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Toluene Degrading Bacteria from the Rhizosphere of *Solanum melongena* Contaminated with Polycyclic Aromatic Hydrocarbon

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A B S T R A C T

The application of hydrocarbon degrading microorganisms in bioremediation applications is a promising approach to accelerate the clean-up of polluted soils. The use of microorganisms to accelerate the natural detoxification processes of toxic substances in the soil represents an alternative ecofriendly and low-cost method of environmental remediation compared to harmful incineration and chemical treatments. Although rhizobacteria found in the rhizosphere possess the ability of promoting plant growth, some rhizobacteria also have the potential of biodegradation polycyclic aromatic hydrocarbons. Keeping this fact in mind this research work was aimed in isolation and characterization of such plant growth promoting bacteria (PGPB) which had the ability to degrade toluene as well as possess plant growth promoting traits. The results obtained revealed that out of seventeen PGPB, three isolates BRB-2, BRB-5 and BRB-9 possessed multiple PGPR traits and had the ability to degrade toluene up to 100-200µL/mL of toluene concentration. The vigour index calculation revealed that the isolates BRB-2, BRB-5 and BRB-9 had vigour index of 1193.49, 1183.5 and 1441.42 respectively which was very high as compared to the control which possessed vigour index 902.4. In conclusion, we reported for the first time the isolation of PGPR from the rhizosphere of *Solanum melongena* with the ability to utilize toluene as a growth substrate. Further, rhizospheric bacteria may have biotechnological value as materials for cleaning of polycyclic aromatic hydrocarbon containing soil sites.

Introduction

Increase in industrialization over the last century has led to elevated releases of anthropogenic chemicals into the environment. Prevalent contaminants include petroleum hydrocarbons (PHCs), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, solvents, metals, and salt. There resulting stresses on human and eco-system health are well documented (CCME, 2001).

Polycyclic aromatic hydrocarbons (PAHs) are the product of incomplete combustion, *i.e.* the burning of fossil fuels, forest fires, coal tar, creosote, diesel and cigarette smoke. These organic chemicals are found almost everywhere and pose a risk to human health because of their potentially carcinogenic nature and bioavailability in water, soil, sediment, and air resources that humans come in contact with daily. Because PAHs are

emitted naturally through forest fires and volcanoes, microorganisms have the ability to break down PAHs and contaminated sites can be remediated through microbial and environmental manipulations.

Toluene is a methyl substitution on the aromatic benzene ring, is distributed in water, soils and industrial effluents (Witzig *et al.,* 2006). Ground water contamination pollution is the major source of toluene (Chang and Lee, 2001). Toluene is used as a solvent for paints, coatings, gums, oils, and resins. Significant bacterial communities with ability to degrade PAH in soil play a critical role in biodegradation in spite of their low bioavailability. Despite these properties, many bacterial strains have been isolated for their ability to transform, degrade and utilize PAH as a source of carbon and energy. Plant-associated microorganisms play key roles in PAH uptake by plants. These microbes can participate in PAH-degrading processes (Vinas *et al.,* 2005; Sheng *et al.,* 2008; Yuan *et al.,* 2001).

Bioremediation method is considered as an economical and safe approach for the environment. Taxonomic groups such as *Sphingomonas, Burkholderia,* and *Pseudomonas* have been dominating the Polyaromatic hydrocarbons (PAH) degradation in soil (Johnsen *et al.,* 2005). The possible fate of PAH in the environment include chemical oxidation, bioaccumulation and adsorption to soil particles, but the principle process for removal of PAH is thought to be microbial transformation and degradation (Heinonsalo *et al.,* 2000). Biological treatment is well known to be feasible and effective than chemical treatment because microorganisms directly degrade contaminants rather than merely transferring them from one medium to another and employ metabolic degradation pathways that can terminate with benign waste products (*e.g.* carbon dioxide and water). Also, microbes derive energy necessary to degrade contaminants from the catabolic degradation of contaminants themselves. Because of all these properties, microbes are used *in situ* to minimize disturbance of the pollutants from contaminated site (Juhasz *et al.,* 1997).

Several naphthalene and anthracene degrading bacteria were isolated from rhizosphere of *Populus deltoides*, which were growing in non-contaminated soil. Among these, four isolates, *i.e. Kurthia* sp., *Micrococcus varians, Deinococcus radiodurans* and *Bacillus circulans* utilized chrysene, benzene, toluene and xylene, in addition to anthracene and naphthalene (Bisht *et al.,* 2010). When a suitable rhizospheric strain is introduced together with a suitable plant, it settles on the root along with indigenous population, thereby enhancing the bioremediation process. In addition, such efficiently root-colonizing, pollutant-degrading bacteria exploit the growing root system and hence this acts as an injection system to spread the bacteria through soil.

Hydrocarbon degrading bacteria and fungi are widely distributed in marine, fresh water, and soil habitats (Atlas and Bartha, 1992), The most prevalent hydrocarbon degrading bacteria in the soil environment are *Pseudomonas, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Rhodococcus, Corynebacterium, Sphingo-monas,* and *Mycobacterium* (Bartha and Atlas, 1977; Atlas and Bartha, 1992; Korda *et al.,* 1997). A number of hydrocarbon degrading *Pseudomonas* species has been isolated from oil contaminated Antarctic soils (Mac Cormack and Fraile, 1997) and Kerry (1990) and Aislabie (1997) found that numbers of Antarctic hydrocarbon degrading bacteria were enhanced in fuel contaminated Antarctic soils compared to pristine Antarctic soils. Moreover, studies conducted by Pratheesh and Jayachandran (2012) revealed that *Pseudomonas* sp. SBCT-17 showed

promising results in biodegradation of toluene hydrocarbon. Therefore, the present work was designed to study the biodegradation ability of toluene by PGPR isolated from the rhizosphere of *Solanum melongena* for soil fertility and its efficacy on plant growth promotion.

Materials and Methods

Collection of soil samples

The soil used for PGPR isolation was collected from the rhizosphere of Brinjal (*Solanum melongena*) grown in Roshnabad village situated near Sidkul, Haridwar. The site was extremely polluted due to the irregular disposal of organic and inorganic contaminants in water bodies which reaches agriculture field by irrigation methods. The rhizospheric soil was collected in sterile polythene bags and stored at the 4˚C until further use.

Isolation of toluene degrading bacteria

Rhizosphere soil samples (10g) were suspended in 90 ml of 0.85% normal saline (pH 7.0) water and shaken vigorously at 150 rpm at 37˚C for 1 hrs. The resulting sample was serially diluted (100µL) to 900 µL of 0.85% normal saline in each eppendorf tube and appropriate dilution (10^{-6}) of this suspension was spread plated in triplicate on MSM medium. Various concentrations ranging from 10-100 µL of toluene was used as sole carbon and energy sources for isolation of strains. Cultures were incubated at 37˚C ± 2 for 2 days. Pure cultures were obtained by successive culturing of colonies picked from these plates on MSM.

