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## **RHIZOSPHERE- ASSOCIATED PLANT GROWTH-PROMOTING BACTERIA FOR SALINE SOIL RECLAMATION**

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### **ABSTRACT**

Agriculture determines the living conditions of the population of a nation. The salinity of soil affects plant growth by inhibiting the process of plant photosynthesis, protein synthesis, and lipid metabolism. According to a study by the United Nations Environment Program approximately 20 % of agricultural land and 50 % of cropland in the world is under the salt-stress. Salinization of soil converts agricultural land to barren land. The rhizobacteria showing traits to improve plant growth, can be further formulated as biofertilizers and check the plant growth by comparing various growth parameters. This study includes the extraction of bacteria from saline soil having growth-promoting properties. The sample was collected from the saline soil of Aurariya remote area in Uttar Pradesh India. The source of living for people in this area is primarily agriculture and most of the land here is saline. The isolated bacteria were tested for the PGPR traits which are the ability of IAA synthesis, Phosphate solubilization, HCN production, and ACC Deaminase activity. The strains showing PGPR traits will be molecularly characterized by 16SrRNA sequencing. The four saline soil samples were tested and the saline-tolerant bacteria were at 3%, 6%, and 9%. The ten isolates were found to be tolerant at such high salt concentrations. The bacterial isolates maximum shown the ACC Deaminase activity. IAA and HCN production test were conducted and

out of 10 isolates, 5 isolates have shown the best positive results. None of the isolates were showing positive results in phosphate solubilization. The two isolates S7 & S3 showing maximum positive results for PGPR were identified by 16SrRNA sequencing and were identified; S7 *Bacillus cereus* and S3 closely resembled *Bacillus licheniformis* and *Bacillus subtilis*. The native Microorganisms in saline soil have the inherent properties to tolerate saline conditions and interact with the plants helping in their growth, by synthesizing compatible solutes and production of plant growth-promoting hormones. The isolation of novel microorganisms may provide an effective way to deal with the problem of salinity. Thus, the competent microorganism can be used as biofertilizers and their positive effects can be spread to farmers to promote sustainable agriculture.

**Keywords: Agriculture, Biofertilizer, Environment, Eco-friendly, Ecosystem, HCN, PGPR, Salinity, Soil**

## INTRODUCTION

Environmental stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides, and soil pH are major limiting factors in crop production because they affect almost all plant functions. The salinity of soil is the environmental stress, reducing cultivated land area, productivity rate, and crop quality [1, 2].

The salt stress has reduced agricultural productivity all over the world. A survey estimated that about 20 % of irrigated and cultivated lands (equivalent to 62 million ha) are negatively affected by salt stress [3]. The salinity of soil is caused by the use of water with high concentrations of ions and minerals like calcium, magnesium, and sodium [4]. The lack of adequate drainage management results in the accumulation of salt in the root area of plants [5]. The use of compost fertilizer is also

responsible for increasing soil salinity as the food waste and municipal organic waste used contain large quantities of sodium chloride. A soil is termed saline when the electrical conductivity of the saturation extract (ECE) exceeds 4 dS m<sup>-1</sup> (approximately 40 mMNaCl) at 25 °C and has 15 % exchangeable sodium [6]. This conductivity is sufficient to reduce crop yield. Plants growing in salt-affected soils exhibit two distinct phases- osmotic (water) and salt stress. The osmotic stress, due to higher water potential in root cells than surrounding soils, prevents water uptake by plant roots resulting in water deficit causing many physiological and biochemical abnormalities that adversely affect plant growth [7].

Elevated Na<sup>+</sup> concentration prevents K<sup>+</sup> absorption which further affects the activities

of key enzymes involved in important metabolic processes such as photosynthesis and protein synthesis [8]. Salinity inhibits root growth in water [3].

