ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF POTENT PLANT GROWTH PROMOTING RHIZOBACTERIA FROM *ASPARAGUS RACEMOSUS*

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Abstract

Present study was aimed to assess plant growth promoting activity of rhizobacteria isolated from rhizospheric soil of Asparagus racemosus. Thirty rhizobacteria were isolated and tested for Plant Growth Promoting Activity, with six of them being employed in further research. Functional plant growth promoting traits such as phosphorus solubilization, zinc and potassium solubilization, indole-3-acetic acid (IAA) production, siderophore production, and growth on N-free media were used to characterize the isolates. Phosphate solubilization was much higher in isolates A and B (84.24 ±0.01 and 86.16 ±0.02 µg/ml respectively) and IAA production (90.11±0.1 and 253.45±0.01 respectively). Both isolates were capable of producing siderophore.

Three of the six isolates were potassium solubilizers, two were zinc solubilizers, and three demonstrated exopolysaccharide production. 16S rRNA gene sequence analysis was used to identify isolates with the highest PGPR performance. Overall potent isolates were identified as Exiguobacterium acetylicum strain RGK and Enterobacter mori strain RGK1 respectively and deposited in GeneBank under accession number ***OL771442 and OL656822*** *respectively. These strains could be used as an PGPR inoculant having multiple PGP-traits for plant growth promotion.*

Key words: *PGPR, Plant Growth Promoting Traits, Exiguobacterium acetylicum RGK , Enterobacter mori RGK1, Asparagus racemosus.*

1. Introduction

Soil contains, many kinds of different microorganisms such as bacteria, fungi, actinomycetes, and algae which contributes in improvement of overall quality and health of the soil. A source of microbial activity can be found in the rhizosphere, which receives nutrition from root secretions. Isolates belonging to other genera, such as *Azotobacter*, *Arthobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Serratia*, and *Azospirillum*, have also been shown to have PGPR (plant growth-promoting rhizobacteria) activity [1-2]. PGPR has the potential to stimulate plant productivity in a variety of different ways, both directly and indirectly. The direct mechanism involved capability to fix nitrogen, synthesis of siderophores and phytohormones, solubilization of phosphate, and the biological regulation of diseased plants [3]. Plant-associated bacteria may provide an indirect benefit to plants by deterring the progress or interaction of plant pathogenic organisms through various mechanisms (such as rivalries for nutrition and space, antibiosis, formation of hydrolytic enzymes, and suppression of pathogen-

produced enzymes or toxins). In addition to this, plant-associated bacteria may induce plant defense mechanisms, which may also benefit plants [4]. PGPR, interact with plants and other microbes that can be either antagonistic or synergistic [5].

PGPR are useful to plants, as they also play a crucial role in sustaining the equilibrium of the environment. In recent years, PGPR has been extremely prevalently used as soil inoculants in environmentally friendly agriculture because they have a smaller negative influence on the surrounding environment and produced the highest possible crop yield [6]. According to [7], PGPR is a constituent of the defensive microflora. They are beneficial to plants because they improve root activities, prevent disease, and speed up growth and development. PGPR also can potentially break down pesticides like endosulfan [8]. In addition to this, they have antifungal properties [9]. According to reports, they play a significant part in the production of secondary metabolites in plants [10]. The effects of PGPR on the phytoconstituents of medicinal plants are also documented [11].

Native medicinal shrubs of the genus *Asparagus* are members of the family Liliaceae and are valued for the therapeutic benefits of their stems, leaves and roots. Around the globe, around 300 different species belong to the genus *Asparagus* [12]. Shatavari is the generic term for the plant that bears the scientific name *Asparagus racemosus* wild. This plant has a long history of usage as a female reproductive tonic because of its ability to protect the health of mothers and the developing fetus and stimulate increased lactation in breastfeeding women [13]. *Asparagus racemosus* wild possesses curative properties that can be applied to treat a diverse range of diseases. According to the Ayurvedic literature (the database of Indian traditional remedies), it is a potent substance that can boost memory and intelligence and retain physical vigor and vitality. Additionally, the plant can be exploited to treat a variety of skin problems, wounds, and a

demulcent to treat dyspepsia [14]. The total phenol and flavonoid content were maximum in the plants from organic manure-treated soil, according to research by [15], with the roots of *Asparagus racemosus* grown under organic manures-cow dung, compost, and vermicompost without using mineral or chemical fertilizer. According to research by [16], PGPR can inhibit fungal infections that reduce asparagus productivity.