Maintenance of isolates

All the isolates were maintained at 4˚C in equal volume (1:1 v/v) of nutrient broth and 30% glycerol.

Identification and characterization of the selected putative toluene-degrading bacterial isolate

The pure cultures obtained were then characterized on the basis of their phenotypic traits. Phenotypic characters of bacteria include morphology and biochemical reactions carrying out by bacteria whose results can be viewed. Morphological characteristics include colony morphology such as color, size, shape, opacity, elevation, margin surface texture, consistency etc. These characters are observed after the incubation period on the cultures on the solid media. In liquid cultures, we can observe the pellicle formation, sediment formation. Biochemical characteristics include enzyme production.

Morphotypic characterization of bacterial isolates

Recovered bacterial isolates were phenotypically (morphotypic and functional) characterized. A total of seventeen isolates were randomly selected morphologically from the pure cultures. Colony morphology of isolates was studied under a microscope. This included shape, edge, elevation, surface and pigmentation. Cellular morphology was based upon cell shape and Gram staining (Agrawal *et al.,* 2015). Bacterial identification was carried out on the isolates by comparing the results obtained with that of Bergey's manual of determinative systematic bacteriology (1986).

Growth and tolerance of isolates to toluene

Overnight cultures were used to inoculate 100 ml sealed bottles containing 5 ml of sterile mineral medium (MM) described by Abu-Ruwaida *et al.,* (1991). The medium was supplemented with different concentrations (50, 100, 150 and 200µl/ml) of toluene to different initial OD_{600}. Cultures were incubated at 28 ± 2˚C and 300 rpm. Growth

and tolerance were analyzed by the appearance of turbidity after five days measured at 600 measuring a UV spectrophotometer.

Biochemical characterization of bacterial isolates

Catalase test

The production of catalase was evidenced by the fact that catalase enzyme breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. A part of the colony of an isolate-grew on Nutrient agar was placed onto a clean microscope slide, whereon 100 µl of an aqueous solution of H_2O_2 30 % (v/v) was added and mixed. A positive result of catalase production was characterized by the rapid evolution of O_2 which was evidenced by bubbles formation.

Oxidase test

Take oxidase discs and moisten it with sterile distilled water. Pick the colony to be tested with wooden or platinum loop and smear on the disc. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds which indicates positive result.

Citrate test

Christensen citrate agar was dissolved in distilled water and heated gently to dissolve. Dispense 4.0 to 5.0 ml into 16-mm tubes. Tubes were autoclaved at 121^{o}C under 15 psi pressures for 15 min. Cool in slanted position (long slant, shallow butt). Isolates were streaked in slants and one uninoculated slants was kept as a control. Incubate at 35 ± 2^{o}C for a week. Positive citrate test shows conversion of yellow color to pink.

Protease production

For the determination of protease enzyme the bacteria were spotted on plates of SMA medium containing 15 g skim milk, 0.5 g yeast extract, 9.13 g agar and 1 L distilled water and incubated at 27˚C for 48 h.

The diameters of colorless halo zone around the bacterial colonies were measured to determine the ability of protease production.

Lipase production

For determination of lipase enzyme the following medium (Peptone 10 g, Calcium chloride 0.1 g, Sodium chloride 5 g, Agar 15 g, distilled water 1 L, 10 mL sterile Tween 20) was used (Omidvari, 2008).

All of bacteria were streaked on this medium and incubated at 27˚C for 48 h. Depositions around the bacterial colonies indicted activity of lipase enzyme.

Amylase test

All isolates were spot inoculated on amylase production medium containing 1% (w/v) starch and incubated at 35 ± 2^{o}C for 24-48 h. Plates were flooded with lugol's iodine for 10 min. Iodine was then drained off; isolates exhibiting a zone of clearance against dark blue background were considered positive.

Evaluation of plant growth promoting

Indole Acetic Acid (IAA) production

Indole acetic acid production was assayed as described by Pant and Agrawal, (2015). A loopful of bacteria was inoculated and incubated into pre-sterilized Peptone broth containing 1% of trypton for 48 hrs at 37 ˚C.

After 48 hrs add 1 ml of Kovac's reagent to all tubes including control and shake after 15 min. The appearance of red ring at the top is the clear indication of indole acetic acid production.

Siderophore production

Bacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium described by Schwyn and Neilands (1987). Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism (l0µl of 10^6 CFU/ml) and incubated at 25±2ºC for 48-72 h. Development of golden yellow-orange halo around the growth was considered as positive for siderophore production.

Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 h at 25±2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of blue to light yellow colour was a positive test for ammonia production.

Production of HCN

All the isolates were screened for the production of Hydrogen cyanide by adapting the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine/1 and bacteria were streaked on modified agar plate.

A Whatman filter paper No. 1 soaked in 2% sodium carbonate in 0.5% of picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 25 ± 2°C for 4 days. Development of orange to red color indicated HCN production.

Phosphate solubilizing activity

Pure cultures of bacteria were spot inoculated at the center of already prepared plates of Pikovskaya's agar medium (Agrawal *et al.*, 2015). The plates were incubated at 30 ± 1˚C for 7-10 days. The colonies forming more than 5.0 mm zone of solubilization were stocked. The zone of phosphate solubilization (mm) formed around colonies was recorded after every 24 hrs for 10 days. The solubilizing efficiency of the microorganisms was calculated using following formula:

$$\text{Solubilizing efficiency (\% S.E)} = \frac{Z-C}{C} \times 100$$

Where; Z = Solubilization zone (mm); C = Colony diameter (mm)

Quantitative estimation of Indole acetic acid (IAA) production from rhizobacteria

IAA production was detected as described by Agrawal *et al.*, 2011; Agrawal and Agrawal, (2013). Each bacterium was cultured in nutrient broth medium and incubated at 28˚C for 48 h in a shaker incubator. Then 50 µL of each bacterial suspension were transferred to nutrient broth containing 100 µg mL^{-1} L-tryptophan. After 48 h, the suspensions were centrifuged at 10000 rpm for 10 min. Consequently, 1mL of supernatant was mixed with 4 mL Salkowski reagent (2 mL of 0.5mol L^{-1} $FeCl_3$ + 98mL 35% $HClO_4$). After 20 min, the samples that turned red were considered as positive and the absorbance of the mixture was measured at 535 nm with a spectrophotometer.