Salinity affects air movement, water holding capacity, penetration of plant roots, and the emergence of seedling and tillage operations [9, 10]. In developing countries, efficient and sustainable practices are needed for the production of adequate nutrition for growing populations. The microscopic organisms can be used as a sustainable solution for present and future agricultural practices [11]. The Plant Growth Promoting Rhizobacteria helps growth in plants and their development by fixing nitrogen, phytohormones, and iron sequestered by bacterial siderophores, solubilizing phosphate, and some by giving resistance to plants for several diseases [12, 13]. The biofertilizer is a material containing living microorganisms that help to promote the growth and production of the plant by increasing the availability of nutrients in the soil. Therefore, the use of biofertilizers is essential and should be promoted as it is an environmentally friendly and ecologically safe alternative [14, 15]. In a study, it was seen that together with organic fertilizer plant growth-promoting rhizobacteria reduces salt stress caused by salinity in rice plants and helps in their growth, [16]. Paul and Nair

(2008) reported that *Pseudomonas fluorescence* MSP-393, a PGPR strain, tolerates salinity and de novo-synthesizes, the osmolytes, alanine, glycine, glutamic acid, serine, threonine, and aspartic acid in their cytosol [17]. Several microorganisms like *Rhizobium*, *Bradyrhizobium*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, etc. isolated from stressed environments like deserts, acid soils, saline, and alkaline areas, are found to be involved in the natural remediation of soil [18, 19].

In India, salt-affected land is about 6.73 million ha. The first ever case of salinity was reported in 1855 in the village Moonak in Haryana. The states like Western Haryana and Uttar Pradesh lack freshwater availability. The rainfall in this area is scanty and the evapotranspiration rate is higher. The highly saline groundwater is the source of water [20]. The area in this study is Auraiya which is in Uttar Pradesh, India. A report submitted by Priya in 2015 claims that Kannauj, Auraiya, Raebareli, Unnao, and Manipuri suffer from salinity to a great degree [21]. The salinity and sodic surface are mostly depending on irrigation water quality. It was reported that the mid-region of Uttar Pradesh in Gangetic Plain is severely degraded by salinity and alkalinity. This study involves the isolation of saline-tolerant rhizobacteria which have the

potential to improve plant growth. The area in this study is very much saline and also unexplored. The people here mainly depend on agriculture for their living. The rhizobacteria isolated can be helpful as biofertilizers for a vast range of salinity. Thus, to fulfill the objective the saline soil sample was to be collected and further tests were to be done for the isolation of desired bacteria.

## MATERIALS AND METHODS

The sample collection involves the soil collection from salt-affected areas in Auraiya which is in Uttar Pradesh India. Collected soil samples were further used to isolate saline-tolerant bacteria using the serial dilution method. The lab work was performed at the chaperone lab Kanpur and Dept. of Biotechnology A.K.S. University Satna (M.P.) from 2019 to 2022. The isolated rhizobacteria were further analyzed on their morphology and were tested for their potential as plant growth-promoting bacteria. with the test involving such as IAA production, HCN production, Phosphate solubilization, and ACC deaminase activity. The PGPR bacteria thus obtained will be identified using 16SrRNA sequencing.

### Collection of soil sample

The soil sample was collected from the saline area of Auraiya 26.47° N 79.52°E in Uttar Pradesh (India). The soil was collected by

uprooting plants and scraping out the soil from their roots. The bacteria were isolated following the serial dilution method. The culture was obtained on LB media at 37°C. The rhizobacteria thus obtained was further isolated and purified by restreaking several times till a pure colony was obtained.

### Isolation of salinity-tolerant bacterial strains

The isolation of bacteria includes the extraction of bacteria from the soil sample by serial dilution method on petri plates of nutrient agar medium prepared with a salt concentration of 3%, 6%, and 9%. Now the plates were incubated for 3 days at 28°C. The bacterial colonies were observed for different characteristic features and each colony was restreaked for isolation of pure colonies and further testing for Plant Growth Plant Rhizobacteria characteristics. The pure cultures so obtained were named HS1 HS2, HS3, HS4, HS5, HS6, HS7, HS8, and HS9.

### 2.2. Characterization of isolates.

The morphological characteristics of the colonies were examined on incubation plates and were observed for characteristics such as shape, size, elevation, surface, margin, color, etc., and gram staining was done for each isolate. The process involves taking a loopful of diluted culture which was smeared and fixed on a glass slide by heating on the flame

of a Bunsen burner. Crystal violet dye and safranin were used in the above process. The slide was observed under a microscope and data were recorded.

