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Isolation and Characterization of Rhizospheric Bacterial Isolates from KAS Plateau and Study of their Plant Promoting Activities

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Abstract
Western Ghats (Sahyadri mountains) India, situated at the edge of deccan plateau are one of the global hotspots and possess extraordinary biotic diversity with endemism. The population density in this rhizospheric soil was ranging from 1.4×10^3 to 8.8×10^3 CFU/ml. In all, bacterial isolates were recovered from rhizosphere. Out of these, 22 isolates were selected for the study on the basis of phenotypic characteristics. Ten isolates showing Gram negative nature were further selected for plant growth promoting traits. As per Bergey's manual of determinative bacteriology, isolate TA1 and SD1 were found to be *Chromobacter* sp. Bacterial isolate TC4, SD2 and TC1, RA3 were found to be the members of Enterobacteriaceae. NA4 isolate was confirmed as *Eikenella* sp. and TC2 as *Zymomonas* sp. Five of these isolates were phosphate solubilizing. The concentration of phosphate solubilization was ranging from 15-42.85 mg/L. These studies revealed that all 10 isolates have potential to fix nitrogen. Two isolates possess siderophore activity. All of these isolates were able to carry out nitrification. None of these isolates showed indole acetic acid (IAA) production. This might be due to the fact that Kas plateau is mostly covered with flowering herbs and IAA mainly play important role in apical dominance. In all, TA1 and RA3 exhibited maximum plant growth promoting activities.

Keywords
Kas plateau, Western Ghat, phosphate solubilization, nitrogen fixation

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INTRODUCTION
Kas plateau of the Western Ghats represents the rare habitat for the various endemic plant species. Kas plateau is situated at 23 Km from Satara district, Maharashtra state. The Kas plateau is covered with red lateritic crusts. Hence, Kas plateau has arid environment throughout the year except the monsoon season (Annual precipitation 2000 mm mainly from

June to September). Monsoonal rain is the important parameter for the endemic and ephemeral flora. The vegetation is preliminarily herbaceous members of dominating families like Acanthaceae, Poaceae, Cyperaceae, Eriocaulaceae, Lentibulariaceae and Balsaminaceae. According to Bhattarai et al.,[1] topographic, hydrological and edapic variations are responsible for overall endemism is observed in both dicots and monocots. Microbial diversity studies are important in order to understand the microbial ecology in soil and other ecosystems [2]. Hence physical, chemical and biological properties of the soil, affecting the plant growth can be studied to get the clear understanding between edaphic factors and plant growth. The soil bacterial diversity of the plateau can be explored to find out the dominant species and their various plant growth promoting activities. Ecological niches are generally explored to get any novel bacterial strains. As a unique ecological niche, we are intended to find out the novel bacterial strain from the Kas plateau soil. Soil microbial communities remain some of the most difficult communities to characterize, because of their immense phenotypic and genotypic diversity [3]. Yet very little of the soil bacterial diversity is explored due to limitations on culture-based studies.[4] Plating studies are incomplete methods for estimating biodiversity, since less than 1% of the total bacterial population has proven to be cultivable on standard media. [3] Rhizospheric bacteria have important role in the growth of the plants as they carry out various plant promoting activities. These involve various direct and indirect mechanisms.[5] So along with the climatic specificities, edaphic factors play important role in the growth of particular flora. These include physical properties like horizonation, color, texture, structure, consistence etc., chemical properties like pH and cation exchange capacity. Similarly, we hypotheses that soil microflora of Kas plateau may have impact and critical role in the growth of the endemic and ephemeral plants. So, we isolated the soil bacterial diversity of the Kas plateau providing suitable and variable conditions. Here, we tried to mimic some natural conditions of Kas plateau. Isolation was followed by the characterization of the isolates using Bergey's Manual of Determinative Bacteriology. On the basis of the identification results we proceeded to study various plant promoting activities of the isolates. Present study was intended to analyze the various plant promoting activities. Further these bacterial strains can be bioprospected for their unique plant promoting activities and may have applications in biotechnology. Plant promoting rhizobacteria are heterogeneous group of bacteria that can be found in rhizosphere, attached to roots and in association with plants which can improve extent and quality of plant growth.[5] Various plant promoting activities of rhizospheric bacteria were studied. These include Ammonification, IAA production, Siderophore production, Phosphate solubilization, antifungal assay,[5] Nitrification,[6] Denitrification etc.

