclaved and non-autoclaved sets. After 90days of experimental period the treatment soils assed for PAHs concentrations. Present study revealed that the bioaugemted strain degraded phenanthrene, anthracene and pyrene upto 73.61%, 70.68% and 70.42% respectively in the treatments of rhizosphere autoclaved soils. However, PAHs degradation is very low in case of non-rhizosphere soils when compared to the rhizosphere soils. Upon the determination of effect of bacterial population on the rhizodegradation, we found that the strain's population was gradually increased with the PAHs degradation and its degradtion started from 0th day and reached maximum by 60days of the experimental period and then after bacterial population and PAHs degradations were gradually decreased. The strain was accomplished nearly 70% of degradation of PAHs compounds in all the soil treatments.

Keywords: PGPR, rhizodegradation, *Bacillus subtilis* SPC14, PAHs, pot culture

INTRODUCTION

Rhizosphere is a dynamic zone of plant-microbe interactions and influences the composition of microflora which helps the plant to adapt to adverse conditions. Plant growth promoting rhizobacteria (PGPR) are one of the important components of rhizosphere microflora and they are beneficial and improve crop productivity and growth by stimulating plant growth or by reducing the damage from soil- borne plant pathogens **[1, 2]**. Apart from this, studies have been focused on PGPR as candidates for good polycyclic aromatic hydrocarbons (PAHs) remediation and improving remediation to several folds than the utilization of general PAHs degraders **[3, 4]**. In recent times, PGPR have been implicated in rhizoremediation of organic toxicants that gave the plants an added advantage to grow even in polluted soils.

Plant and microorganisms play a complementary role in plant based remediation systems of PAHs contaminated soils and it is much more dependent on the microbial diversity of the plant rhizosphere. Rhizodegradation today, considered as a realistic, low-cost alternative, highly efficient than other classical remediation methods like physical, chemical methods and importantly proved as best suited for even treating extensive areas polluted by organic chemicals. Hence, the present work is taken up for the study of remediation of PAHs degradation using a potent PAHs degrading strain Bacillus subtilis and blackgram species.

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 at least 24h to constant weight before use. Concentrations of nitrogen, phosphorus and potassium were estimated by standard methods **[5, 6, 7]**.

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 was 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. 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.

Experimental design and analytical method

The method for evaluating rhizodegradation of PAHs by the strain *B. subtilis* SPC14 was adopted from Chouychai *et al* **[8]**. In the present study, the treatments were categorized and set to following seven types:

I. Non-rhizosphere soils

(a) Autoclaved soil(ACS) - control

(b) Non-autoclaved soil(NACS)

(c) *B. subtilis* SPC14 in autoclaved soil

(d) *B. subtilis* SPC14 in non-autoclaved soil

II. Rhizosphere soils

(e) Plant in non-autoclaved soil

(f) Plant and *B. subtilis* SPC14 in non-autoclaved soil

(g) Plant 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* **[9]**. Two grams 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 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 vacuum evaporator under low pressure conditions. The remnants of 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 found 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 HPLC(SHIMADZU, model RF-10AXL). The instrument consists of dual pump system and connected with UV detector (SPD-20A). Instrument was equipped with column C18 (250mm×4.6mm, 5A° particle size) of Phenomenex Co. Mobile phase was consisted of 75% acetonitrile and 25% of de-ionized water. Detector was set at 250nm and mobile phase was maintained at 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 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* **[10]**. 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 results are presented in **Table 1**.

Table 1: Extraction efficiency of PAHs from the soils

PAH Name	Amount of PAH added(ppm)	Extracted concentration(ppm)	Extraction efficiency (%)
Phenanthrene	200	119.31 ± 1.00	59.65
Anthracene	200	141.19 ± 0.91	70.59
Pyrene	200	51.39 ± 1.01	25.69

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 like N, P and K were estimated. The N, P and K concntrations are 112-120, 120-150, and 35-40 kg/ha soil respectively.

Effect of *B. subtilis* SPC14 on rhizodegradation of phenanthrene, anthracene and pyrene

The PGPR strain, *B. subtilis* SPC14 showed an enhancement effect on degradation of phenanthrene, anthracene and pyrene in pot culture with black gram after 90 days of incubation(**Fig. 1** and **2**). High degradation of phenanthrene, anthracene and pyrene was reported in the rhizosphere soils than the non-rhizosphere soils and the results are presented in **Table 2**.