### **Selection of PGPR strains**

The isolated bacterial strains were tested for their plant growth-promoting activity. The plant growth-promoting activity evaluation involves tests such as IAA production, HCN production, Phosphate solubilization, and ACC deaminase activity.

### **IAA production**

Indole-3-acetic acid acts as an essential plant-promoting hormone [22]. The peptone broth enriched with tryptophan was used to culture the isolates obtained above to check indole acetic acid production which is a precursor of auxin, an important plant hormone.

The Salkowski method is used for the quantitative estimation of IAA. This method involves the Salkowski reagent is 0.5 M FeCl<sub>3</sub> and 35% HClO<sub>4</sub> [23]. The isolated bacteria were grown in 50 ml yeast extract broth supplemented with 50 mg/ ml-1 of L-Tryptophan and incubated in the dark on an orbital shaker at 200 rpm for 72 h. One ml of culture supernatant and 1 ml of Salkowsky's reagent were mixed and incubated in the dark for 30 min and the pink color was observed., which was then estimated on a spectrophotometer at 536 nm.

### **ACC Deaminase**

The enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase is the enzyme that degrades ACC which is a precursor of ethylene that increases stress in all higher plants [24]. The isolated cultures were grown on, DF salt (Dworkin and Foster) media supplemented with 3mM ACC. Then cultures were incubated for 3 to 4 days and checked for the inhibition zone.

### **HCN**

HCN production was tested by Morrison and Askeland (1983) method [25]. The plates of Kings B media along with 0.4% (w/v) glycine were prepared for the identification of HCN production. The plates with culture were incubated for 24 hours. The alkaline picric acid solution (2% Na<sub>2</sub>CO<sub>3</sub> in 0.5% picric acid) was added to the What Man filter paper and placed on the upper lids of Petri plates. These plates were monitored for 6 days for the development of red-brown from the yellow color of filter paper.

### **Phosphate solubilization**

Phosphorus is only second to nitrogen in mineral nutrients which is most commonly limiting in plant growth. Many soil microorganisms can solubilize unavailable forms of bound phosphorus [26]. The plates were prepared with Pikovaskya's medium and the culture was incubated in the above media.

The incubation of cultures was done on a spot method and was incubated at 28°C for 3-5 days. The formation of a clear zone around the microbial colonies indicated phosphate solubilization [27].

### **Molecular characterization of Bacterial isolates**

The PGPR bacteria obtained is further identified by sequencing of DNA sample of the desired isolates using 16SrRNA. The DNA was obtained from the isolated competent bacteria.

### **Isolation of genomic DNA sample**

The genomic DNA was extracted using the phenol-chloroform method.

### **Amplification and sequencing of 16S rRNA**

The amplification of the 16S rRNA sequence was done of the selected salt-tolerant PGP isolates using universal primers. The amplification and sequence identification were done by Biokart Genomic Lab India Pvt Ltd Bengaluru. The sequence obtained was analyzed and identified using BLAST search and was compared against bacterial 16S rRNA sequence available on the NCBI database. The sequences were aligned by using Clustal W 1.74 followed by the construction of a neighbor-joining phylogenetic tree, using MEGA4.

## **RESULTS AND DISCUSSION**

### **Screening of salt-tolerant bacterial strains**

The crop plants show a wide range of responses to salinity, and thus, this reduces the yield of the crop. The salinity of soil negatively affects many physiological, metabolic, and biochemical processes, like photosynthesis, respiration, and transpiration, [28].

