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Restoration of Petrol Contaminated Soil by PGPR Consortium Producing Rhamnolipids and Enhancement of Growth and Antioxidant activity of *Withania somnifera*

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Abstract

Oil spills may considerably damage the soil quality. Increased contamination of agricultural soil by different hydrocarbons due to increased extraction and refinement of hydrocarbon are a serious environmental hazard due to their persistence in nature for a long time. Scope of bioremediation strategies is limited due to poor hydrocarbon accessibility and their low solubility which can be overcome by use of rhamnolipids, a class of biosurfactants. In the present study, pseudomonas strains (RK 4 and RK 3) isolated from contaminated soil identified by 16S rRNA were ascertained for PGPR as well as biosurfactants (rhamnolipids) property and checked for the compatibility to form consortium. Inoculating the strains and consortium of both the strain in petrol hydrocarbon contaminated soil and its interaction with *Withania somnifera* in presence of petrol oil reveals that rhamnolipids property of the strains helped in lowering the impediment effect of petrol engine oil hydrocarbons and the growth promotory activities enhanced the growth and antioxidant activity of *Withania somnifera*. Consortium of the both the strains showed positive results as compared to the individual strain and the interaction were found to be beneficial. The selected consortium of the strain could be further used for restoration of hydrocarbon contaminated soils for growing *Withania sominfera*.

Keywords: Rhamnolipids; Hydrocarbon; PGPR; *Withania somnifera*; Antioxidant activity; Restoration

Introduction

Petroleum hydrocarbons pollution cause major changes in the chemical and physical properties of soil resulting in impediment effect on plant growth. The damage to plant is attributed to hydrostatic and anaerobic condition that interferes with soil plant water relation [1]. Various microorganisms have adopted various mechanisms to thrive and grow in oil containing environment and showed immense role in treatment of this pollutant [2-4]. One of the limiting factors in this process is the bioavailability of many fractions of oil. This limitation can be whelming by the biosurfactants of diverse chemical nature and molecular size produced by hydrocarbon degrading microorganisms [5]. These surface active materials increases the surface area of hydrophobic water insoluble substrates and increase their bioavailability, thereby enhancing the growth of bacteria and rate of bioremediation through emulsification. Many strains of genus Pseudomonas inhabiting plant roots has the capability to promote plant growth under stress condition. The Plant Growth Promoting Rhizobacteria (PGPR) promotes plant growth directly and indirectly by various mechanisms. And the mechanisms are Nitrogen fixation, phosphate solubilization, siderophore production, Indole Acetic Acid production, ACC deaminase production, Ammonia and HCN production etc [6,7]. *Withania somnifera* is known to augment defense against diseases, arrest aging, increase the capability of the individual to resist adverse environmental factors and create a sense of mental wellbeing [8]. It is also used as anti-stress, anti-inflammatory, antioxidant and anti-tumor agent [9]. Due to its immense importance, the crop was selected for the present study to know its interaction with the surfactant producing PGPR bacterial strains in hydrocarbons contaminated soil. In this study, a consortium of fluorescent pseudomonads was developed and used for enhancement of growth and antioxidant activity of *Withania somnifera* in petrol oil contaminated soil.

Materials and Methods

Collection of soil sample and isolation of bacterial strains in pure form

Sub-surface soil sample was collected from oil contaminated sites of Pantnagar, Uttarakhand, India (29˚N latitude and 79˚E longitude). Using serial dilution technique, bacterial strains were isolated on King's B agar medium at 300C in 48 hours. Ten strains were isolated in pure form and characterized morphologically and biochemically. Bacterial strains were screened for plant growth promotory properties such as siderophore, phosphate solubilization and Indole Acetic Acid (IAA) production [10-12]. Further, bacterial strains were screened for biosurfactant production on Mineral Salt Medium.

Development of bacterial consortia

To develop consortia the strains were checked for compatibility by using agar diffusion technique on King's B solid medium. The developed consortium was again checked for compatibility in liquid media (50 ml MSM broth) in presence and absence of petrol oil. The absorbance was taken at periodic interval of 24 hours from 0 hours up to 120 hours in

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spectrophotometer (Beckman DU 340B model) and finally, based on growth pattern, consortia was prepared. Two strains named as RK3 and RK4 were selected for the present study on the basis of PGPR as well as biosurfactant property and on the ability to form consortia.