MATERIALS AND METHOD

Soil Sample Collection

The soil samples were collected on the 26th September 2016 from Kas plateau. It was a peak flowering season of the Kas plateau. Plateau was covered with shrubs and different types of the flowering at different places. Five different sites were chosen on the basis of the soil color, texture, and flowering. The rhizospheric soil was collected from 10 cm depth around the flowering mats. These all samples were labelled as A, B, C, D, E. These all samples were collected using sterile sickles and sterile polythene bags. Soil samples were then processed after 20 hrs. Until then samples were stored at 4^{o}C.

Isolation of bacterial strains

Soil samples were processed on next day at 9 am. Four different media were used for the isolation of the maximum diversity. These include Nutrient agar, Thioglycollate agar [7], R_2A [3], Soil extract agar medium [3]. Sterile saline was used for the serial dilution of the soil samples. 1 g of each soil sample was weighed and further serially diluted upto to the 10^{-6}. Dilutions ranging from the 10^{-2} to 10^{-6} for each soil sample were plated on the each medium. These all plates were incubated at 28^{o}C for 72 hrs. Forty-five morphologically distinct bacterial isolates that were observed then subcultured on to the nutrient agar for the further study. These all isolates were labelled as per medium, collection site and number of particular isolates viz., RA3, TA1, NA4, SD2 etc.

Characterization of the isolated bacterial strains

Amongst all isolates, 22 isolates were selected for the study on the basis of morphological distinctness. These 22 isolates were subjected to morphological characterization by Gram staining. Ten isolates

showing Gram negative nature were further selected for the study of plant promoting activities and identification using Bergey's Manual for Determinative Bacteriology. These include NC1, SD1, RA3, TC2, TA1, TC4b, TC1, SD2 and NA4. As per following Bergey's Manual for Determinative bacteriology, all of these cultures were subjected for the detection of the aerobic and anaerobic growth (under the group of Gram-negative rods and cocci). Based on these results further biochemical tests were carried out for all the ten isolates viz., glucose utilization, nitrate reduction, catalase, oxidase and indole production.

A) Glucose Fermentation: All the isolates were inoculated into 10mL of sterile nutrient broth containing 1% glucose and incubated at 28°C for 24 hrs. Both positive (*E.coli*) and negative controls were maintained [9].

B) Indole Production: All the isolates were inoculated into sterile tryptone water base. After 48 hrs of incubation Kovac's reagent was added to observe the cherry red colored ring at the top of the broth. Both positive (*E.coli*) and negative controls were maintained[9].

C) Nitrate Reduction: Some aerobic and facultative anaerobic microorganisms can utilize the nitrates as the terminal electron acceptor which is further converted to the nitrites. Some organisms possess further ability to reduce nitrites to the gaseous nitrogen. All the isolates were inoculated into sterile nitrate broth medium containing 0.1% potassium nitrate (KNO_3). After 48 hrs of incubation, nitrate production was detected by using Solution A (Sulphanilic acid) and Solution B (alpha naphthylamine). Immediate cherry red color after addition of the both solutions indicates nitrate reduction. Negative control was maintained.

D) Catalase test: Catalase test for all the ten isolates were performed using 3% hydrogen peroxide. Immediate effervescence of oxygen was observed for the positive test.[9]

E) Oxidase test: Oxidase test for all the ten isolates were performed using 1% oxidase reagent (N,N,N,N-tetramethylparaphenyldiamine, TMPD). Immediate violet coloration by the streaked colony was considered as the positive test. [9]

F) Methyl Red Test, Vogus Prousker's Test, Citrate Utilization Test: Based on the results of primary characterization, MR, VP and Citrate Utilization Test were carried out. For MR and VP test, MR-VP broth was used. Methyl Red test detects the acid production by the organism at pH 4. Bacterial isolates were inoculated into MR-VP broth and incubated at 28^{0}C for 24 hrs. Methyl red indicator was used to detect the acid production. VP test was carried out to detect the presence of non-acidic end products such as acetyl methyl carbinol. Bacterial isolates were inoculated into MR-VP broth and incubated at 28^{0}C for 24 hrs. Acetyl methyl carbinol was detected using Barritt's Reagent.[9] Citrate utilization test was carried out to check the ability of organism to utilize citrate as sole carbon utilization. Simmon's citrate agar slants were used for the test. Change to blue coloration was considered as positive test. [9]