Quantitative estimation of phosphate soulubilization

Quantitative estimation of inorganic phosphate solubilization was done as per methodology described by Nautiyal, 2001 and Jackson, 1973. The phosphate solubilization was carried out using National Botanical Research Institute's Phosphate-Bromo Phenol Blue (NBRIP-BPB) broth (Pradhan and Sukla, 2005) containing 0.5% tricalciumphosphate (TCP). The flask containing 50ml medium was inoculated with

500µl bacterial cultures and incubated at 30˚C at 300 rpm for five days. Simultaneously, the uninoculated control was also kept under similar conditions. The cultures were harvested by centrifugation at 10,000 rpm for 10 min. The absorbance of the resultant color was read after 10 min at 430nm in UV/Visible spectrophotometer.

Effect of bacterial isolates on seed germination

To study the effect of the isolates on germination rate, 20 seeds of Brinjal were prepared for each treatment. For sterilization, seeds were soaked in 2% sodium hypochlorite for 3 min and then they were washed by sterile distilled water for 5 times. Incubation of the seeds was done with 24 hrs old cultures with cell concentration of 10^8 ml^{-1}. In control, seeds were germinated in water. These seeds were then taken and twenty seeds were placed in each Petri dish. In control, only water was added. Each test was performed in triplicates. Germination took place in an incubator at 25˚C, in the dark. The seeds were considered to be germinating at the moment of radicle emergence. The number of germinated seeds was recorded and the final germination percentage was calculated after 5 days.

Efficacy of toluene degrading bacterial isolates on plant growth promotion using pot experiment

The seeds were surface sterilized by soaking it in 2% sodium hypochlorite for 3 min and then they were washed by sterile distilled water for 5 times. After drying seeds were soaked in the suspension of the bacterial isolates separately for a few minutes in order to coat the seeds with the test organism then the seeds were removed and allowed to dry. The soil was sterilized by autoclaving and seeds were sowed in soil as a test (inoculated seeds) and control (uninoculated seeds). Water was added in the pots as per requirement daily and observed for shoot length, root length and seed germination with respect to control after 20 days.

Results and Discussion

Isolation of bacterial isolates

The rhizospheric soil samples of brinjal were collected from the agricultural land of Raoshnabad village situated near sidkul, Haridwar. The soil samples of two different varieties *i.e.* “Chechu” and “Bati” were collected in triplicates. Bacterial isolates were isolated by the serial dilution method in Minimal Salt Media (MSM) containing toluene as sole carbon source. The viable bacterial colonies were counted and the CFU so found were $4.5X10^6$ CFU/gm for Chechu variety and $5.5X10^6$ CFU/gm for Bati variety (Fig 1).

Purification and maintenance of pure cultures

Primary screening resulted in about 52 isolates. All bacterial colonies obtained were further purified by continues sub-culturing in Minimal Salt Media. After purification about seventeen bacterial isolates were obtained. The pure cultures, so obtained were preserved in slants and glycerol stocks for further use.

Morphological identification of the isolates

The seventeen pure isolates obtained were characterized based on their morphological characteristics. The analysis of morphological characteristics included colony morphology such as shape, margin, elevation, pigmentation and surface whereas cell morphology included cell shape, size, gram staining and arrangement of cells. The bacterial isolates exhibited a broad range of morphological variation. Most of the isolates

were circular in shape. Their surfaces were flat, umbonate or convex in elevation. Maximum of the obtained isolates were gram negative with dry texture, pale white in color and smooth in appearance (Table 1).

Biochemical characterization

All the seventeen bacterial isolates were subjected to biochemical characterization. The biochemical analysis of the bacterial isolates was done to better comprehend the working, growth and metabolism of microorganisms which helps in using media or conditions to be used for bacterial cultures further. The different tests performed included gram staining, endospore staining, catalase activity test, oxidase test, lipase production, protease activity, citrate test and amylase production test (Table 2).

Gram staining results showed that number of gram positive bacteria was dominating among all the isolates. These gram positive bacteria were then subjected to endospore staining. Endospore staining helps in differentiating between microorganisms which can produce endospores and those which cannot. The presence of endospore is the characteristic feature of *Bacillus*. After endospore staining the endospores can be seen as bright green color spores and vegetative cells are brownish red or pink in color.

The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source. A positive diagnostic test rests on the generation of alkaline by-products of citrate metabolism. The subsequent increase in the pH of the medium is demonstrated by the color change of a pH indicator. The visible presence of growth on the medium and the change in pH indicator color due to the increased pH are the signs that an organism can import citrate and use it as a sole carbon and energy source; such organisms are considered to be citrate positive. Here ten isolates were found to be positive for citrate test.

Protease is an enzyme used to degrade proteins in amino acids. Casein is a large protein that is responsible for the white color of milk. Proteolytic bacteria use the enzyme caseinase to hydrolyze casein and form soluble nitrogenous compounds displayed as a clear zone around colonies. This clearing is much more pronounced on agar containing milk if the bacteria are able to produce acid from fermentable carbohydrates in the medium. A total of seven out of seventeen isolates were positive for protease test.

The Lipase Test is used to detect and enumerate lipolytic bacteria, especially in high-fat dairy products. The lipase enzyme is secreted and hydrolyzes triglycerides to glycerol which is converted into glycolysis intermediate. Out of seventeen isolates three were positive for lipase activity test.

Amylase test is done to determine if a bacterium can use starch, a complex carbohydrate made from glucose, as a source of carbon and energy for growth. Use of starch is accomplished by an enzyme called alpha-amylase. Iodine turns blue-black in the presence of starch. Absence of the blue-black color indicates that starch is no longer present in the medium. Bacteria which show a clear zone around the growth produce the exoenzyme amylase which cleaves the starch into di- and monosaccharides. *Bacillus* species are known to produce the exoenzyme, amylase. Eight isolates showed positive results for amylase test.

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome

oxidase enzyme, the reduced colorless reagent becomes an oxidized colored product. If bacterium contains cytochrome oxidase, it can use the oxygen for energy production with an electron transfer chain. The oxidase reagent will turn blue or purple within a short time. The reaction is positive. If the bacterium does not contain cytochrome oxidase, the reagent will remain colorless. The reaction is negative. It is commonly used to distinguish between oxidase negative *Enterobacteria-ceae* and oxidase positive *Pseudomadaceae*. Here six out of seventeen isolates showed positive test for oxidase test.

The catalase test facilitates the detection of the enzyme catalase in bacteria. The catalase test is also valuable in differentiating aerobic and obligate anaerobic bacteria, as anaerobes are generally known to lack the enzyme. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2$ + Catalase $\rightarrow$ $2H_2O$ + O_2). This reaction is evident by the rapid formation of bubbles. Out of seventeen isolates seven isolates showed positive results for catalase activity test.