In this study, the soil samples were collected from the Auraiya region of Uttar Pradesh. (**Figure 1** shows the saline patches of the research area). The soil samples collected have a pH of 6.5 with 6% Electrical conductance. This pH and conductivity are responsible for the saline behavior of the soil in this region, [20]. As evident from **Table 1** a total of 9 isolates were found to be saline tolerant at different salt concentrations. HS3, HS7, and HS9 have shown moderate growth at 6% salt concentration. The isolate HS 1 and HS6 has shown very little growth at 6% while HS2, HS4, and HS8 show no growth at 6% salt concentration. The isolate HS9 has shown growth at 9% salt concentration while no other isolate has shown growth at 9% salt concentration. (**Figure 2 a, b, and c** showed the bacterial strains at salt concentration respectively). These isolates were designated as HS1, HS2, HS3, HS4, HS5, HS6, HS7, HS8, HS9 (**Figure 2**). A similar study was conducted by Mahmood and his co-workers in the common ice plant *Mesembryanthemum*

*crystallinum* L. They isolated, screened, and characterized rhizosphere bacteria. They isolated 152 strains and 80 of them show saline-tolerant behavior. Bacterial strains of *Streptomyces* sp. PR-3 and *Bacillus* sp. PR-6

was effective against soil salinity [29]. However, in our study, we isolated some 9 bacteria that showed salinity to the higher ranges and these strains were further tested for PGPR traits.



Figure 1: Saline patches of Auraiya

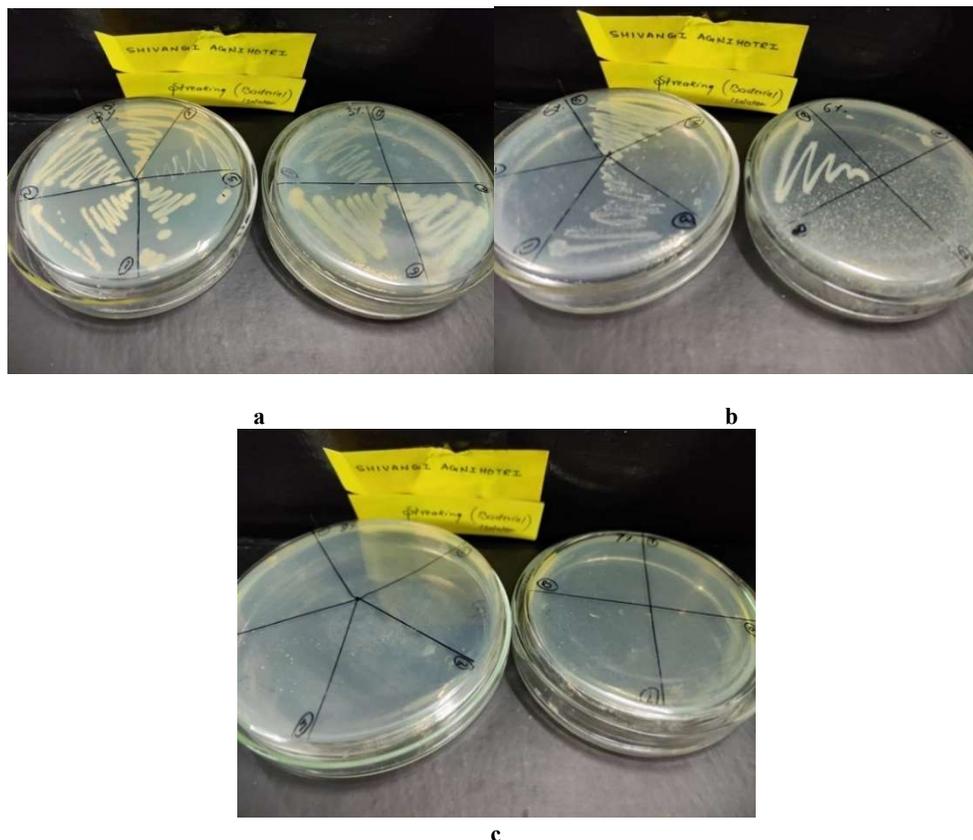


Figure 2 a, b, c: Bacterial Growth at 3%, 6%, and 9% respectively

Table 1: Growth of Nine isolates in different salt concentrations

S. No.	Strains	3%NaCl Conc.	6% NaCl Conc.	9%NaCl Conc.
1	HS1	Abundant	Very little	No growth
2	HS2	Abundant	No growth	No growth
3	HS3	Abundant	Moderate	No growth
4	HS4	Abundant	No growth	No growth
5	HS5	Abundant	Abundant	No growth
6	HS6	Abundant	Very little	No growth
7	HS7	Abundant	Moderate	No growth
8	HS8	Very little	No growth	No growth
9	HS9	Abundant	Abundant	Slightly Growth

The morphological characteristics of the colonies obtained are mentioned in **Table 2**. The HS4, HS7, and HS8 have an irregular shape and the others are round, all the colonies were white only HS6, and HS7 are light

yellow. The HS1 is umbonate in elevation while HS2, HS3, HS4, HS6, HS8, and HS9 are having flat colonies. The isolate HS6 is raised and HS7 is convex in elevation.