The current investigation demonstrates that inoculation of PGPR is an important agricultural approach that plays a significant role in protecting crops and promoting plant development in control of the diseases. As these isolates can tolerate high salt concentrations, they can be used as a biofertilizer in saline soil. They provide an option in place of conventional agricultural practices that rely on synthetic fertilizers, antibiotics, herbicides and insecticides.

2. Material and method :

2.1 Isolation of PGPR from soil:

Soil samples (rhizospheric area of *A. racemosus)* were collected from different locations in Kolhapur and Satara district. Isolation of PGPR was carried out, for that 1 g of soil was added in 100 ml sterile nutrient broth for enrichment in 250 ml Erlenmeyer flasks separately. These flasks were kept on a rotary shaker for 24 hrs (at 120 rpm) at 30 °C. Then a serial dilution technique was used for bacterial isolation and a 0.1 ml aliquot of 10^{-5} to 10^{-8} dilution was spread on sterile nutrient agar plate and plates were incubated at 30 °C for 24 hrs.

2.2 Screening for Plant Growth-Promoting Activities

2.2.1 Phosphate Solubilization

To assess their phosphate solubilization potential, all bacterial isolates were streaked on Pikovskaya's agar plates [17]. After incubation, the transparent zone around the growth suggested that inorganic phosphate had been solubilized. Bacteria growing in Pikovskaya's broth were quantified, with a sterile uninoculated medium serving as a control. The culture was collected after 48 hours by centrifugation at 6000 rpm for 15 minutes, and the amount of soluble phosphate in the supernatant was determined utilizing the [18]. The quantity of soluble phosphate was determined using the KH_2PO_4 standard curve.

2.2.2 Production of indole-3-acetic acid

Culturing the PGPR in yeast extract-mannitol-mineral salts broth enriched with various concentrations of tryptophan, at 28±1˚C with constant shaking and it was used to quantify IAA production. Further, 5 ml of cultures were centrifuged for 15 minutes at 10,000 rpm at 4±1˚C after 48 hours, and the supernatant was extracted [19]. Two drops of orthophosphoric acid and 4 ml of Salkowski reagent were added to the supernatant (2 ml) (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). IAA production is signified by the appearance of the cherry red color. A UV–Vis spectrophotometer was used to assess color at 540 nm. The concentration of IAA was determined from a standard curve of IAA (50–300 µg/ml).

2.2.3 Ammonia Production

A freshly grown culture of PGPR was inoculated in 30 ml of peptone water. The mixture was then placed in an incubator at 30˚C for 48 hours. Following by the completion of the bacterial growth, 0.3 ml of Nessler's reagent was applied to each flask. The appearance of a color ranging from brown to yellow is indicative of a successful assay for the generation of ammonia [20].

2.2.4 Siderophore Production

The chrome azurol S agar (CAS) was used to test the siderophore synthesis of isolates [21]. All isolates were inoculated on chrome azurol S agar plates and incubated for 3 days at 30°C. The appearance of a yellow to orange halo zone around the colony after the incubation time was regarded as positive for siderophore synthesis.

2.2.5 Hydrogen Cyanide Production

Using King's B medium, the isolates were tested for cyanide formation [22]. Each bacterial isolate was placed on King's B agar plates ammended with 1% glycine. The Petri plates were coated with parafilm and incubated at 30°C with a covering containing a piece of filter paper saturated in 1% picric acid and wet with a few drops of 10% $NaCO_3$ [23]. Without inoculation, control plates were made. HCN generation was claimed to be facilitated by a shift in filter paper color from yellow to brown.