Molecular characterization of the Bacterial strains

The PCR product of the both bacterial strains was sequenced using 16 S rRNA sequencing. The sequences obtained were blasted using NCBI site and similarity coefficient was used to establish the genus and species based on the homology.

Screening of the isolated bacterial strains for biosurfactant production

Both the bacterial strains were tested for biosurfactant production on minimal salt medium, MSM (composition g/l $NaNO_3$ -2.5, K_2HPO_4 -1.0 , $MgSO_4$ (anhydrous) -0.5, KCl -0.1, $FeSO_4$ -0.01, $CaCl_2$ -0.1, Na_2HPO_4 -5.67, $MnSO_4$ -0.002, NH_4NO_3 -0.39, dextrose -30, pH 7.0; dextrose autoclaved separately), incubated at 30°C for 72 hours with shaking at 150 rpm. Biosurfactant produced by the bacterial strains was characterized by different methods mentioned below:

Foaming

Bacterial strains showing foaming in the medium as compared to negative controls were observed for reflected light which scattered like rainbow colour. Contents of the test tubes of minimal salt medium with the test strains (bacterial strains) showing foaming were subjected to centrifugation at 10,000 rpm for 20 minutes (4°C). Cell free supernatant (crude biosurfactant) was used for further experiments.

Surface tension

Cell free supernatant was subjected to surface tension determination using Du Nuoy Ring detachment method with Fischer Autotensiomat Model-21 [13].

Drop collapse test

Drop collapse test was performed in the 96 microwell polystyrene plates [14]. Thin coats of 10 W-40 oil was applied to each well and were equilibrated at 23°C for 24 h. Five microlitre of supernatant aliquot was deported into the center of each well. Beaded drop indicates negative results while spread drop which collapse indicates positive results for the presence of biosurfactant.

Emulsification index

2 ml of petrol engine oil hydrocarbons was taken in test tubes. They were then overlaid with 2 ml of crude biosurfactant. Height of oil, crude biosurfactant and total height of the column was recorded. The resulting mixture was vortexed for 2 minutes to enable proper mixing of petrol engine oil hydrocarbons and crude biosurfactant. Tubes were allowed to stand for 24 hours without any disturbance. After 24 hours, height of the emulsified layer was recorded to calculate emulsification index [15]. The equation used to determine the emulsion index E24 (%) is as follows:

$$E_{24}(\%) = \frac{H_1}{H_2} \times 100$$

H_1=the height of emulsion layer, H_2=the height of total solution

Where, 24 stands for emulsification taking place in 24 hours.

Characterization and detection of the Rhamnolipids

Detection of rhamnolipids by cetyl-trimethyl ammonium bromide-methylene blue test (ctab): For screening for rhamnolipid production the isolates were spot incoulated on the plates composed of the mineral salts medium with the addition of 200 µg/ml cetyl-trimethyl ammonium bromide (CTAB; Himedia), 5 µg/ml methylene blue, and 1.5% agar.

Quantification of rhamnolipids: Seventy two hours grown bacterial culture in Mineral salt medium with 150 rpm shaking on rotatory shaker was centrifuged at 10,000 rpm, 4°C temperature for 20 minutes. Approximately 40 ml of culture supernatant was acidified with 6M HCl to obtain a pH of 2.0 and kept overnight at room temperature. The extraction was performed twice with an equal volume of diethyl ether using a separating funnel and vigorous shaking for 60 minutes. The upper organic layer was collected and transferred to pre –weighed empty beakers. Diethyl ether was allowed to evaporate for 24 hrs and then dried in an oven at 60°C for 1 hr. Brown coloured surfactant obtained was transferred to dessicator to remove any traces of moisture and dissolved in 0.5 ml of deionised water. 900 µl of a solution containing 0.19% Orcinol (in 53% Sulphuric acid) was added to100 µl of each sample. For 30 minutes samples were heated at 80°C in a water bath and cooled at room temperature for 15 minutes and the absorbance were measured at 420. The rhamnolipid concentration were quantified from the standard L-rhamnose calibration curve between 0 and 50 µg/ml and the result were expressed as Rhamnose Equivalents (RE) (mg/ml) by multiplying rhamnose values by a coefficient of 3.4, obtained from the correlation of pure rhamnose/rhamnolipids [16,17].

Characterisation of rhamnolipids by infrared spectroscopy

For FTIR Spectroscopy, one milligram of rhamnolipids was grinded with 100 mg of KBr and percentage transmission was recorded using FTIR model Bruker, Vertex 70.