The strain degraded phenanthrene to a maximum level (73.61%) in autoclaved rhizosphere soil. It also recorded moderate degradation of phananthrene (30-40%) in autoclaved non-rhizosphere soil while low degradation (15-30%) was recorded in non-autoclaved non-rhizosphere soil. However, non-autoclaved rhizophere soil showed very low degradation of phenanthrene (0-15%).

Degradation of anthracene by the strain was maximum (70.68%) in autoclaved rhizosphere soil. It also recorded low degradation (15-30%) of anthracene in autoclaved and non-autoclaved non-rhizosphere soils. However, non-autoclaved rhizophere soil treatment showed very low degradation of anthracene (0-15%).

The maximum degradation of pyrene (70.42%) by the strain was observed in autoclaved rhizosphere soil while low degradation of pyrene(15-30%) by the strain was recorded in autoclaved and non-autoclaved non-rhizosphere soils. However, very low degradation (0-15%) of pyrene was noticed in non-autoclaved rhizophere soil.

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

Addition of *B. subtilis* SPC14 to the soils amended with PAHs showed an apparent effect on bacterial population in pot culture (**Fig. 3** and **Table 3**). Higher bacterial population was observed in rhizosphere soils over non-rhizosphere soils. Gradual increase noted from 0 day to 60days of incubation in all treatments and afterwards it was declined. Autoclaved treatments showed low bacterial population than non-autoclaved soils. However, autoclaved rhizosphere treatments recorded high population increase than non-autoclaved non-rhizosphere treatments. Maximum bacterial population was observed

in non-autoclaved rhizosphere soil. The mean differences between treatments and time intervals were statistically significant at 0.01 and 0.05 probabilities.