Qualitative screening of the isolates for their toluene biodegradable ability

The present study focused on the isolation and characterization of efficient bacterial strains which are capable of toluene biodegradation as well as possess PGPR properties. Seventeen morphologically different strains were successfully screened from the rhizosphere of Brinjal plant most of which showed ability of tolerating toluene up to 100µL/ml of toluene concentration (Table 3). These seventeen isolates were screened for its growth capacity in minimal salt media, which consisted of toluene at increasing concentrations as a sole carbon source and the most efficient strain was selected and used for further studies. However, studies conducted by Pratheesh and Jayachandran (2012) revealed that *Pseudomonas* sp SBCT-17 showed promising results in biodegradation of toluene hydrocarbon.

In vitro qualitative evaluation of plant growth promoting activities

All the seventeen toluene degrading isolates were evaluated for their PGPR properties. For rhizobacteria to qualify as a PGPR it should have the following five attributes such as phosphate solubilization, IAA production, ammonia production, HCN production and siderophore production. These isolates were also subjected to the above tests (Table 4). Phosphorus (P) is one of the major essential macronutrients for plants, which is applied to the soil in the form of phosphate manure. However, a large portion of the applied phosphorus is rapidly immobilized, being unavailable to plants. The growth of P-solubilizing bacteria (PSB) often causes soil acidification, playing a key role in P-solubilization. A total of nine out of seventeen isolates showed positive result for P solubilization.

IAA production is one of the direct mechanisms which PGPR uses for plant growth promotion. IAA functions as an important signal molecule in the regulation of plant development including organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, and gene regulation. The test showed that out seventeen isolates eight isolates were potent IAA producers.

Then the isolates were screened for their ammonia production ability. Production of ammonia is an important attribute of PGPR that influences plant growth indirectly. Among seventeen isolates five isolates

produced this secondary metabolite. Cyanide production is one of the possible ways by which rhizobacteria may suppress plant growth in soil. Cyanide production by the bacteria will affect plant root growth and other rhizospheric process. Here twelve isolates showed negative results for HCN production. Siderophore are low molecular weight compounds which have high affinity for iron. Under iron limited areas these isolates produce siderophore which chelate iron and make it available to the plants. Appearance of hollow orange zone CAS dye media indicates the production of siderophore. Among seventeen isolates nine isolates showed this property.

The percentage of PGP traits shown by isolates was also evaluated. Among all the seventeen isolates, about 47.05% isolates showed positive IAA production, 70.5% showed negative HCN production activity, 52.94% isolates tested positive for siderophore production, only 29.41% isolates produced ammonia and 52.94% were potent phosphate solubilizers (Fig 2).

Quantitative estimation of plant growth promoting traits

Taking the results of qualitative results of PGP activities into consideration out of seventeen isolates seven isolates were chosen for further quantitative estimation. The seven isolates taken were BRB-2, BRB-4, BRB-5, BRB-8, BRB-9, BRB-16, and BRB-17.

Table.1 Morphotypic characterization of bacterial isolates obtained from brinjal rhizosphere

Name of isolate	Color	elevation	margin	surface	form
BRB-1	Pale White	Flat	Undulate	Smooth	Circular
BRB-2	Pale white	Convex	Undulate	Smooth	Circular
BRB-3	White	Flat	Entire	Dry	Irregular
BRB-4	Glistening	Convex	Entire	Smooth	Circular
BRB-5	White	Umbonate	Serrate	Dry	Circular
BRB-6	White	Umbonate	Lobate	Dry	Irregular
BRB-7	Pale White	Flat	Entire	Smooth	Circular
BRB-8	White	Umbonate	Serrate	Dry	Circular
BRB-9	Pale white	Flat	Lobate	Dry	Irregular
BRB-10	Pale white	Flat	Entire	Smooth	Circular
BRB-11	White	Convex	Entire	Smooth	Circular
BRB-12	Pale white	Flat	Undulate	Dry	Irregular
BRB-13	White	Convex	Lobate	Smooth	Circular
BRB-14	Glistening	Umbonate	Entire	Dry	Irregular
BRB-15	White	Convex	Lobate	Dry	Irregular
BRB-16	Glistening	Flat	Undulate	Smooth	Circular
BRB-17	Pale white	Flat	Serrate	Smooth	Circular

Table.2 Biochemical characterization of the bacterial isolates

Name of Isolates	Gram staining	Endospore staining	Citrate test	Protease test	Lipase test	Amylase test	Oxidase test	Catalase activity	Probable Organism
BRB-1	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-2	Gram -	-	-	+	+	+	+	+	*Pseudomonas*
BRB-3	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-4	Gram -	-	-	+	+	+	+	+	*pseudomonas*
BRB-5	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-6	Gram -	-	-	+	+	+	+	+	*Pseudomonas*
BRB-7	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-8	Gram -	-	-	+	+	+	+	+	*Pseudomonas*
BRB-9	Gram -	-	-	+	+	+	+	+	*Pseudomonas*
BRB-10	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-11	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-12	Gram -	-	-	+	+	+	+	+	*Pseudomonas*
BRB-13	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-14	Gram +	+	+	+	+	+	+	+	*Bacillus*
BRB-15	Gram -	-	-	+	+	+	+	+	*Pseudomonas*
BRB-16	Gram -	-	-	+	+	+	+	+	*Pseudomonas*
BRB-17	Gram +	+	+	+	+	+	+	+	*Bacillus*

Table.3 Screening of isolates based on their ability to degrade toluene

ISOLATES	10µL/ml	20µL/ml	30µL/ml	40µL/ml	50µL/ml	70µL/ml	100 µL/ml
BRB-1	+	+	+	+	+	+	+
BRB-2	+	+	+	+	+	+	+
BRB-3	+	+	+	+	+	+	+
BRB-4	+	+	+	+	+	+	+
BRB-5	+	+	+	+	+	+	+
BRB-6	+	+	+	+	+	-	-
BRB-7	+	+	+	+	+	+	+
BRB-8	+	+	+	+	+	+	+
BRB-9	+	+	+	+	+	+	+
BRB-10	+	+	+	+	+	+	+
BRB-11	+	+	+	+	+	+	+
BRB-12	+	+	-	-	-	-	-
BRB-13	+	-	-	-	-	-	-
BRB-14	+	+	+	+	+	+	+
BRB-15	+	+	+	+	+	+	+
BRB-16	+	+	+	+	+	+	+
BRB-17	+	+	-	-	-	-	-