Study of Plant Promoting Activities

A) Phosphate Solubilization: All the selected isolates were subjected to the screening for phosphate solubilization on Pikovskaya's Agar medium [5,10], these plates were incubated at 28° C for extended incubation upto 7 days. Further, the quantitative estimation of the positive isolates was carried out using method mentioned by Karpagam and Nagalakshmi.[10] Each positive isolate was inoculated into 100 ml / L of Pikovskaya's broth (0.3% tricalcium phosphate) incubated for 7 days at 28°C on rotary shaker. After incubation broths were centrifuged at 10,000rpm for 30min. The solubilized phosphorous was determined using spectrophotometry at 410nm using standard potassium dihydrogen phosphate.

B) Denitrification Test: All the isolates were inoculated into sterile nitrate broth medium containing 0.1% KNO_3. After 48 hrs of incubation nitrate production was detected by using solution A (Sulphanilic acid) and solution B (alpha naphthylamine). Immediate cherry red color after addition of the both solutions indicates nitrate reduction. Negative control was maintained. [9]

C) IAA Production: Indole acetic acid production was detected by using Salkowaski's method.[5,11] All the isolates were inoculated in nutrient broth supplemented with the 0.1g/L tryptophan and incubated at 28° C for 48 hrs. All the tubes were centrifuged at 3000 rpm for 30 minutes. The IAA production was determined using orthophosphoric acid (2 drops in 2 ml supernatant+ 4 ml Salkowaski's reagent).

D) Nitrogen Fixation: Nitrogen fixation ability of the isolates was tested using nitrogen free semisolid

malate medium containing BTB indicator [12]. The cultures were incubated at 28C for 48 hrs. The presence of the growth along with color change was considered as the positive test for the nitrogen fixation. Further, the results were confirmed by the growth of the isolates on the Ashby's agar (nitrogen free medium).[13]

E) Siderophore Production: Chrome Azurol S medium described by the Schwyn and Neilands.[16] was used to detect the ability of the isolates to produce the siderophore. All the test cultures were spot inoculated onto the plates. These medium plates were incubated for prolonged time. The production of yellow to brown halo around the growth was considered as the positive test. [5]

F) Gibberellin Production: Gibberellin production was detected by the protocol mentioned by the Pandya and Desai (2014). [14] All isolates were grown in nutrient broth for 48 hrs. After incubation the broth was centrifuged at 10000 rpm for 20 minutes. Supernatant was transferred to fresh tubes and pH was adjusted to 2.5 using 3.75N HCl. Solvent extraction was carried out using ethyl acetate. Amount of gibberellin in ethyl acetate was measured by UV-V spectrophotometer at 254 nm against standard gibberellin. Further, gibberellin production was confirmed by thin layer chromatograph [15] using silica gel papers. Isopropanol: Ammonia: Water (10:1:1) solvent system was used as mobile phase. The plates after drying were sprayed with 50 mg $FeCl_3$ and heated in oven at 80°C for 10 minutes. Then plates were observed under UV light for the black spot of gibberellins [16] . The figure shown below displays the assembly of extraction of gibberellin.

G) Ammonia Production (Ammonification): Ammonification is one of the important phases in nitrogen cycle. This process involves the degradation of nitrogenous biopolymers and subsequent release of ammonia. Ammonification is initiated by excretion of extracellular proteolytic enzymes that is produced by soil microflora. Enzymes produced by these soil microorganisms hydrolyse the proteins of plant and animal origin into their constituent amino acids. The amino acids subsequently enzymatically deaminated, with the release of ammonia. Freshly grown bacterial isolates were inoculated into 10ml peptone water in each tube and incubated for 48-72 h at 28°C. Nessler's reagent was used for the detection of ammonia. Brown to yellow color was positive test for ammonia production. [9]

H) Nitrification: Nitrification is carried out by soil bacteria into two steps. First, in which ammonia produced by ammonification is further oxidized to nitrites. Second, these produced nitrites are further oxidized to nitrates. *Nitrosomonas* and *Nitrobacter* play important role in nitrification process. Nitrates released in the soil are highly soluble and assimilated by terrestrial plants and microorganisms for the biosynthesis of cellular proteins. [9]

Determination of nitrite production: Ammonium sulfate broth was inoculated with bacterial isolates and incubated at 28°C for 48 h. Presence of nitrites in broth was detected by using Trommsdorf's reagent. Blue-Black color indicates the presence of nitrite. [6]