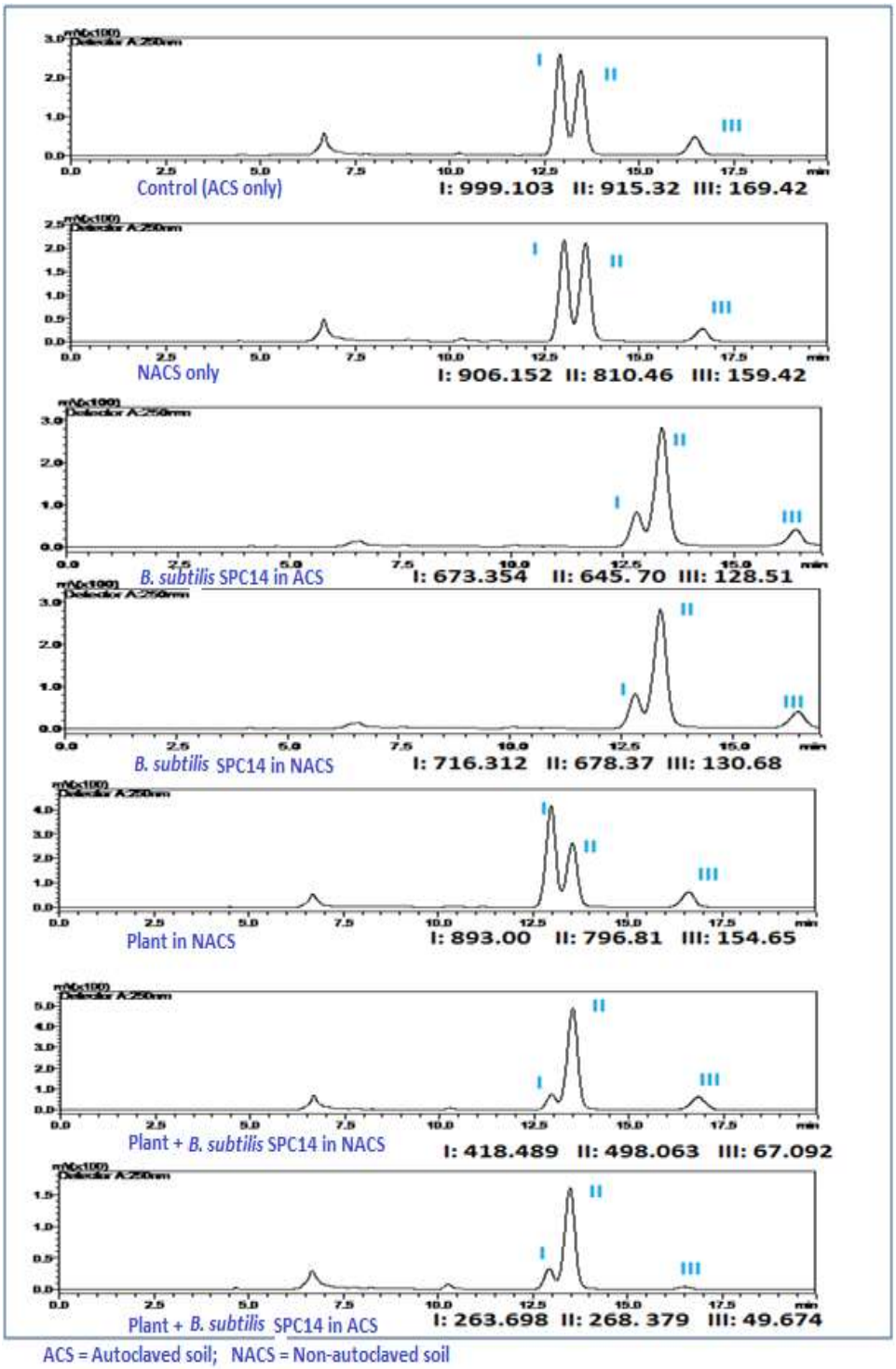

Fig. 1: HPLC chromatograms showing the effect of *B. subtilis* SPC14 on rhizodegradation of PAHs in pot culture studies(I= Phenanthrene; II= Anthracene; III= Pyrene)

Table 2: Effect of *B. subtilis* SPC14 on rhizodegradtion of phenanthrene, anthracene and pyrene in pot culture

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	0	141.19	0	51.39	0
2	Non-autoclaved soil		108.21	9.3	125.10	11.4	48.24	6.11
3	*B. subtilis* SPC14 in autoclaved soil		80.41	32.64	99.60	29.46	38.98	24.15
4	*B. subtilis* SPC14 in non-autoclaved soil		85.54	28.21	104.64	25.89	39.64	22.87
5	Plant in non-autoclaved soil	Rhizosphere soils	106.64	10.62	122.91	12.95	47.91	8.72
6	Plant + *B. subtilis* SPC14 in non-autoclaved soil		49.98	58.11	76.83	45.58	20.30	60.5
7	Plant + *B. subtilis* SPC14 in autoclaved soil		31.49	73.61	41.40	70.68	15.20	70.42

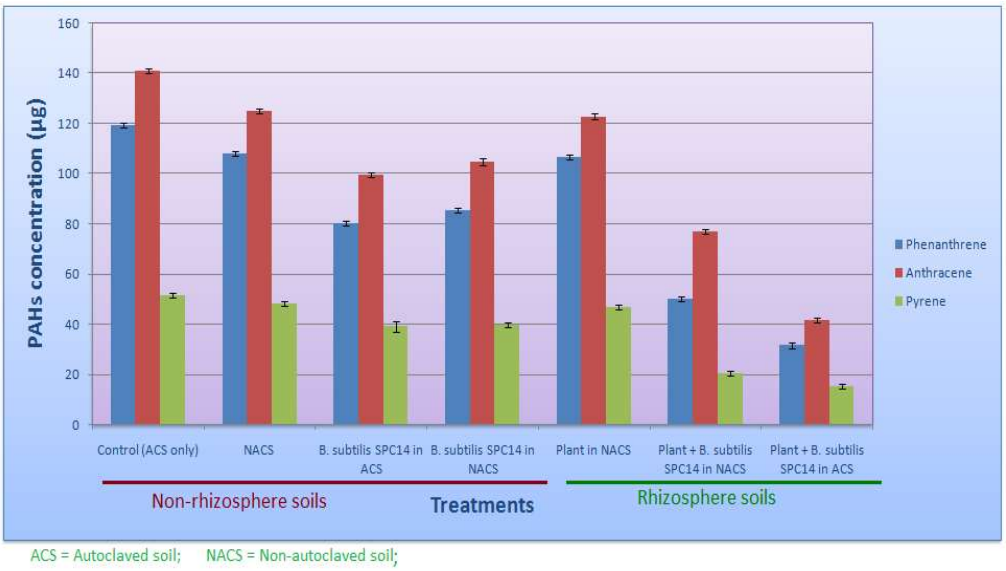

Fig. 2 Effect of *B. subtilis* SPC14 on rhizodegradation of phenanthrene, anthracene and pyrene in pot culture after 90days(Error bars represent standard deviation of three replicates)