2.2.6 Exopolysaccharide Production

According to Nicolaus and team (1999), the isolate's production of exopolysaccharides was evaluated qualitatively [24]. Bacterial strains were cultivated in 250 mL Erlenmeyer flasks at 30°C for 48 hrs under shaking conditions in 100 ml medium supplemented with 1g of yeast extract, 0.75g casamino acids, 0.3g trisodium citrate, 0.2g KCl, 2 g $MgSO_4.7H_2O$, 0.036 mg $MnCl_2.4H_2O$, 5 g $FeSO_4.7H_2O$ (120 rpm). The supernatant was extracted by centrifuging for 15 minutes at 4°C at 8000 rpm. The development of a precipitate was deemed positive for the synthesis of exopolysaccharides after adding cold 100% ethanol dropwise under agitation [25].

2.2.7 Potassium solubilization

Potassium solubilizing isolates were inoculated in a modified Alexandrov's medium (Glucose- 5 g; Magnesium sulfate- 0.5 g; Ferric chloride- 0.005 g; Calcium carbonate- 0.1 g; Tri-calcium phosphate- 2 g; Potassium aluminosilicate- 2 g; agar 15-20 g; Double distilled water- 1000 ml) The test organisms were seeded on the media and incubated for 48-72 hours at 28˚C. The colony's color variation and the diameter of the zone around it were both measured [26].

. 2.2.8 Zinc solubilization

The isolates were spot inoculated on an agar medium having 0.1% insoluble zinc compounds, such as ZnO. This media containing plates with test microorganisms were incubated at 30°C for 48 hours. Further, the clearing zone diameters around the colonies were evaluated [27].

2.2.9 Salt tolerance

The isolated plant growth-promoting bacteria were used to check their intrinsic resistance to salt stress. For this purpose, the isolates were grown in flask containing nutrient broth supplemented with various concentrations of NaCl (1-7%). The flasks were incubated at 30 °C for 48 h and after the incubation period, growth in NaCl-supplemented medium was observed [28].

2.3 Biochemical Characterization and Identification of isolates

A carbohydrate utilization test kit (KB 009, Hi-Media) was used to determine the PGPR's capability to consume various carbs. 16S rRNA gene sequence analysis was used to identify isolates with the highest PGPR performance. Employing the neighbor-joining approach, the

evolutionary history was determined. MEGA X was used for evolutionary analysis [29]. Under the accession codes OL771442 and OL656822, the partial 16S rRNA gene sequences were registered in the Gene Bank database.

2.4 Statistical analysis

The data are reported as means ± SD (standard deviation) for three replicates. The results were compared by analysis of variance (ANOVA) according to Tukey comparison test ($p < 0.05$) using the graph pad software.

3. Results

3.1 Isolation of rhizobacterial strains PGPR

PGPR strains were isolated from soil attached to *Asparagus* roots employing the culture-dependent standard plate method. Twenty rhizobacterial isolates were chosen based on distinct colony morphologies and biochemical assays. Two PGPR (A and B) isolates with the highest plant growth promotion activity were preferred for physiological and biochemical investigation among the 20 isolates.

3.2 Phosphate solubilization

Phosphate solubilization was tested on all isolates. In Pikovskaya's agar plates, six isolates displayed a distinct zone, but the diameter of the zone was significant in (A and B) isolates. In a continuous culture medium, Quantitative phosphate solubilization was carried out for 48 hrs in continuous culture medium. After 48 hours of incubation, A and B had the highest phosphate solubilization of 84.24 ±0.01 and 86.16 ±0.02 µg/ml. Data are shown as mean ± SD of three replicates. (Table 1 figure 1,2)

3.3 IAA production

Rhizobacterial strains were examined for IAA quantification in tryptophan levels of 25, 50, 150, 200 and 250 µg/ml concentrations. The colorimetric investigations revealed that distinctive *Rhizobia* isolates differed substantially in their ability to produce IAA in the broth; isolates A and B produced the most IAA (Table 1, Figure 2).

3.4 Siderophore, Ammonia, Hydrogen Cyanide Production

Six isolates can produce siderophores on CAS agar medium, as illustrated in figure 3. A and B generate ammonia and hydrogen cyanide, respectively.