Table 2: Morphology characteristics of Isolates

Strains	Shape	Elevation	Colour
HS1	Large, opaque, irregular	Umbonate	White
HS2	Round, smooth	Flat	White
HS3	Round, undulated edge	Flat	White
HS4	Irregular	Flat	Wheat
HS5	Round, lobate irregular edge	Raised	White
HS6	Irregular, undulated edge	Flat	Light yellow
HS7	The round, smooth entire edge	Convex	Light yellow
HS8	Irregular, lobate irregular edge	Flat	White
HS9	Round, Smooth	Flat	White

The salt-tolerant rhizobacteria were further screened for their plant growth properties. These strains were tested for tests like Inode-3-Acetic Acid production, Phosphate solubilization, 1-Aminocyclopropane-1-carboxylate deaminase synthesis, and HCN production.

As illustrated in **Table 3**, The HS1 strain shows positive results for the IAA production test and HCN production test only. This strain shows negative results for ACC deaminase activity and phosphate solubilization. The HS2 strain shows positive results only for

ACC deaminase activity and negative results for IAA production HCN production and phosphate solubilization test. The HS3 strain shows positive results for IAA production, HCN production, and ACC deaminase activity and this strain shows negative test only for the phosphate solubilization test. The HS4 shows positive results only for the HCN production test while it shows negative for all other tests. The HS5 strain shows positive results only for ACC deaminase activity and remains negative for all other tests. The HS6 strain shows negative for all tests. The HS7

strain shows positive for IAA production, HCN production, and ACC deaminase activity and negative for the phosphate solubilization test. The HS8 strain shows positive results for only HCN production while remaining negative for the all-other tests. The HS9 strain shows positive results for IAA production and ACC Deaminase activity and remains negative for the HCN production and phosphate solubilization test. Thus, from the above result, we conclude that the HS3 and HS7 strain shows maximum plant growth-promoting activity and can be further analyzed.

It is observed in our study that HS3 & HS7 strain shows IAA production which is also seen in previous research done on PGPR bacteria. According to Rani *et al.*, (2012) the most important signal molecule in the regulation of plant development is auxin [30]. Auxin (IAA) production by PGPR stimulates the production of flavonoids by plants and improves nitrogen fixation, nodulation, and nutrient uptake which in turn reduces the harmful effects caused by salinity [31]. Mitigation of salinity by PGPR inoculants has been shown in rice, wheat, maize, cotton, lettuce, tomato, and pepper [32]. In a study conducted by Maharana in 2019, in the marigold plant they isolated, characterized, and identified indole acetic acid-producing

rhizospheric bacteria. The isolated bacterial strains *Azotobacter*, *Pseudomonas*, and even *Spirillum* (Gram-negative bacteria); show positive tests in IAA, catalase, oxidase, urease, and starch hydrolysis testing respectively. The IAA-treated marigold seeds were found to show more growth of root and shoot when compared with the control, [33]. Thus, we see that rhizobacteria producing IAA are competent plant growth-promoting bacteria and can be formulated as biofertilizer. The two strains HS3 & HS7 isolated in our study can also be used as biofertilizer.

The HS3 & HS7 strains in our study show HCN production which is also a marker test for identifying plant growth-promoting bacteria and the same is also found in previous research. Blumer and Hass (2000) reported that IAA production promotes plant growth and HCN production has been proposed as a defense regulator against phytopathogens [34]. A study conducted by Rijavec and Lapanjae proposed that the level of HCN produced by the rhizobacteria in oligotrophic alpine environments, and in also any other place, HCN contributes to the sequestration of metals and thus facilitates the availability of nutrients, which is beneficial for the rhizobacteria and their plant hosts [35]. Hence, HS3 & HS7 strains isolated in our study can be used as biofertilizer.