Determination of nitrate production: Nitrite broth was inoculated with bacterial isolates and incubated at 28° C for 48h. Presence of nitrates in broth was detected by diphenylamine amine reagent and sulfuric acid. Deep blue color indicates the presence of nitrates. [6]

RESULTS

Isolation of bacteria from rhizosphere soil of Kas plateau: Bacterial colonies with Different colors (orange yellow, pale green etc.) and shapes were obtained from five different soil samples. In all, 22 isolates were recovered from these soil samples. Out of these, 10 isolates were found to be Gram negative

rods, 10 were Gram positive rods and 02 were Gram positive cocci. The details are shown in table 1.

Table 1. Colony characters of the isolates

Isolates	Size (mm)	Shape	Color	Margin	Elevation	Opacity	Consistency	Gram nature*
NC1	2	Circular	Off white pigmented	Entire	Low convex	Opaque	Dry	GN rods
SA6	2	Irregular	Orange	Undulated	Effuse	Opaque	Moist	GP rods
SA5	3	Circular	White	Erose	Low convex	Opaque	Moist	GP rods in chain
RC1	3	Circular	White	Entire	Raised	Opaque	Moist	GP rods, endospore
SD1	2	Circular	White	Entire	Raised	Translucent	Moist	GN short rods
RA3	3	Circular	Pale orange	Entire	Effuse	Translucent	Dry	GN rods
RB4	2	Circular	White	Entire	Effuse	Opaque	Moist	GP rods
TC2	2	Circular	Cream	Entire	Raised	translucent	Moist	GN rods
NA6	3	Irregular	Yellow	Undulated	Pulvinated	Opaque	Moist	GP rods, endospore
TA1	2	Circular	Dark yellow	Entire	Effuse	Opaque	Moist	GN short rods
TC4a	1	Circular	White	Entire	Effuse	Opaque	Moist	GP cocci
C3	3	Circular	Off white	Entire	Pulvinated	Opaque	Moist	GP rods
TC4b	<1	Punctiform	White	Entire	Raised	Opaque	Moist	GN rods
SD1	<1	Circular	White	Entire	Pulvinated	Opaque	Dry	GN rods
RA1	<1	Circular	White	Entire	Effuse	Opaque	Dry	GN short rods
TC1	1	Circular	Pale green	Entire	Effuse	Opaque	Moist	GN short rods
SC2a	1	Circular	white	Entire	Raised	Opaque	Moist	GP short rods
NC2	2	Circular	Pale yelllow	Entire	pulvinated	Translucent	Dry	GP cocci
NA4	3	Circular	Pale yellow	Entire	Effuse	Translucent	Moist	GN short rods
SD2	4	Circular	white	Entire	Pulvinated	Translucent	-	GN short rods
RB1	2	Irregular	white	Entire	translucent	Opaque	Moist	GP rods
NA3	1	Circular	white	Entire	raised	Opaque	Moist	GP rods

***GP, Gram Positive; GN, Gram Negative**

Characterization of Isolates

Various biochemical tests were performed by using Bergey's manual of Determinative Bacteriology. [17] All the isolates were capable to grow under aerobic and anaerobic conditions. Isolate TA1, SD2, TC4b, SD1, TC1 were able to produce acid and gas in peptone water base containing glucose medium. None of the isolates showed IAA production. Except TC2 and NA4 all others were capable to reduce nitrate. All 10 isolates were catalase and seven isolates were oxidase test positive. Results are showed in Table no: 2 and fig A, B.C.

Table 2: Biochemical Characterization

Isolates	Aerobic	Anaerobic	Glucose fermentation	Indole production	Nitrate reduction	Catalase test	Oxidase test
TA1	+	+	Acid &Gas	-	+	+	+
TC2	+	+	-	-	-	+	+
SD2	+	+	Acid &Gas	-	+	+	-
TC4b	+	+	Acid &Gas	-	+	+	-
RA3	+	+	-	-	+	+	+
NC1	+	+	-	-	+	+	+
SD1	+	+	Acid &Gas	-	+	+	+
NC2	+	+	-	-	+	+	+
NA4	+	+	-	-	-	+	+
TC1	+	+	Acid &Gas	-	+	+	-

Fig 1: **Biochemical characterization of the isolates**. **A) Glucose fermentation; B) Indole production; C) Nitrate reduction**

As per Bergey's Manual of Determinative Bacteriology (9th Edition) isolate TA1 and SD1 were found to be *Chromobacter* sp. Bacterial isolates TC4b, SD2 and TC1 were found to be members of family Enterobacteriaceae. Among these SD2 was found to be either *Enterobacter* sp, *Erwinia* sp, or *Klebsiella* sp. While TC4b was found to be either *Citrobacter* sp., *Salmonella* sp. or *Enterobacter* sp. NA4 was probably confirmed as *Eikenella* sp. and TC2 was confirmed as *Zymomonas* sp. However, further biochemical tests should be performed for to confirm the identification.