Experimental design and treatment

Withania somnifera used in the present study belongs to the family Solanaceae. *In situ* studies were conducted to study its interaction in petrol oil contaminated soil with surfactant producing PGPR strains and to assess the effect of contaminant on its medicinal properties. The pot experiment was conducted in poly house at room temperature for 90 days. The whole experiment was performed in presence of petrol oil and replicated thrice with suitable control.

Culture treatment in pots

Experimental soil was dried and sieved with 2 mm sieve and autoclaved for 1 hour at 121°C for three successive days. 1.5 kg of autoclaved soil was shipped to each pot and mixed with (30ml) of petrol oil. The mixture was allowed to stabilize and evaporate for 10 days. Fifty ml of 72 hours grown culture as well as consortia in MSM on a rotary shaker (150 rpm) having bacterial population approx. 1×107 cells of the test strains were applied to respective pots. The inoculated pots were allowed to stabilize for 7 days. The seeds of ashwagandha (*Withania somnifera)* variety Jawahar-20 obtained from MRDC (Medicinal plants Research and Development Centre), Pantnagar, and India were washed with 0.1% $HgCl_2$ followed by repeated washings with sterile distilled water (8-10 times). The treated seeds were sown in the pots after 7 days. Twenty seeds were sown in each pot just below the oil surfaces (2.5 cm).

Plant health parameter

The effect of inoculated strains and consortium of both the strains on plant health in petrol contaminated soil were monitored by taking the seedling germination percentage, plant height, root length, chlorophyll content and average yield per pot [18].

Test strain	Accession no	Siderophore production	IAA production	Phosphate solubilization
RK-3	HM771642	++	++	++
RK-4	HM771643	+	+	+

(+), moderate; (++), good; (-), negative results

Table 1: Plant growth promotory properties of the test strains.

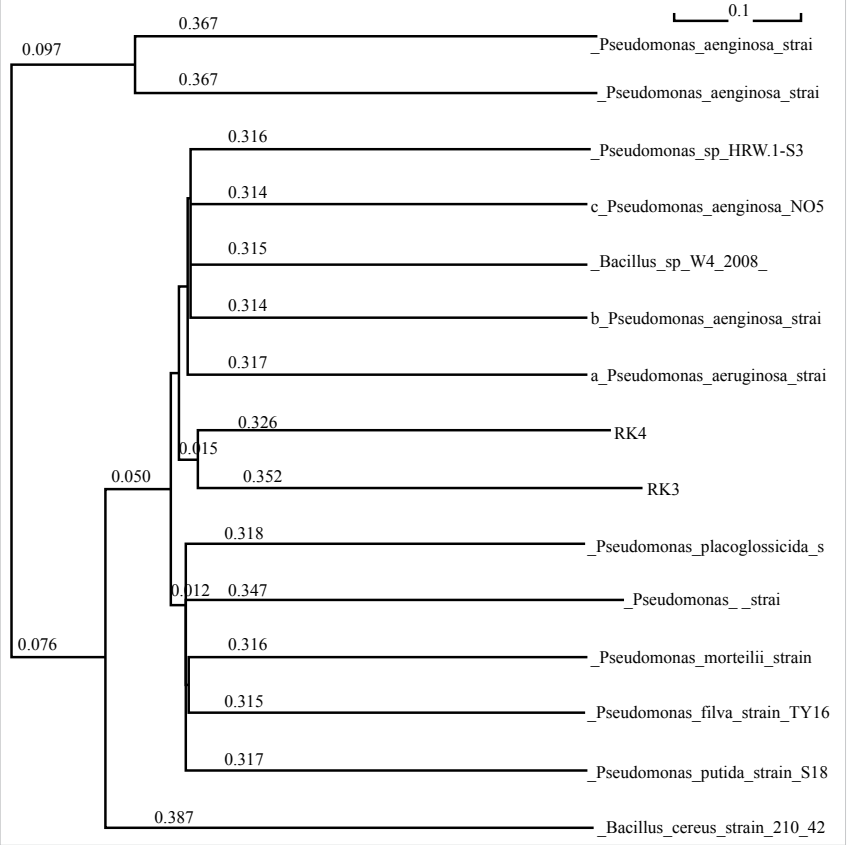

Figure 1: Phylogenectic tree of the test strains.