Phosphorus is typically insoluble or poorly soluble in soils under salt-stressed conditions and these PGPRs help in the solubilization of insoluble P and thus improve plant growth and development [24]. However, in our study, no strains show phosphate solubilization.

ACC deaminase activity is a crucial property needed by a potent rhizobacteria to be a biofertilizer. Ethylene acts as an inhibitor when released during stress by plants and causes a reduction in legume nodulation. In plants, the pathway of production of ethylene is Yang cycle in which the ACC oxidase enzyme is responsible for the conversion of ACC into ethylene [36]. Plant growth-promoting rhizobacteria (PGPR) can produce enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACC deaminase), which is a precursor of ethylene and reduces the level of ethylene during salt stress [24]. The ACC deaminase produces PGPR attached to the root of plants and uptakes ACC which is released from plant roots and then hydrolyzes it Glick *et al.*, (2014.) Many scientists have reported in reduction of salinity stress through ACC Deaminase containing PGPR improving plant growth [24]. In our study, HS3 & HS7 strain shows positive ACC deaminase activity. In a study, conducted by Mosqueda and his coworkers on the ability of ACC Deaminase containing PGPB endophytes

*Pseudomonas fluorescens* YsS6, *Pseudomonas migulae* 8R6, and their ACC Deaminase deficient mutants help in promoting tomato plant growth in the absence of salt and under two different levels of salt stress was assessed. It was found that strains having ACC Deaminase activity facilitate better growth of plants [37]. The major mechanisms utilized by PGP bacteria to reduce stress include lowering the level of ethylene via hydrolyzing 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC deaminase. ACC is the immediate precursor of the hormone ethylene in plants. It is widely reported that certain PGPR possesses ACC deaminase enzyme that can degrade ACC to ammonia and ketobutyrate, thus reducing the level of ethylene inside the plants [38]. Therefore, the PGPR containing ACC deaminase has the potential to curb abiotic stress-induced ethylene production and its associated adverse effect on plants.

Thus, it is observed that the two strains obtained HS3 & HS7 show PGPR activity test which correlates with previous research done, and hence, these two strains can be used as biofertilizers.

The PGPR test results of the salt-tolerant strains obtained above are represented in (Table 3). The IAA test is positively shown

by strains HS1, HS3, HS7, and HS9. HCN production is shown by HS1, HS4, HS8, HS3, and HS7. ACC Deaminase is seen in HS2,

HS5, HS3, HS7, HS9. Phosphate solubilization is not shown by any of the isolates.

**Table 3: PGPR Test of salt tolerant Bacterial Strains**

Sample	IAA production test	Phosphate solubilization	ACC Deaminase activity	HCN Production
HS1	+	-	-	+
HS2	-	-	+	-
HS3	+	-	+	+
HS4	-	-	-	+
HS5	-	-	+	-
HS6	-	-	-	-
HS7	+	-	+	+
HS8	-	-	-	+
HS9	+	-	+	-

As observed from the results out of 9 strains two of them HS3, HS7 showed maximum positive results for PGPR traits, and hence HS3 & HS7 were finalized for molecular characterization. The 16S rRNA gene of the selected isolates was successfully amplified using PCR and approximately 1500bp of the amplified product was sequenced. (Fig 3a & b shows the identification of HS3 & HS7 respectively). The BLAST -N comparison of the searched sequences in the NCBI nucleotide database revealed 99.87% similarity of the isolate HS7 with *Bacillus cereus* MN 793064.1. The sequence obtained is submitted in NCBI and the accession number that has been allotted is OP895692 (Fig 4a showing phylogenetic analysis of HS7) and HS3 shows 99.87% similarity with *Bacillus subtilis* and *Bacillus licheniformis* MF765317.1. The sequence obtained is submitted in NCBI and the accession number

that has been allotted is OP895707 (Fig 4b showing phylogenetic analysis of HS3).