Table 3: Bacterial population in *B. subtilis* SPC14 augmented soils in pot culture

S. No.	Bacterial population($\times 10^4$ CFU/g soil)						
	Treatment		0^{th} day	15days	30days	60days	90days
1	Control(Autoclaved soil)	Non-rhizosphere soils	0	0	0	0	0
2	Non-autoclaved soil		73	76	76	79	76
3	*B. subtilis* SPC14 in autoclaved soil		3.3	5	6.6	7	6.6
4	*B. subtilis* SPC14 in non-autoclaved soil		160	200	200	200	160
5	Plant in non-autoclaved soil	Rhizosphere soils	73	86	93	130	100
6	Plant + *B. subtilis* SPC14 in non-autoclaved soil		160	200	260	330	300
7	Plant + *B. subtilis* SPC14 in autoclaved soil		33	70	90	260	200

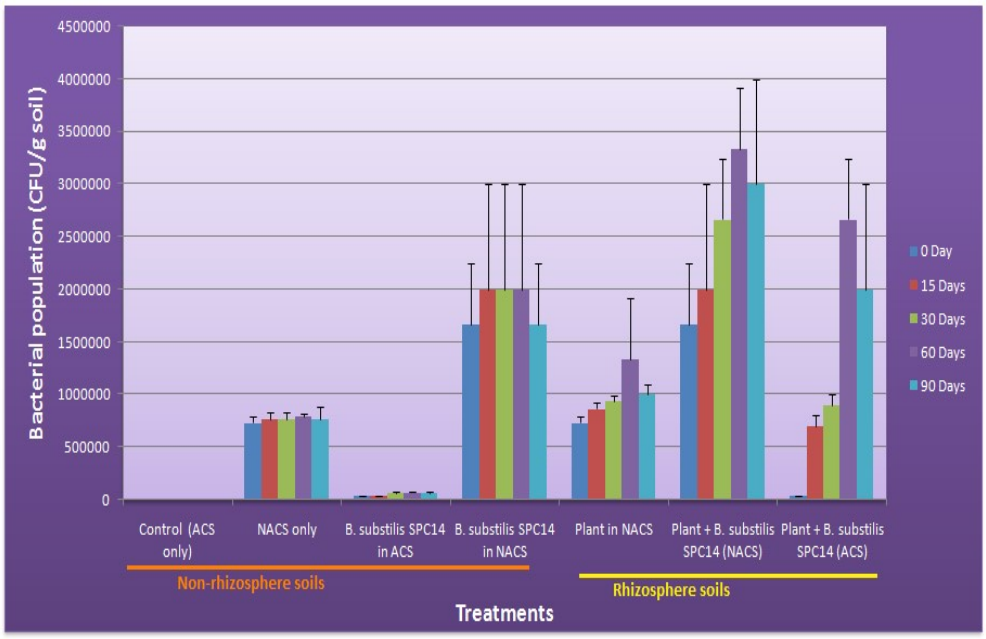

Fig. 3: Total bacterial population in *B. subtilis* SPC14 augmented rhizosphere and non-rhizosphere soils during rhizodegradation of PAHs in pot culture(Error bars represent standard deviation of three replacates)

DISCUSSION

PGPR strains play a imperative role in adaptation of the host plant to a changeable environment due to pollutant contamination and they posses mechanism of plant development *via* synthesis of phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores, biocontrol agents and uptake of nutrients. Along with these, phytohormones released by PGPR, induce plant growth and are also accountable for pollutant remediation as well as activating plant defense response against pollutant induced stresses **[11, 12]**. Hence the present study is taken up to determine the PAHs degradation in the rhizosphere soils of blackgram augmented with potent PAHs degrading PGPR strain in the duration of 90days.