Table.4 Bacterial isolates having PGPR properties

Isolates	IAA Production	HCN production	Siderophore production	Ammonia production	Phosphate solubilization
BRB-1	-	++	+	-	+
BRB-2	+	-	+	+	+
BRB-3	+	+	+	-	+
BRB-4	+	-	+	+	+
BRB-5	+	+	+	+	+
BRB-6	-	-	+	-	-
BRB-7	-	++	-	-	+
BRB-8	+	-	+	-	+
BRB-9	+	-	-	+	+
BRB-10	-	+	-	-	-
BRB-11	-	-	-	-	-
BRB-12	-	-	+	-	-
BRB-13	-	-	-	-	-
BRB-14	-	-	-	-	-
BRB-15	-	-	-	-	-
BRB-16	+	-	-	+	+
BRB-17	+	-	+	-	-

Table.5 Efficacy of PGPR isolates on root and shoot length, germination percentage and seedling vigour

Isolates	Percentage seed germination	Root length (cm)	Shoot length (cm)	Vigour index
Control	80	4.33±0.7	6.95±1.3	902.4
BRB-2	89	6.3±1.8	7.11±0.7	1193.49
BRB-4	80	5.6±0.8	6.91±0.5	1001.6
BRB-5	90	5.8±2.1	7.35±0.6	1183.5
BRB-8	82	5.4±2.1	7.28±0.4	1039.76
BRB-9	97	7.16±1.6	7.7±0.8	1441.42
BRB-16	82	5.28±0.8	7.03±0.6	1009.42
BRB-17	86	5.23±0.6	7.15±0.9	1064.68

Fig.1 Total CFU/gm of two varieties of brinjal rhizospheric soil

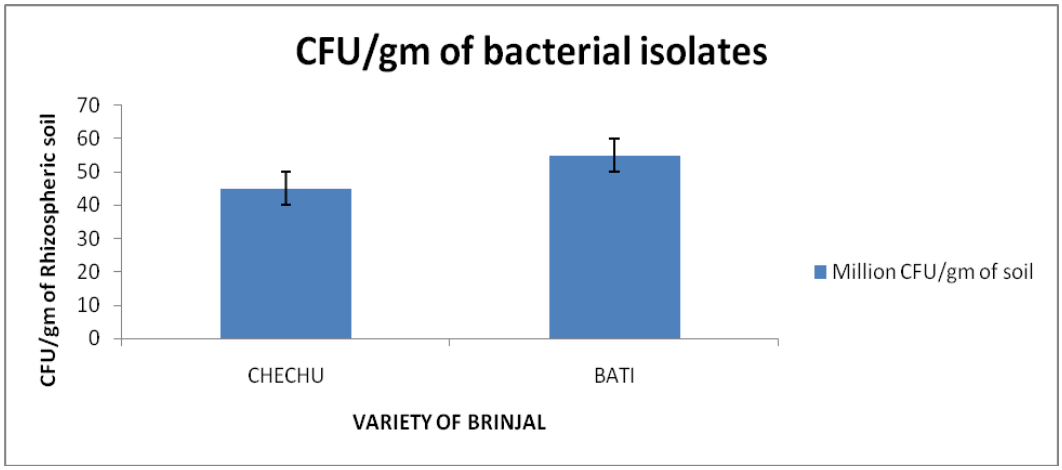


Fig.2 Percentage of PGPR trait present in the isolates

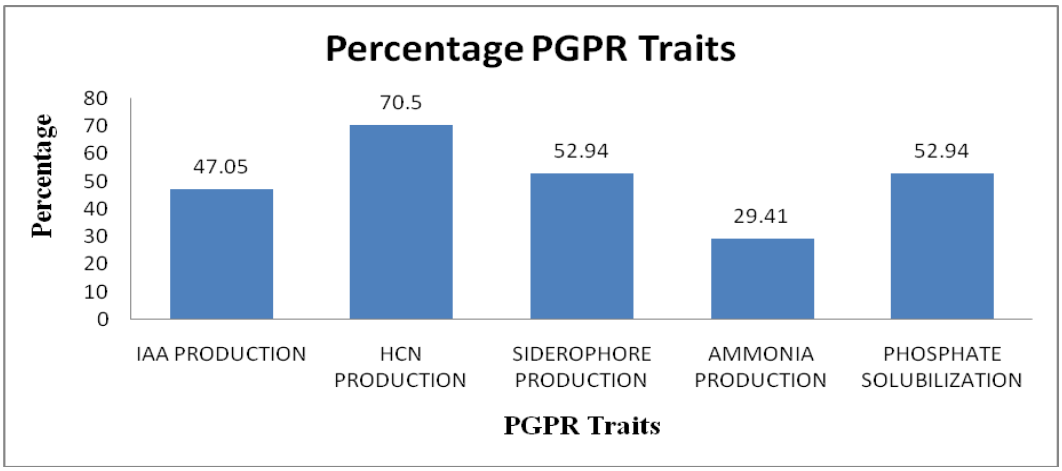


Fig.3 Quantitative estimation of IAA produced by the rhizobacterial isolates

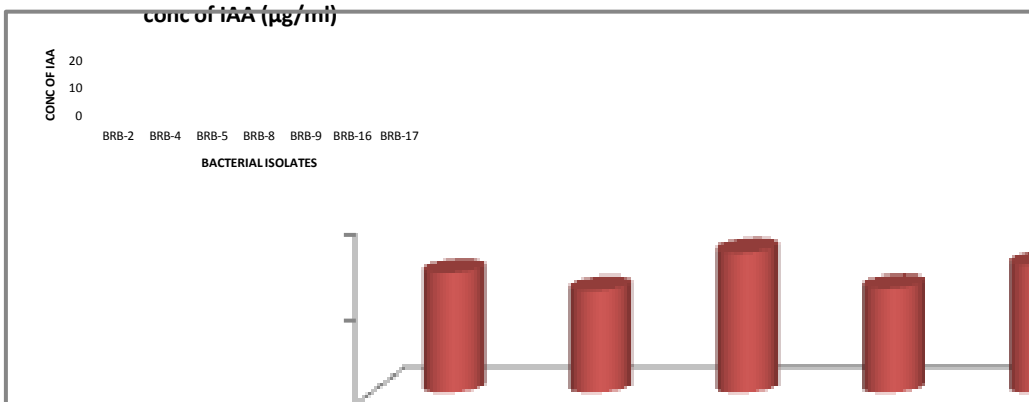


Fig.4 Quantitative estimation of phosphate solubilization activity of Rhizobacterial isolates