The *Bacillus* strain has been earlier also reported to be a potent plant growth-promoting rhizobacteria. Ayaz and his coworkers isolated the halophilic *Bacillus* strains bacteria from the rhizosphere of the extreme environment of the Qinghai-Tibetan plateau region of China and analyzed the capability of these isolates to reduce salt stress in wheat plants [39]. Sultana and her coworkers conducted a similar study in which three salt-tolerant bacterial isolates viz; *Bacillus aryabhattai*, *Achromobacter denitrificans*, and *Ochrobactrum intermedium* were identified through comparison of 16S rRNA gene sequences. These bacteria demonstrated plant growth-promoting activities like high atmospheric nitrogen fixation, phosphate solubilization, and indoleacetic acid production at a

concentration of 200 mmol/l salt [40]. The isolates we identified are also from the genus *Bacillus*. In a different study, *Bacillus* SB1 and *Halobacillus* SB2 strains were tested for salt tolerance in combination with metals such as zinc, aluminum, and lead in the growth of *Arachishypogaea* L under saline stress [41]. In our study, the isolated bacteria are *Bacillus* sp.

Our results are related to the work of many other scientists in the literature. Thus, we conclude that salt-tolerant bacteria with PGPR activities are much better at managing salt-affected agricultural fields and crop improvement can be achieved, [42-44].

PGPRs have different pathways to ameliorate salt stress in the vicinity of plants [36].