Preparation of extract to assess impact of hydrocarbons on medicinal properties

After 90 days plants were uprooted and roots were taken for preparation of extract. They were washed with distilled water thrice, surface sterilized with 0.1% mercuric chloride for 20 seconds, followed by repeated washings with sterile distilled water (3 times). Roots were then air dried for four weeks at room temperature and grounded to powder form, using an agate pestle and mortar.

Determination of Antioxidant Activities

DPPH assay

Free radical activity was determined using stable 1, 1-diphenyl-2-picrylhydrazyl (DDPH) [19]. Two hundred fifty microgram of extract mixed with 100 ml of absolute ethanol was prepared and 3 ml of this solution was mixed with 1 ml of 0.01 mM solution of DDPH prepared in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min in dark. The absorbance was measured at 517 nm in a spectrophotometer. Ethanol was used as a blank. The DPPH radical concentration was calculated using the following equation:

$$DDPH\ scavenging\ activity = \frac{Abs.of\ control - Abs.\ of\ sample}{Abs.of\ control} \times 100$$

Metal chelating activity

The chelating of ferrous ions by the extract and standards was estimated using method reported by Decker and Welch 1990 with slight modification [20]. Different amount (250 µg approx.) of extract were mixed in 1 ml of methanol and diluted with 3.7 ml of deionised water. The mixtures were shaken vigorously and left for reaction with $FeCl_2$ (2 mM, 0.1 ml) and ferrozine (5 mM, 0.2 ml) for 10 min. The absorbance was measured at 562 nm using uv-vis spectrophotometer (Visican, Indian). The chelating activity was calculated by the following equation:

Chelating activity (%)=[1-(At/A0)] X 100 where it is the absorbance of the sample and A0 is the absorbance of the control.

Reducing power activity

Reducing power was estimated according to the method of Oyaizu [21]. Root extracts were mixed with 2.5 ml of the phosphate buffer and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated at 50°C, followed by addition of 2.5 ml of 10% trichloroacetic acid to

the mixtures. The mixture was centrifuged at 650 rpm for 10 min. The upper layer (5ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride and the absorbance of the resultant solution were measured at 700 nm using vis-spectrophotometer (Visican).

Statistical analysis

Experimental results were analyzed by using one-way-analysis of variance (ANOVA). Statistical analysis Data were expressed as means ± S.D and differences were considered significant at $p<0.05$.

Results and Discussion

Isolation and characterization of the bacterial strains

Of the isolated ten bacterial strains RK3 and RK4 were the promising strains. Both the bacterial strains were Gram negative and showing green fluorescence on King's B agar and based on the 16S rRNA studies, isolated bacterial strains were found similar to Pseudomonas aeruginosa. Both the strains were found positive for phosphate solubilization, siderophore and indole acetic acid production (Table 1). Result obtained from compatibility test indicates that both strains have a great tendency to form consortia. Sequences of the both test strains RK3 and RK4 have been deposited in the NCBI database and accession number allotted to them are HM771642 and HM771643 (Figure 1).

Characterization of biosurfactant produced by the test strains

Both the test strains produced biosurfactant on Mineral Salt Medium (MSM) and produced foam with surfactants. Surfactant produced by the test strains reduced surface tension upto 29 dynes cm^{-1}. Both the strains were able to reduce surface tension (Table 2). The results of drop collapse test were positive for surfactant production as the drop spread and collapsed.

Detection and quantification of rhamnolipids

Cetyl-trimethyl Ammonium Bromide-methylene blue test blue dark blue colonies were from by the strains which indicates the production of rhamnolipids. Rhamnolipids consist of a polar head and non-polar tail group like synthetic surfactants and as a result they combine with cationic substances, like Cetyl-trimethyl Ammonium Bromide to form insoluble ion pairs in aqueous solution and precipitates as dark blue zones against a blue black ground [22]. The amount of rhamnolipids produced by the strains RK3 and RK4 was estimated using the Orcinol method which indicates production occurred during the stationary phase of growth.

Fourier transform infrared spectroscopy of extracted rhamnolipids

FTIR spectra of biosurfactants obtained from both the test strains are summarized in Figure 2a and 2b. On comparison, FTIR spectra of rhamnolipids produced by both bacterial strains have some peaks in common. One is hydrogen stretching (2778-2779), second is –OH stretching frequency at 2671-2694; third pH bonding between 2411-2413, fourth carbonyl stretching frequency 1734-1738, fifth CH deformation frequency 1456-1464,sixth is secondary amide

Sr. No.	Test strain	Surface tension in dynes cm^{-1} with 6 cm ring	Emulsification index (E_{24}) for petrol engine oil hydrocarbons
1	Standard	72.80	-
2	RK3	30.50	40.00
3	RK4	29.00	42.30

Table 2: Surface tension and emulsification index by crude surfactant after 72 hours.