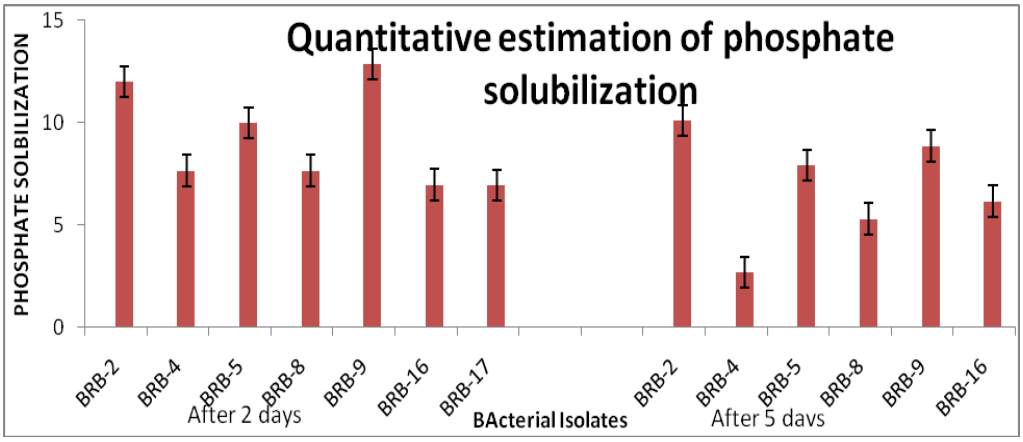


Fig.5 Estimation of zone of siderophore produced by the isolates

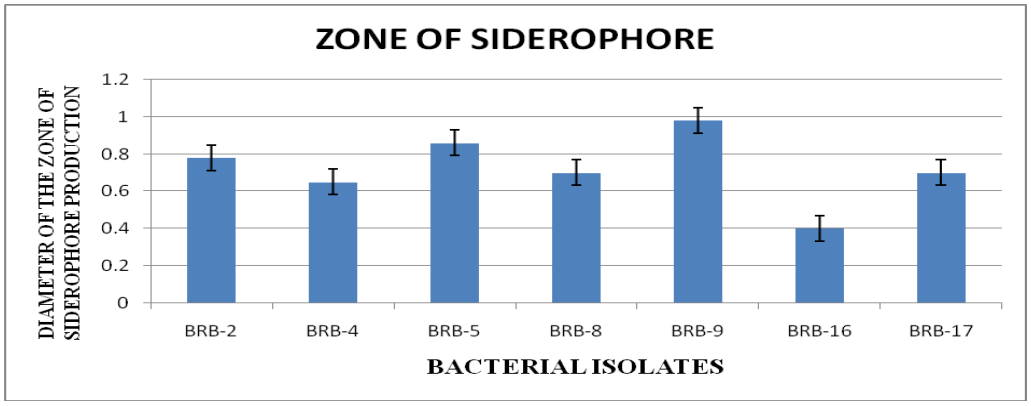
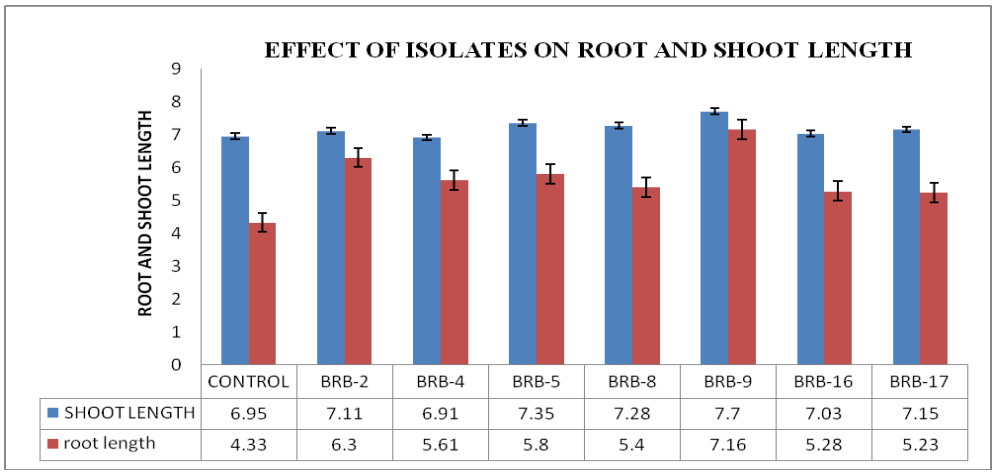


Fig.6 Effect of bacterial treatment on plant growth promotion of Brinjal plant romotion of Brinjal plant



	CONTROL	BRB-2	BRB-4	BRB-5	BRB-8	BRB-9	BRB-16	BRB-17
SHOOT LENGTH	6.95	7.11	6.91	7.35	7.28	7.7	7.03	7.15
root length	4.33	6.3	5.61	5.8	5.4	7.16	5.28	5.23