3.5 Exopolysaccharide Production

After 72 hours, isolates were able to produce exopolysaccharides in the minimal medium. A and B were two of the six isolates that produced exopolysaccharides. Data represents in (Table 2)

3.6 Potassium and Zinc solubilization

Potassium releasing capacity was found in A and B isolates. The colour of the pH indicator changes as potassium was solubilized, and the resulting solubilization zone was recorded. After 72 hours of incubation at 28±2˚C, a range of diameter zone 20 mm to 30 mm was noted. The zinc solubilizing isolates were examined for effectiveness on TRIS minimal medium enriched with zinc source ZnO. The maximal solubilization zone of A and B was 18 mm and 22 mm. As a result, both isolates were capable of solubilizing potassium and zinc. Data presented in (Figure 4., Table 2)

3.7 Salt tolerance

In the presence of NaCl, six out of twenty bacteria showed a 3 percent salt tolerance capacity. A, on the other hand, could withstand up to a 5% salt concentration, whereas B could tolerate up to a 7% salt concentration.

3.8 Biochemical Characterization and Identification of isolates

The most efficient plant growth-promoting rhizobacterial isolates were A (*Exiguobacterium acetylicum* strain RGK) and B (*Enterobacter mori* strain RGK1) (Table 3). summarizes the biochemical profile of the isolates. 16S rRNA sequencing analysis identified the isolates as *Exiguobacterium acetylicum* strain RGK and *Enterobacter mori* strain RGK1. (Figure 5)shows the evolutionary tree of both the organisms.

4. Discussion

Plant symbiosis with rhizospheric microorganisms is an essential and critical component of environmentally friendly and efficient agriculture systems. Many bacteria found in the rhizosphere (the area around plant roots) can help plants thrives. Phosphorus (P) is the second most important macronutrient after nitrogen (N), and it plays an important function in plant growth and productivity. Due to insoluble forms of phosphorus, even in phosphorus-rich soil, the majority of the P is inaccessible to the plants [30]. *Pseudomonas, Bacillus, Enterobacter*, and endosymbiotic *Rhizobium* strains have been found to be highly efficient P-solubilizers in soil microbial flora. We observed that our two PGPR strains, *Exiguobacterium acetylicum* strain

RGK and *Enterobacter mori* strain RGK1, had distinct P-solubilization zones in the current study.

Plant hormones have a significant function in regulating plant growth and development. In numerous herbaceous plants, PGPR generating IAA in the rhizosphere soil plays an important role in growing the number of root tips and root surface area [31]. Both rhizobacterial strains were found to generate IAA in the range of 90 to 253 μg/mL. in the current study. Ghosh et al. (2013) reported that increasing L-tryptophan concentration increased symbiotic growth and IAA production, but further elevated tryptophan concentration decreased IAA production [32]. Ammonia, hydrogen cyanide, exopolysaccharide synthesis, phosphate solubilization, and IAA formation were also observed in both strains. PGPR converted organic nitrogen residues into soil organic matter, such as ammonia nitrifiers. Through ammonification, this PGPR releases ammonia [33]. Hydrogen cyanide is a secondary metabolite that can be used to manage weeds biologically. The ability of HCN to block essential metalloenzymes, such as cytochrome c oxidase, impacts its toxicity [34]. Exopolysaccharides generated by this PGPR have been proven to impact plant growth and drought tolerance significantly. Exopolysaccharides have important roles in desiccation resistance, microbial aggregation, plant-microbe interaction, surface adhesion, and bioremediation [35]. In the current study, both PGPR isolates can synthesize ammonia, IAA, HCN, and exopolysaccharides.

Iron is one of the most important elements for plant and microorganism development and appropriate functioning. Bacteria, fungi, and plants synthesize siderophores, which are low-molecular-weight chelating agents (200-2000 Da) that helps bacteria, fungi, and plants to absorb iron. According to Singh et al. (2019), *Azotobacter vinelandii* nitrogen-fixing bacteria synthesize siderophores that are also utilized in the acquisition of nitrogenase co-factors such as