Plant growth promoting traits of test isolates

Various plant growth promoting traits were studied and it was found that all isolates have ability to fix Nitrogen. Qualitative and quantitative analysis of phosphate solubilizing activity was done and isolate TA1,TC2 ,SD2 ,SD1 ,NC2 ,TC1 showed larger zones which were selected for quantitative study (Fig. 2A). In quantitative study isolate SD1 was recorded to possess highest (42.85mg/L) concentration to solubilize phosphorous (Table 4). None of our isolate showed gibberellin production. All isolates shows ammonia production as tested by Nessler's reagent (Fig. 2D). Presence of nitrites in the broth indicated by blue colour, all isolates were capable to show nitrites (Fig. 2C). Isolate RA3 and NA4 was found to be siderophore producing bacterium, the presence of yellow halo zone around colony on CAS medium indicates positive test (Fig. 2 B). All the results of PGPR traits are shown in in Table 3.

Table 3: Plant growth promoting traits

Isolates	Phosphate solubilizing activity	Siderophore production	Nitrogen Fixation	Nitrification	Denitrification	Ammonification	Gibberellin production
TA1	+	-	+	+	+	+	-
TC2	+	-	+	+	+	+	-
SD2	+	-	+	+	+	+	-
TC4b	-	-	+	+	+	+	-
RA3	-	+	+	+	+	+	-
NC1	-	-	-	+	+	+	-
SD1	+	-	+	+	+	+	-
NC2	+	-	+	+	+	+	-
NA4	-	+	-	+	+	+	-
TC1	+	-	+	+	+	+	-

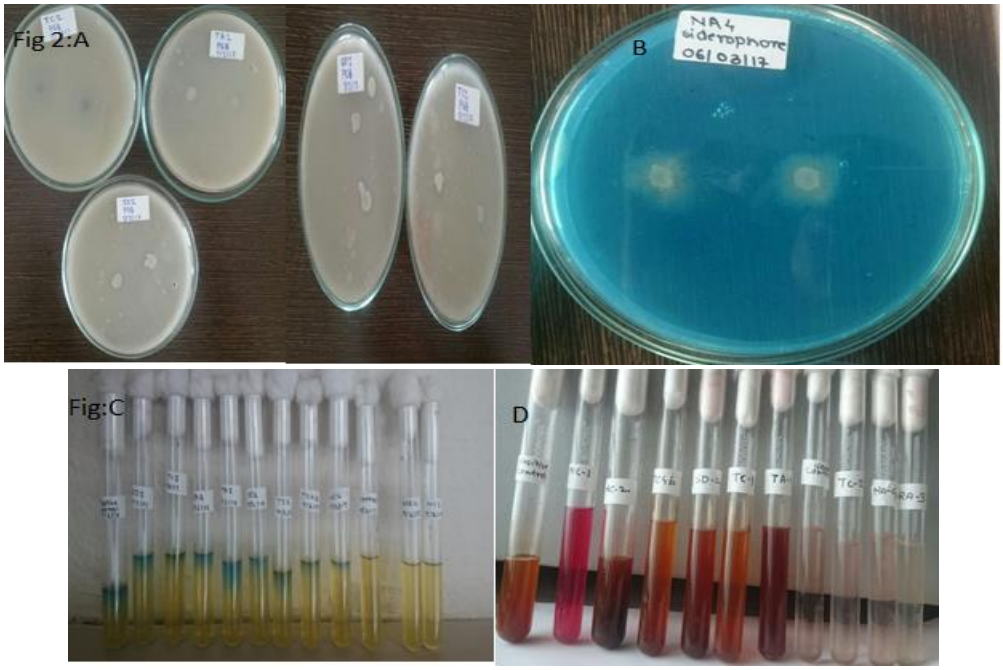

Fig 2: Plant growth promoting traits of rhizospheric bacterial isolates. A) Qualitative analysis of PSB (zone of clearance around colony); B) Siderophore production (Yellow halo zone around colony on CAS medium); C) Nitrification test (Presence of Blue colour); D) Denitrification test (Presence of cherry red colour)