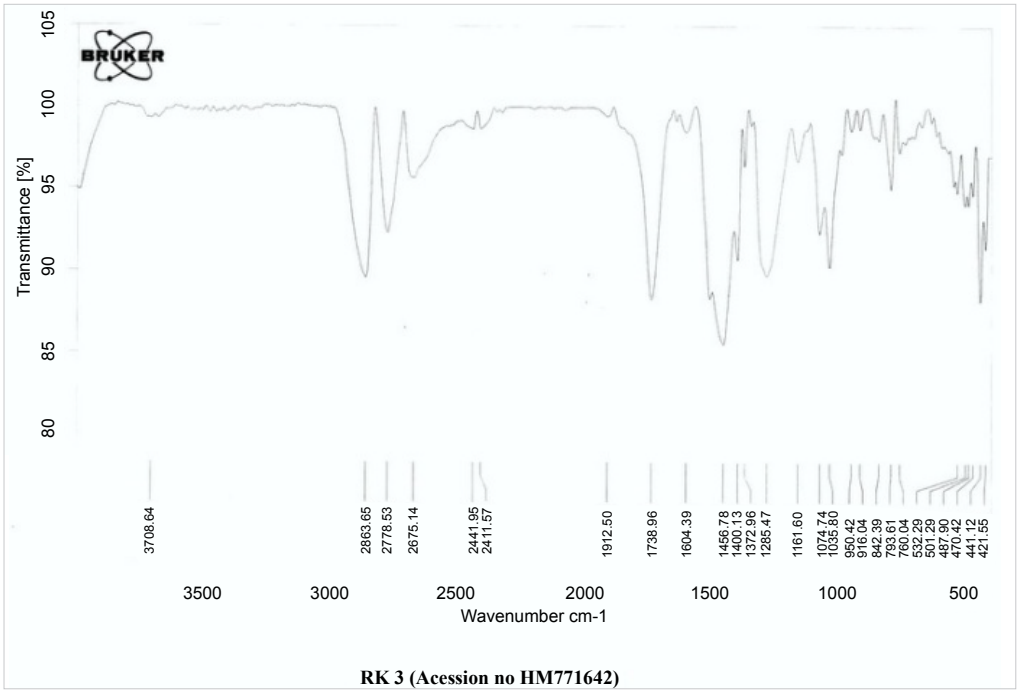

Figure 2a: FTIR Spectra of RK3.

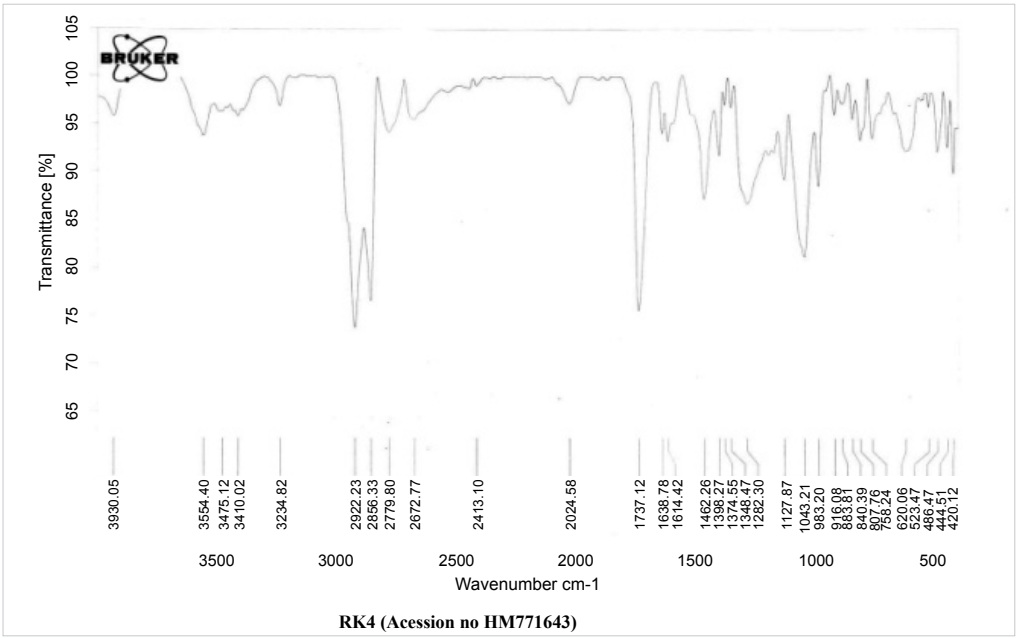

Figure 2b: FTIR Spectra of rk4.