molybdenum (Mo) and vanadium (V) [36]. Many studies have found that siderophore-producing bacterial strains play an important role in growth promotion and biocontrol [37]. Venkat et al. (2017), found that *Bacillus* sp. and *Enterobacter* sp. isolates from iron-enriched soil were good candidates for producing siderophores [38]. On TRIS minimal medium enriched with zinc oxide, potassium-releasing isolates were tested for their ability to solubilize zinc. According to Singh et al. (1998), increasing potassium application rates had a favorable and significant influence on fresh rhizome output [39]. *Burkholderia*, *Bacillus* spp., *Enterobacter* spp., *Paenibacillus mucilaginosus*, and other rhizospheric bacteria have been described as K-solubilizers and have a high capacity for mobilizing and solubilizing K from minerals. The salt stressor is abiotic stress that damages soil characteristics, making it unsuited for isolate growth and also, inhibiting the plant growth and development [40]. In the current investigation, isolates showed resistance to a salt stress level of 7%. In saline soil, where plant development is impossible, these isolates are useful. The current study demonstrates the ability of PGPR to solubilize zinc and potassium, generate siderophore, and tolerate up to 7% NaCl.

In recent decades, the isolation and identification of potent isolates as inoculants have escalated [41]. Several bacterial genera, including *Azotobacter*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Pseudomonas*, *Azotobacter Serratia*, and others, have been utilized as biofertilizers in recent decades [42]. PGPR increases rhizosphere fertility in five ways: improving nutritional status in the immediate vicinity of roots, biological nitrogen fixation, encouraging beneficial host plant symbiosis, growing root surface area, and combining all of the above mechanisms of action. The usage of commercial biofertilizers containing the best PGPR strains is fast expanding, and as a result, the hunt for PGPRs and their method of action is becoming increasingly important [43]. PGPR was isolated from *Asparagus*

rhizospheric soil for this investigation. Isolation was carried out on various culture media plates. Furthermore, the other physiological properties of two screened isolates were examined based on several plant growth-promoting attributes. Because the isolates have multiple properties related to plant growth promotion, such as phosphate solubilization, IAA production, zinc and potassium solubilization, nitrogen fixation, exopolysaccharide synthesis, siderophore synthesis, ammonia production, and salt tolerance, they will be very useful for developing bio inoculants and assisting in the sustainable cultivation of crops.

5. Conclusion

This study revealed that bacteria *Exiguobacterium acetylicum* strain RGK and *Enterobacter mori* strain RGK1 have a wide range of plant growth-promoting properties, including phosphate, zinc and potassium solubilization, auxin production, HCN and ammonia production, siderophore synthesis, and high salt tolerance. The PGPR are appealing as biofertilizers and biopesticides as well as a cost-effective solution to sustainable agriculture. PGPR protects plants from phytopathogens and helps them grow and perform better. Chemical fertilizers and pesticides are generally effective and convenient for plant production and disease management, but they pose a risk to soil, plant, human health and the environment. As a result, using these isolates as an inoculant can promote plant development. In conclusion these PGPR could also be used as a biofertilizer in the future.

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Figures :

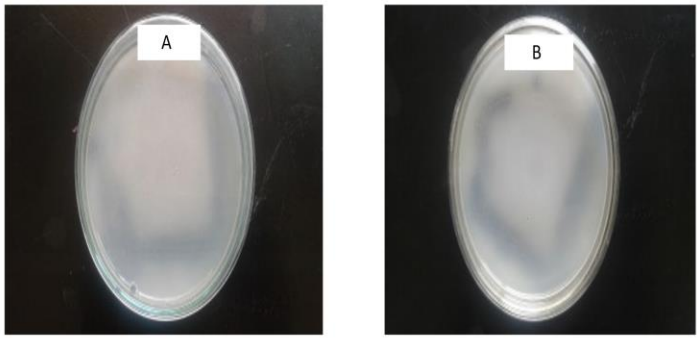
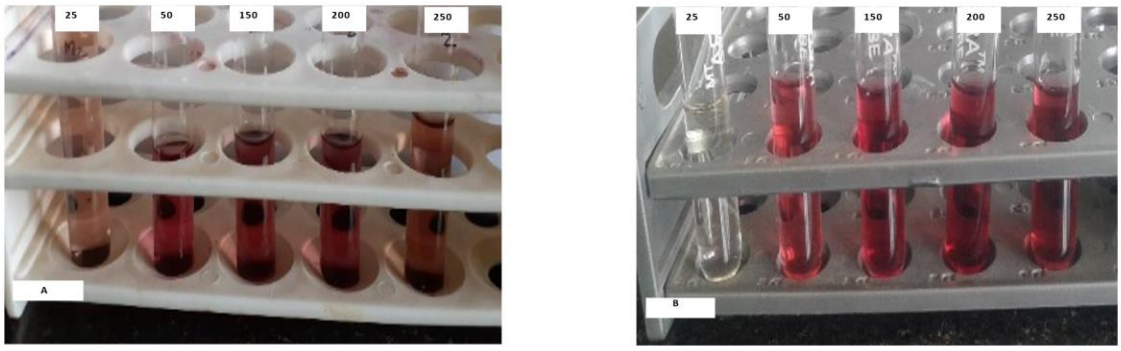