Fig.7 Growth tolerance of isolate BRB-2 in increasing toluene concentration

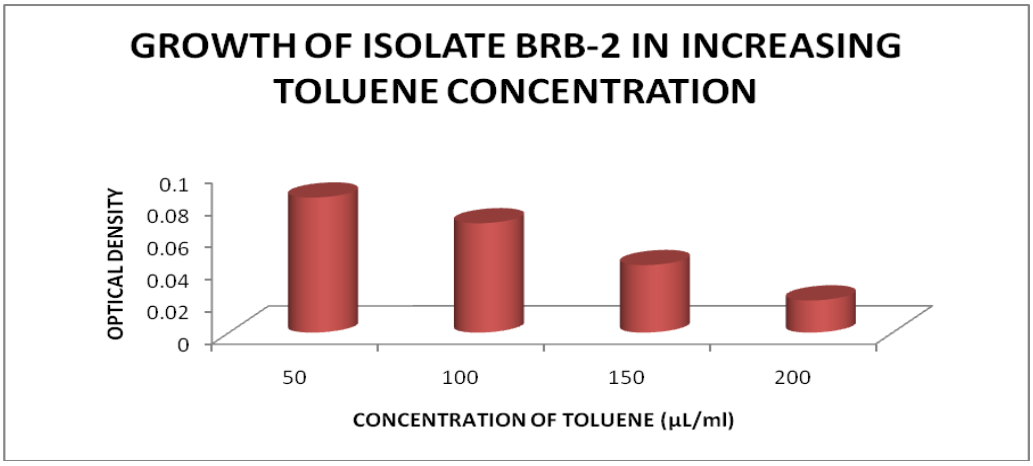


Fig.8 Growth tolerance of isolate BRB-5 in increasing toluene concentration

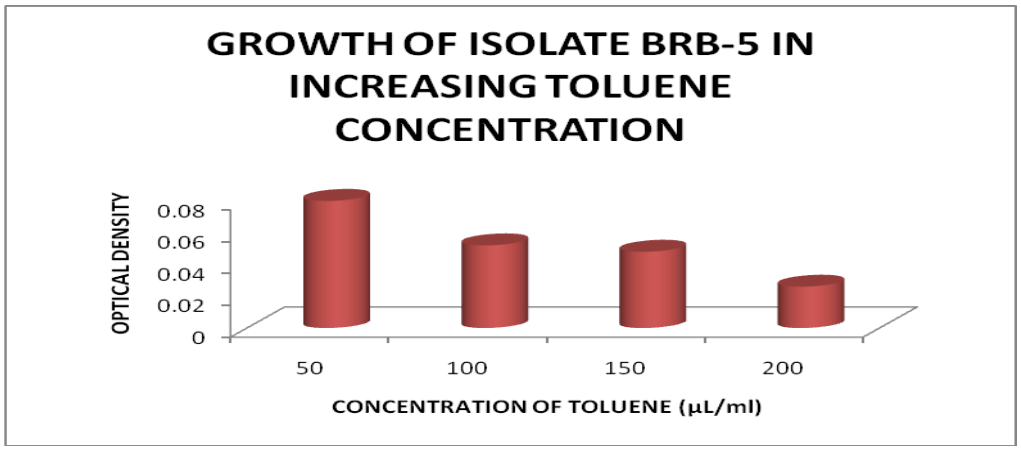
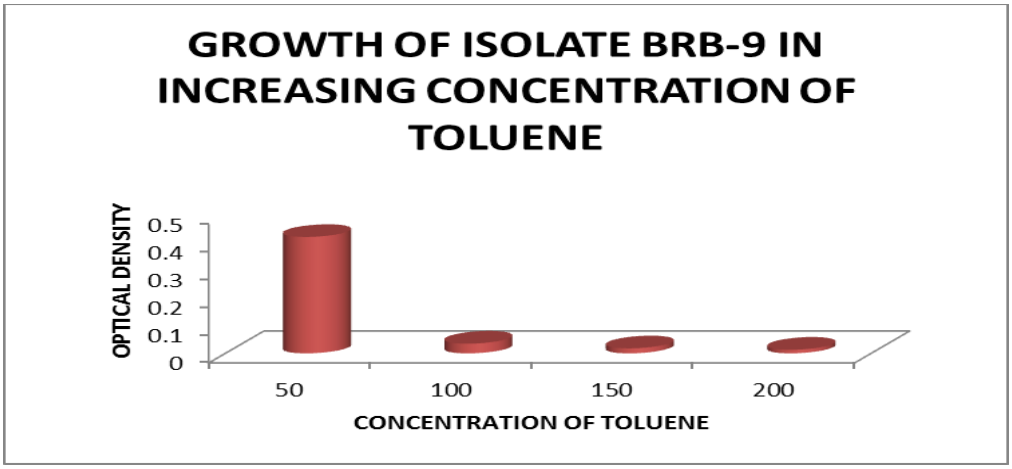


Fig.9 Growth tolerance of isolate BRB-9 in increasing toluene concentration



Quantitative analysis of IAA production

The use of the technique for the detection of IAA using the Salkowski reagent is an important option for qualitative and semi-qualitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of bioinoculants. The productions of indole acetic acid (IAA) by all rhizobacteria were investigated as important mechanism for plant growth stimulation. All rhizobacterial isolates produced IAA in vitro by the addition of L-tryptophan, in the culture medium (Agrawal and Agrawal, 2013). The quantitative estimation of IAA production was calculated in the presence of 100 µg/ml tryptophan. The amount of IAA produced was calculated by comparing with the standard calibration curve. The estimated amounts of IAA produced by all seven isolates are shown in figure 3. The isolates BRB-2, BRB-5 and BRB-9 showed best results by producing 14µg/ml, 16.44µg/ml and 15 µg/ml concentration of IAA. The amount of IAA produced by the rhizobacterial isolates were within the detection limits of Salkowski reagent **(**Ehmann 1977**).** The reagent gives reaction with IAA and does not interact with L-tryptophan and Na-acetyl-L-tryptophan and used by and large (Vaghasiat *et al.,* 2011).

Agrawal and Pant (2015) reported PGPB from rhizosphere of *Withania somnifera* showed red colour with Salkowaski reagent indicating production of IAA by the organisms. A total of six isolates were able to produce auxin ranging from 5 to 11 µg ml^{-1} in the presence of the precursor L-tryptophan in the medium. Agrawal *et al*., (2011) reported PGPB isolate MFB-1 R-3 produced highest level of IAA in liquid broth (33.55 µg ml^{-1}) and FA_2K100^3, the least (1.75 µg ml^{-1}). In a study carried by Shukla *et al.,* (2012) it was reported that an isolate of *Pseudomonas* sp designated as KS51 showed the properties of PGPR such as production of IAA (8 µg ml^{-1} day^{-1}) and was also a potent degrader of naphthalene (78.44%) and anthracene (63.53%) as determined by HPLC analysis.

Quantitative estimation of phosphate solubilization

Quantitative estimation of phosphate solubilization was done by inoculating the isolates in National Botanical Research Institute's Phosphate - Bromo Phenol Blue (NBRIP-BPB) broth and measuring absorbance at OD_{600}. The media contained tricalcium phosphate as the sole source of insoluble phosphorus source. The isolate BRB-9 showed maximum phosphate solubilization after 2 days of incubation whereas the isolate BRB-2 showed maximum solubilization after 5 days of incubation (Fig 4). Studies revealed that several bacterial strains (*Pseudomonas, Bacillus, Rhizobium* and *Enterobacter*) and fungal strains (*Aspergillus* and *Penicillium*) have been recognized as powerful phosphate solubilizers and potential bioremediation agents as well (Jain and Khichi, 2014).

Zone of siderophore production

The isolates were also subjected to siderophore production test. Siderophores are low molecular weight, extracellular compounds with a high affinity for ferric iron, that are secreted by microorganisms to take up iron from the environment (Sharma and Johri, 2003; Agrawal *et al.,* 2011) and their mode of action in suppression of diseases were thought to be solely based on competition for iron with the pathogen (Bakker *et al.,* 1987; Duijff *et al.,* 1997). The siderophore production potential was measured by calculating the siderophore production index from the clear zone formed in CAS dye media. The isolate BRB-9 showed maximum zone of siderophore

production with a diameter size of 0.9 mm (Fig 5). Gaonkar *et al.,* (2012) revealed that a pigmented bacterial culture TMR2.13 identified as *Pseudomonas aeruginosa* showed growth on mineral salts medium (MSM) with 2% of sodium benzoate and produced a yellowish fluorescent siderophore identified as pyoverdine.