The degradation of selected PAHs was investigated with eight prairie grasses namely *Andropogon gerardi*, *Sorghastrum nutan*, *Panicum virgatum*, *Elymus canadensis*, *Schizachyrium scoparius*, *Bouteloua curtipendula*, *Agropyron smithii*, *Bouteloua graciis* and found that chrysene, benzo(a)pyrene, benzo(a)anthracene, dibenz(a,h) anthracene degradation was high in the rhizosphere of grasses compared to the soils without grasses in 219days **[13]**. Binet *et al* **[14]** recorded the dissipation of a mixture of eight PAHs ranging from 3-6rings in the rhizosphere of ryegrass (*Lolium perenne*) and reported consistent and significantly higher degradation of PAHs in

the rhizosphere of aged polluted soil. As the above our results indicated degradation of phenanthrene, anthracene and pyrene in the rhizosphere soils of blackgram than rhizosphere soils. Similarly, the degradation of pyrene in rhizosphere and non-rhizosphere soils of maize in a green house study and reported that after 45days of incubation the ratio of remained pyrene were 61.25% and 35.58% in rhizosphere and non-rhizosphere soils **[15]**. In our study the augmented strain, *B. subtilis* SPC14 in autoclaved rhizosphere soils degraded the PAHs phenanthrene up to 73%, anthracene by 70% and pyrene up to 70% in the rhizosphere of blackgram in pot culture. Here degradation of pyrene was slightly less than phenanthrene. These results are coinciding with the findings of many researchers **[16, 17, 18]**.

The varying degradation rates of PAHs in 90days of study may be depended on their complexity natures and bioavailability. The PAHs having more aromatic rings are more resistant to microbial attack and degradation due to low solubility **[19, 20]**. Mohan *et al* **[21]** recorded pyrene has lower biodegradability and higher toxicity that may affect soil microflora and reduce their degradation. However in our study slightly low degradation of pyrene achieved by the application of more adaptable bacteria **[22]**. However, the degradation levels are much lower in non-rhizosphere soils revealing the importance of rhizoremediation that means rhizodegradation of PAHs was accelerated by the addition of PAHs degrading microorganisms.

Rhizodegradation is a type of mutual relation in that root exudation stimulates survival, multiplication and action of bacteria. In turn, bacteria decrease pollutant concentration and their toxic effects on plants. Additionally, plants root system helps bacteria to spread over the soil because the root exudates are a mixture of nutrients consisting of amino acids, organic acids, enzymes, carbohydrates, and sugars**[23]**. Hence, the rhizodegradation of PAHs is primarily depends on the microbial population and their metabolic activity. In view of this significance of bacterial population in rhizodegradation bacterial count was taken up for important criterion for the evaluation. Our results recorded that rhizosphere soils supported maximum PAHs degraders and natural microflora and their metabolic activities. The population increase in bio-augmented soils didn't show major differences but degradation potential of each was much different and varied. This data suggests that variation in PAHs degradation rates is mainly due to the PAHs degrading potentials of *B.*

subtilis SPC14. However, bacterial population slightly decreased after 60days of the study. As Cerniglia **[24]** reported that the PAH degrading bacterial counts was may be decreased with the depletion of inorganic nutrient concentration in soil. This reduction may be due to substrate reduction or toxic intermediate production **[25,26]**. We found an inverse relationship with microbial population of *B. subtilis* SPC14 and the degradation of PAHs as the population increased with the degradation of phenanthrene, anthracene and pyrene or with incubation period. Our findings are in conformity with earlier findings of Wiltse *et al* **[27]** and Nichols *et al* **[28]**.

CONCLUSIONS

The impact of PAHs degraders on rhizodegradation of phenanthrene, anthracene and pyrene is more conspicuous and much more significant. This strain became key factor for high degradation of PAHs than natural bacterial communities in the soil. As the assumption, variability in PAHs compounds degradation, mainly for pyrene was low and that might be depended upon it physic-chemical properties. The population of the augmented strain and other bacterial communities played key role in the rhizodegradation. Yet more insights have to concentrate on this aspect with further studies for clear-cut discriminations.

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