Figure 1: solubilization of phosphate on Pikovskaya's agar after 48 hrs where A) *Exiguobacterium acetylicum* RGK B) *Enterobacter mori* RGK1



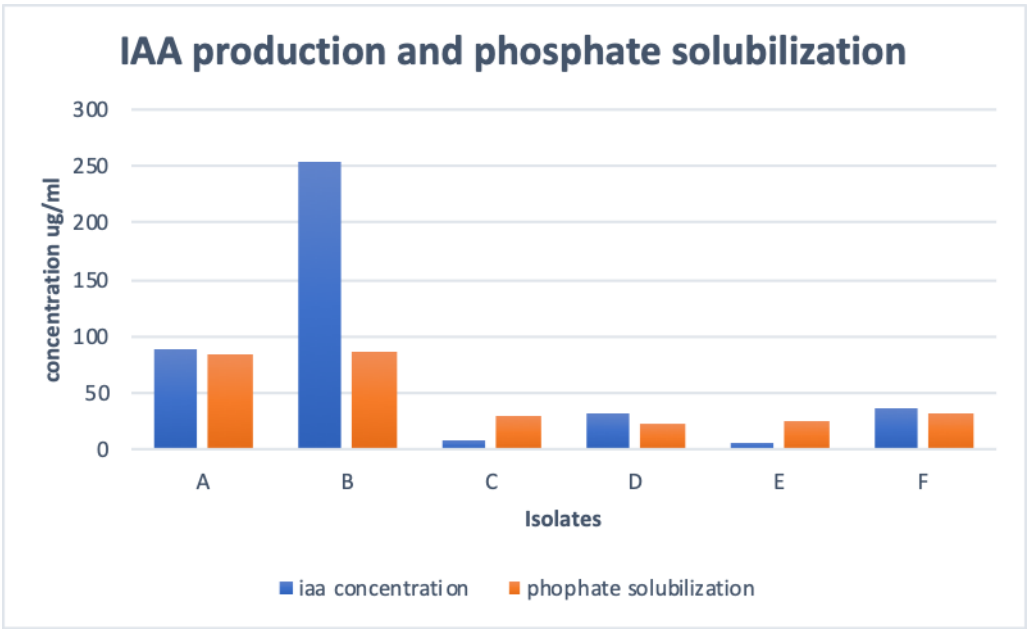


Figure 2: Production of IAA in tryptophan containing medium after 48 hrs A) *Exiguobacterium acetylicum* RGK B) *Enterobacter mori* RGK1