Efficacy of PGPR isolates on plant growth

Effect on seed germination and seed vigour index

These isolates were then assessed to know their influence on the seed germination by measuring the shoot and root length. The growth promoting activity of isolates of PGPR was tested for seed germination and seedling vigour. The effect of the isolates was evaluated on the percentage seed germination and on seed vigour index. Seed Inoculation significantly enhanced seed germination and seedling vigour of brinjal. The isolate BRB-9 showed maximum seed germination percentage of 97% (Table 5).

Effect of isolates on root and shoot length

To evaluate the efficacy of isolates on plant growth pot trials were carried out using seven selected isolates. Brinjal seeds were treated with this isolates individually and were planted in pots and their effect of root length and shoot length were calculated after 21 days. The PGPR isolates significantly affected the length of brinjal seedlings. Results revealed that both root and shoot length increased in PGPR treated plants over uninoculated control. The highest root length 7.16 cm was recorded in treatment of BRB-9 isolate and highest shoot length was also seen in treatment of isolate BRB-9 of 7.7 cm as compared to the control whose root and shoot length was recorded as 4.33 cm and 6.95 cm respectively (Fig 6). The isolate BRB-9 showed not only good results in toluene degradation but also possessed potent PGPR properties.

Many researchers have done quite a good number of researches on plant growth promoting rhizobacteria. Agarwal *et al.,* (2015) reported that the strains isolated from the rhizosphere of tomato plant significantly improved seed germination when compared to the uninoculated control. The strains GKS-V, HPR-I and HPR-III significantly increased shoot and root length as well as enhanced vigour index of 124.54 and 741.45 after 6 and 16 days respectively. The biochemical characterization proved that all the three strains belonged to *Pseudomonas* species. Bacterized lentil seeds showed improved plant growth compared to untreated control (Agrawal et al, 2011). Isolates exhibiting improved seed germination also supported improved root and shoot length and a significant difference in percentage of germination was observed compared to control; values ranged between 14.18 to 38.36%. Also experiment carried by Gholami *et al.,* (2009) revealed that the strains *P.putida* strain R-168, *P.fluorescens* strain R-93, *P. fluorescens* DSM 50090, *P. putida* DSM291, *A. lipoferum* DSM 1691, *A. brasilense* DSM 1690 increased plant height and leaf area significantly as compared to the control.

In the work was carried by Ventorino *et al.,* (2014) it is stated that strain *M. populi* VP2 demonstrated multiple plant growth promotion activities and simultaneous showed potential biodegradation of xenobiotic organic compounds of industrial origin. Research of Sun *et al.,* (2014) also confirmed that after ryegrass (*Lolium multiflorum Lam.*) roots inoculation, *Pseudomonas* sp. strain Ph6-gfp actively and internally colonized plant roots and transferred vertically to the shoots. Ph6-gfp had a natural capacity to cope with phenanthrene *in vitro* and in planta. Ph6-gfp degraded 81.1% of phenanthrene (50 mg L^{-1}) in a culture solution within 15 days.

Quantitative estimation of toluene degradation

The seventeen isolates obtained from brinjal rhizosphere which had toluene degradability properties were further analyzed for presence of PGPR properties. Out of seventeen isolates three isolates designated as BRB-2, BRB-5 and BRB-9 showed best PGPR activity as well as good efficacy on plant growth. Considering these qualities, the above three isolates were quantitatively analyzed for their range of biodegradability of toluene. Isolate BRB-2 showed highest growth of 0.084 (OD_{600}) at 50 µL/ml concentration of toluene. Isolate BRB-5 and BRB-9 also showed the highest growth at 50 µL/ml of 0.08 (OD_{600}) and 0.0421 (OD_{600}) respectively (Figure 7, 8 and 9). However, as the concentration of toluene was increased all the three isolates showed decrease in its growth. This inability to utilize the hydrocarbons may be attributed to membrane toxicity and non-possession of the necessary enzymes. Moreover, the lipophilic hydrocarbons accumulated in the membrane lipid bilayer and may affect the structural and functional properties of the membrane. It may also lead to loss of membrane integrity, increase in permeability to protons and consequently, dissipation of the proton motive force, and impairment of intracellular pH homeostasis (Sikkema *et al.*, 1995). Huang *et al.,* (2005) have reported that combined use of PGPR and specific PAH degrading bacteria for successfully removal of complex contaminants. The application of certain rhizobacteria can increase the uptake of Ni from soils by changing its phase (Abou-Shanab *et al.*, 2006). Also, the manipulation of genetic engineering technologies greatly expands the extension and degree of bioremediation.

In conclusion the recent researches of PGPR on the remediation of contaminated soils show a brilliant prospect for the successive studies. The most important processes and causes of soil degradation are water–wind erosion, salinization, alkalinization, acidification, and leaching and soil pollution. The rate of soil degradation is directly related to unsuitable land use. While growers routinely use physical and chemical approaches to manage the soil environment to improve crop yields, the application of microbial products for this purpose is less common (Turan *et al.*, 2011). However, plant growth promoting rhizobacteria (PGPRs) can prevent the deleterious effects of one or more stressors from the environment. These beneficial microorganisms can be a significant component of management practices to achieve the attainable yield in degraded soil. In such soils, the natural role of toluene-tolerant PGPRs in maintaining soil fertility is more important than in conventional agriculture.

Remediation with PGPRs is called bioremediation in degraded soil and is another emerging low-cost in situ technology (Cohen *et al.,* 2004) employed to remove or alleviate pollutants from the degraded land. The efficiency of bioremediation can be enhanced by the judicious and careful application of appropriate toluene tolerant, and plant growth promoting rhizobacteria. This manuscript may be result of studies on the recent developments in the utilization of PGPR for direct application in soils degraded with poly cyclic aromatic hydrocarbon and consequently, promote crop productivity in degraded soils across the globe and their significance in bioremediation.

The effectiveness of the selective ecological strategy employed in this study allowed for the isolation of indigenous strains that are naturally present in the contaminated soils of Roshnabad village, Sidkul, Hardwar. A total of seventeen bacterial isolates were obtained from the rhizosphere of the fields of Brinjal plant. To my knowledge this was the first

work carried on toluene biodegradaton by Plant growth promoting rhizobacteria recovered from rhizosphere of Brinjal plant. As future prospect of this work molecular studies could be done so as to get better understanding of the mechanism of the working of isolates for biodegradation and PGPR properties.

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