Plant Health parameters		RK3	RK4	Consortium	C1	Control
Germination Percentage (%)	15 DAS	35 ± 0.5	26.5 ± 0.11	58.3 ± 0.5	18 ± 0.91	70 ± 0.66
	30 DAS	51.5 ± 1.5	50 ± 0.8	67 ± 1.2	38 ± 1.6	91.5 ± 0.97
Chlorophyll Content index	45 DAG	0.19 ± 0.05	0.14 ± 0.043	0.22 ± 0.043	0.04 ± 0.043	0.24±0.026
	90 DAG	0.24 ± 0.04	0.17 ± 0.052	0.28 ± 0.030	0.038 ± 0.027	0.33 ± 0.11
Plant height(cm) Day after Germination (DAG	30 DAG	1.42 ± 0.20	1.73 ± 0.20	2.4 ± 0.48	1.48 ± 0.21	6.03 ± 0.12
	60 DAG	4.076 ± 0.24	1.91 ± 0.06	7.25 ± 0.75	1.80 ± 0.30	8.38 ± 0.49
	90 DAG	7.21 ± 0.61	3.5 ± 0.5	9.63 ± 0.71	2.17 ± 0.59	11.62 ± 1.26
Root length(cm) Day after germination (DAG)	30 DAG	1.65 ± 0.32	1.4 ± 0.36	3.76 ± 0.55	1.25 ± 0.28	5.54 ± 0.45
	60 DAG	4.47 ± 0.88	1.71 ± 0.36	6.03 ± 0.25	1.26 ± 0.25	7.45 ± 1.05
	90 DAG	6.45 ± 0.63	2.83 ± 0.80	10.62 ± 1.02	1.64 ± 0.23	12.79 ± 0.44

RK3, *Withania somnifera* + culture RK 3 + Petrol oil+ soil ; RK4, *Withania somnifera* + culture RK 4 + Petrol oil +soil ; Consortium, *Withania somnifera* + consortium of RK3 and RK4 + Petrol oil +soil ; C1, *Withania somnifera* + No culture + Petrol oil+ soil ; Control, *Withania somnifera* + No culture+ No petrol oil+ soil.

Table 3: Health parameters of *Withania somnifera* in petrol engine oil contaminated soil Day after germination (DAG), Results are mean of three replicates with standard deviation (Means ± S.D, n=3) and differences were considered significant at $p<0.05$.

deformation in the range 1279-1285 and Seventh, Carbonyl stretching frequency.

Effect on germination: There was a delay in germination in all the treatments and the germination percentage was low in presence of petrol engine oil as compared to control. However better germination was observed in case treatment with bacterial consortium RK3 and RK4 as compared to individual treatment RK3 and RK4 (Table 3). The lower percentage of germination in C1 control pots can be attributed to the toxicity of petrol engine oil hydrocarbons which cause empident effect to the plants. Similar findings have been reported by Kulakow in 2000 where the legumes tested succumbed to hydrocarbons toxicity in absence of any growth promoting bacterial inoculums [18].

Effect on chlorophyll content: Chlorophyll content was again better in plant treated with consortium as compared to the control (Table 3). But chlorophyll content of RK4 treatment plant was lower than RK3 and this may due to amount of siderophore produced by the bacterial strain RK4 was less as compared to other test strain RK3 that might be the reason for low content of chlorophyll as iron deficiency inhibits both chloroplast development and chlorophyll biosynthesis under abiotic stress [23,24].

Effect on growth: With the passage of time all the inoculated plants showed increase in plant height. The increase plant height was less in pots treated with RK3 and RK4 but plant height was better in the pot treated with the consortium (Table 3). Root length was also found better in all the treatment while in case of C1, the root growth was much decelerated.