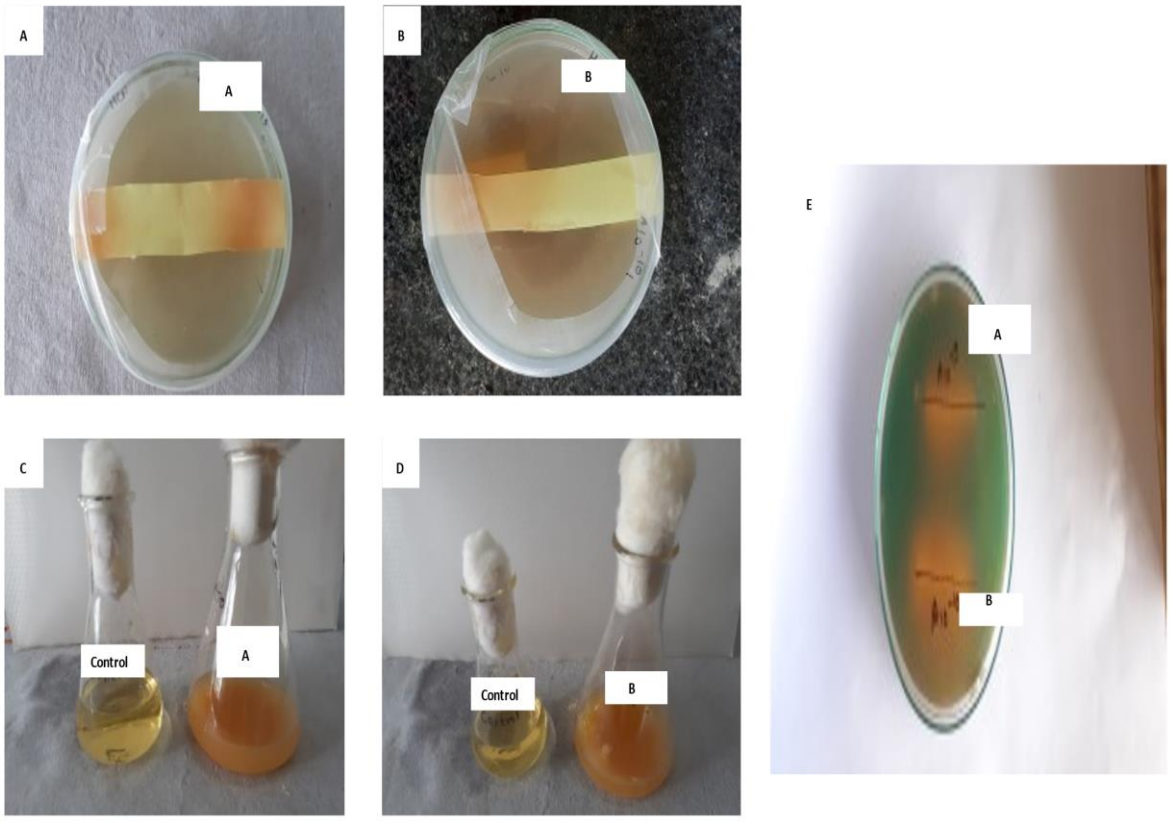


Figure 3: A, B are HCN production, C,D are ammonia production and E is siderophore production by *Exiguobacterium acetylicum* strain RGK (A) and *Enterobacter mori* strain RGK1(B) respectively after 48 hrs of incubation.

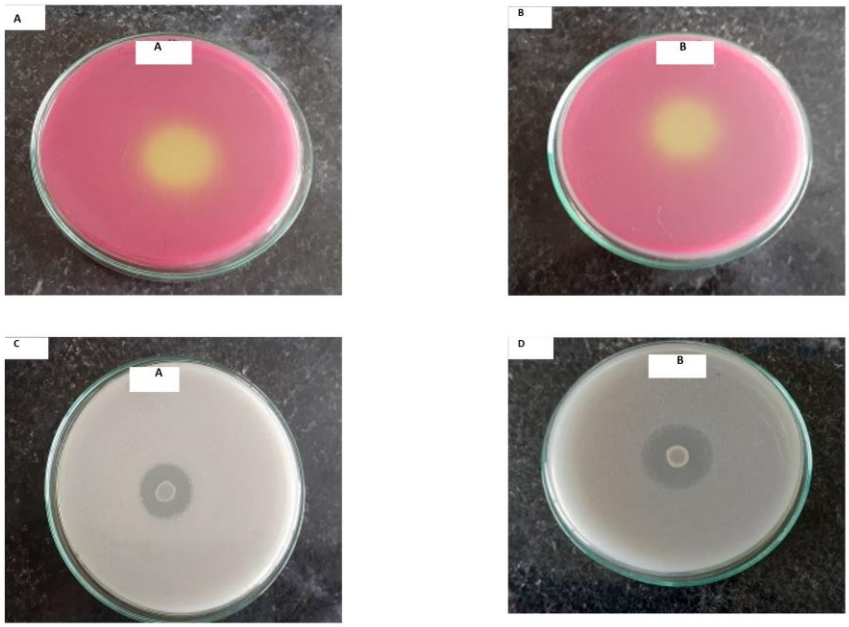


Figure 4: A, B are solubilization of potassium on Modified Aleksandrovsk medium and C,D are Zinc solubilization by *Exiguobacterium acetylicum* RGK (A) *Enterobacter mori* RGK1(B) respectively after 72 hrs of incubation.