Table 4: Phosphate solubilization quantification

Isolate	OD at 410nm	Concentration of solubilized phosphate mg/L
TA1	0.011	15.71
TC2	0.021	30.0
SD1	0.030	42.85
SD2	0.023	32.85
TC1	0.014	20.0

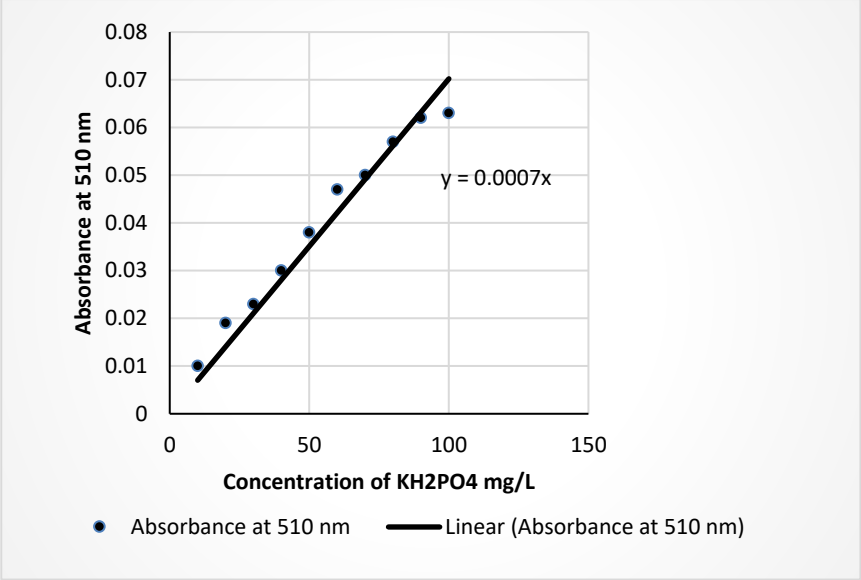

Fig 3: Standard graph of potassium dihydrogen Phosphate

DISCUSSION

As rhizospheric soil of plant is rich in various nutrient, in our studies all our isolates were capable to fix nitrogen. [19,6] However, in our studies RA3 and NA4 shows siderophore production, which influence the plant growth indirectly. Considerable fact is that, some of the isolates showed good role in the nitrogen cycle. Some of them are nitrogen fixers, some are nitrifying bacteria converting ammonia to nitrites are further to the nitrates. [5] Also some of these isolates showed denitrification ability reducing nitrates again to nitrites. Some release ammonia from organic matter. All these properties are important in the growth of the plant at the same time equally important for the recycling of the nitrogen through the ecosystem. [18] In the present studies, five isolates were showing phosphate solubilization activity. These were further investigated by quantitative method and phosphate solubilization concentration was found to be from 15.71 to 42.85 mg /L and it was maximum. [10]

Remarkably none of the isolate produce gibberellin as per our protocol of extraction and detection. As less literature is available for the gibberellin production from bacteria [about media, precursor etc), we consider some precursors should be incorporated in the medium for the gibberellin for the gibberellin production. Similarly, none of the isolates showed IAA production. This might be due to fact that Kas plateau is mostly covered with flowery herbs. IAA mainly play important role in the apical dominance i.e foot and shoot development. The investigated Kas soil was found to be rich in microbial flora. Wide diversity was found after the isolation of soil rhizospheric bacteria. The isolated strains also exhibit some remarkable plant promoting activities.

CONCLUSION

The investigated Kas soil was found to be rich in microbial flora. Wide diversity was found after the isolation of soil rhizospheric bacteria. The isolated strains also exhibit some remarkable plant promoting activities. Isolated rhizospheric bacteria exhibits good activity in nitrogen cycle. Isolate TA1 *Chromobacterium* sp. exhibits maximum rhizospheric activity including phosphate solubilization, nitrogen fixation and nitrification. Isolate RA3 also exhibits good rhizospheric activity with siderophore production. Kas soil can be exploited as good source of the microflora for bioprospecting purposes. Remaining isolates that could not be identified might possess some novel characters.

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