Effect on antioxidant activity: There was a variation in the antioxidant properties of the plants treated with different treatments (Table 4). Control pot and the pot treated with consortium showed decrease in absorbance of DPPH radical than RK3 and RK4 treated plants. This is due to the reaction inoculated plants between antioxidant molecules and DPPH radical, which results in the scavenging of the radical by hydrogen donation. Decrease in absorbance of the reaction mixture indicates higher free radical scavenging activity of control plant and consortium treated plant, whereas un-inoculated plant (C1) had poor DPPH activity.

Antioxidant Activity	RK3	RK4	Consortium	C1	Control
DPPH Activity(µg/ml)	80.96 ± 2.95	89.46 ± 2.15	79.06 ± 3.05	95.93 ± 1.2	81.26 ± 2.31
Metal chelating activity(µg/ml)	41.78 ±1.65	41.93 ± 2.07	39.32 ± 2.17	52.37 ± 1.48	37.94 ± 1.66
Reducing power activity(µg/ml)	62.16 ± 3.25	61.96 ± 1.95	68.23 ± 2.45	55.1 ± 1.01	71.5667 ± 2.28

RK3, *Withania somnifera* + culture RK 3 + Petrol oil+ soil ; RK4, *Withania somnifera* + culture RK 4 + Petrol oil +soil ; Consortium, *Withania somnifera* + consortium of RK3 and RK4 + Petrol oil +soil ; C1, *Withania somnifera* + No culture + Petrol oil+ soil ; Control, *Withania somnifera* + No culture+ No petrol oil+ soil

Table 4: Antioxidant Activity of *Withania somnifera* after 90 Days of treatment, Results are mean of three replicates with standard deviation (Means ± S.D, n = 3) and differences were considered significant at $p < 0.05$

Metal chelating capacity is important since it reduces the concentration of the catalyzing transition metal in lipid per oxidation. The metal chelating activity of consortium treated and control plant were nearly same. RK3 and RK4 showed low metal chelating activity then their consortium while treatment C1 showed very low metal chelating activity.

Increase in absorbance indicates increase in reducing power. Reducing activity of consortium treated plant and control plant is highest as compared to the RK3 and RK4 treatments plants. C1 plants showed very poor reducing activity.

Petrol oil has a toxic effect on the growth of the plants due to the hydrocarbons [25]. Petroleum oil spillage on soil generally decelerates soil productivity and plant growth and dilutes aeration by blocking air spaces between soil particles as a result creates a condition of an aerobiosis which cause root stress in plants and reduces leaf growth [24]. Deceleration of plant growth parameters such as plant height, germination, and biomass is due to toxic low molecular weight hydrocarbons compounds present in petroleum hydrocarbons [26]. They enter and transmit to cell membrane and reduced membrane integrity or cause death of the plant cell [27]. Many studies have revealed association between poor growth parameters (germination rate, chlorophyll content, growth rate) in hydrocarbon contaminated soil [28]. In this present study, without treatment pot showed very poor growth parameters and antioxidant activity and the treated pot with the bacterial strains and their consortium did not have a significant adverse effect. This is due to the biosurfactant produced by the bacterial strains. Biosurfactants behave as an interface between water-air. The hydrophobic part contributes towards pollution and attaches to it and the hydrophilic part forms a hydrogenic bond with water and defecates polluter detach from pollution level and thus cause it to remove [25]. This increases the rate of hydrocarbon dissolution and their utilization by microorganisms. Rhamnolipids are best studied biosurfactants and have been identified predominantly from Pseudomonas sps [29]. The strains (RK 3 and RK 4) produces rhamnolipids as well as showed plant growth promoting activity and has the capability to restore the petrol contaminated soil and enhance plant growth, but the present experiment showed that their capability increases when they are use in form of consortium [30].

Conclusion

Microbes isolated from petroleum hydrocarbons affected sites, which are more adapted to peculiar soil environment and can be commercial and effectively used for raising medicinal plants like *Withania sominifera* in hydrocarbons contaminated soils. The study was found to be beneficial as strains helped in plant growth promotion of *Withania somnifera* in petrol oil contaminated soil while surfactant property of the strains helped in lowering the toxicity of hydrocarbons to the plant. The growth parameters and the antioxidant activity of the plants inoculated consortium of the test strain RK3 and RK4 were approximate to the control plants where no petrol engine oil hydrocarbons were present while test strains (RK and RK 4) inoculated plants of *Withania somnifera* had low antioxidant activity but still better than the plants where no bacterial culture was inoculated. Hence further these strains can be used for restoration of petroleum hydrocarbons contaminated soils.

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