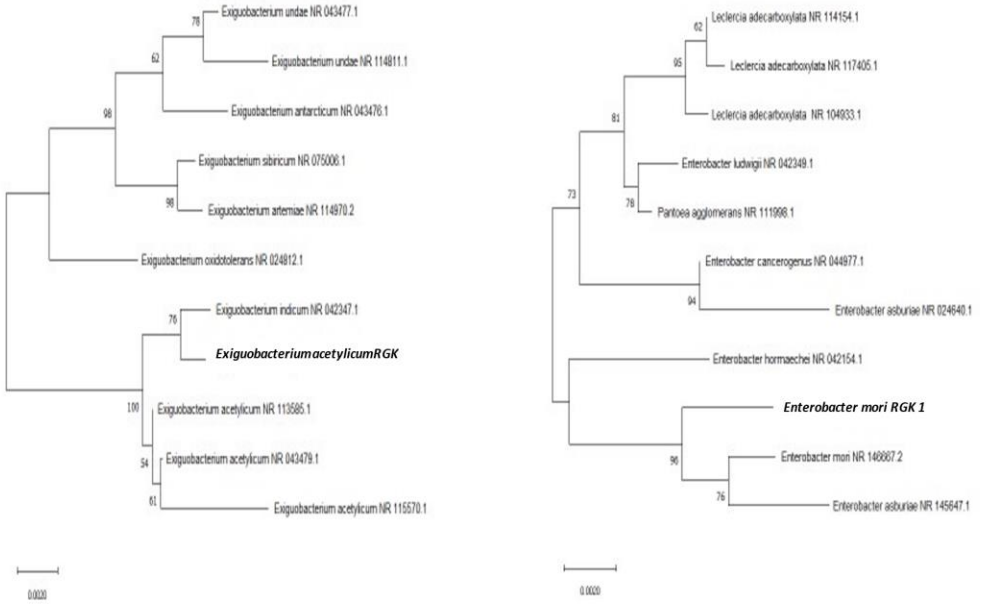


Figure 5: Neighbor-joining phylogenetic tree based on16S rRNA gene sequence of the closely related isolates of (a.) *Exiguobacterium acetylicum* strain RGK (b.) *Enterobacter mori* strain RGK1 , bootstrap values on each branch point indicates 1000 pseudo replicates.

Tables:

Table 1. Solubilization of phosphate and IAA production by *Exiguobacterium acetylicum* strain RGK (A) and *Enterobacter mori* strain RGK1 (B) after 48hrs. Data are shown as mean ± SD of three replicates.

Organism names	**Solubilization of Phosphate µg/ml**	**IAA Production in µg/ml**
A	84.24 ±0.01	90.11 ±0.1
B	86.16 ±0.02	253.45 ±0.01
C	31.35 ±0.01	8.45 ±0.02
D	24.30 ±0.03	33.45 ±0.01
E	25.90 ±0.01	6.55 ±0.03
F	31.67 ±0.02	38.45 ±0.02

Table 2 . Exopolysaccharide synthesis, Solubilization of potassium and solubilization of zinc by *Exiguobacterium acetylicum* strain RGK (A) and *Enterobacter mori* strain RGK1(B) after 48hrs.

Organism names	**Exopolysaccharide production**	**Solubilization of potassium**	**Solubilization of zinc**
A	+	+	+
B	+	+	+

C	+	-	-
D	-	-	-
E	-	+	-
F	-	-	-

+ present , - absent .

Table 3 . Biochemical characters of *Exiguobacterium acetylicum* strain RGK (A) and *Enterobacter mori* strain RGK1(B) .

Biochemical activity	***Exiguobacterium acetylicum* strain RGK**	***Enterobacter mori* strain RGK1**
citrate utilization	+	-
catalase	+	-
Gelatin hydrolysis	+	-
Oxidase	+	-
starch utilization	-	-
Sucrose utilization	+	+
Fructose utilization	+	-
Maltose utilization	+	-
Raffinose utilization	-	+
Nitrate reduction	-	-

Gram nature	Gram positive	Gram negative

+ present , - absent .