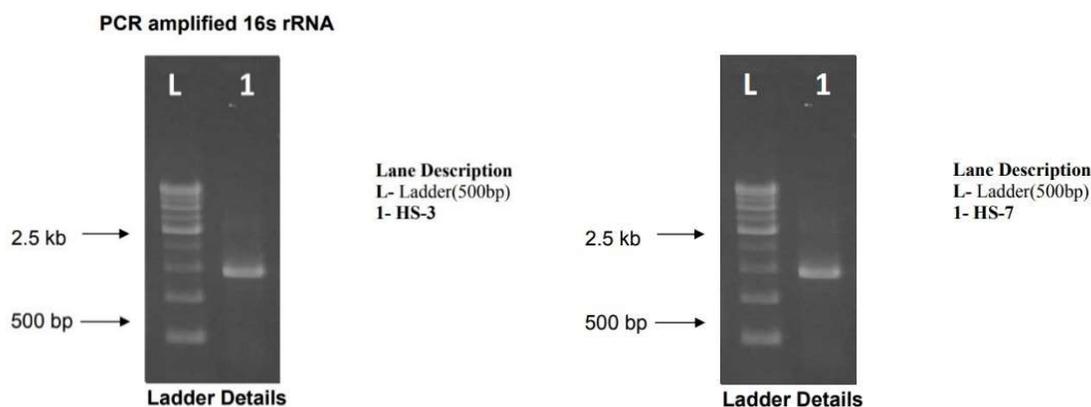


Figure 3a & b: Shows the identification of HS3 & HS7 respectively

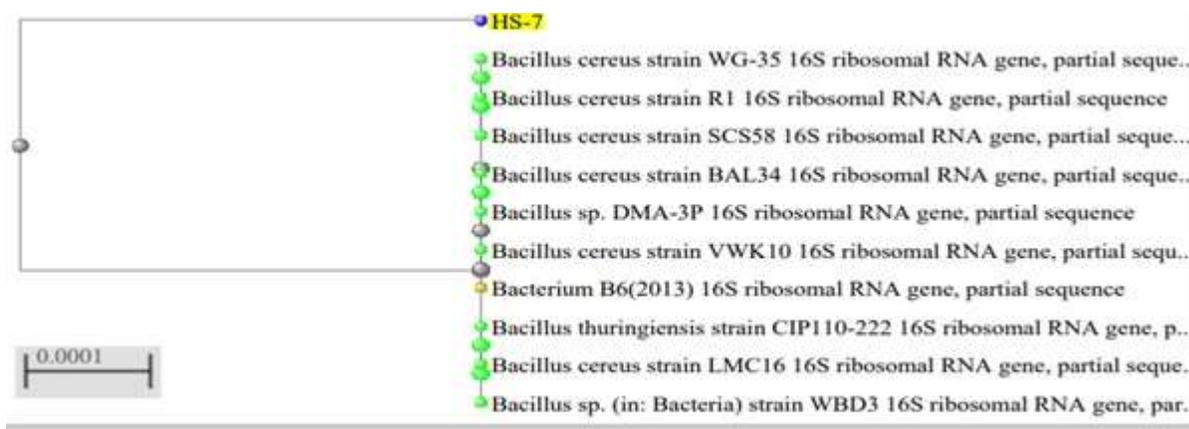


Figure 4a: Shows the phylogenetic analysis of HS7

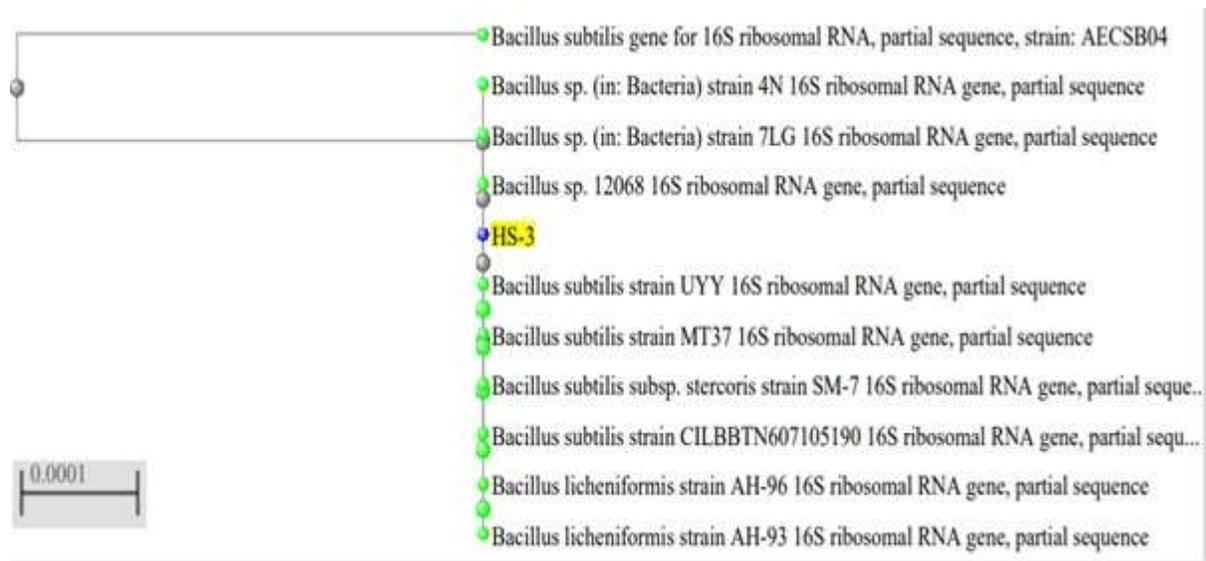


Figure 4b: Shows a phylogenetic analysis of HS3

## CONCLUSION

As evident from the results obtained from the present research strains HS3 and HS7 showed the maximum positive results of the PGPR test and thus these two strains can be formulated as biofertilizers. The use of chemical fertilizers harms the environment, animals, and human beings and disturbs the ecological balance. The increase in the human population has increased the demand for food production which is a problem for the agricultural sector. Moreover, biofertilizer is a material containing living microorganisms that help to promote the growth and production of the plant by facilitating the available nutrients in the soil. These products play a role in increasing crop production by varying mechanisms. Therefore, the use of

biofertilizer products is essential because it is environmentally friendly.

Salinity is a big threat to agriculture around the world. The agricultural land in India is rapidly converting to saline land which in turn makes the fields less productive and even turns barren land. The rhizobacteria found in saline soil enable plant growth with direct and indirect mechanisms. These bacteria can be further formulated into biofertilizers. Thus, these can be a better alternative to the alleviation of the stress caused by salinity.

Thus, this study concludes that salinity can be alleviated using rhizobacteria with plant growth traits. The above study observed the two bacterial isolates HS3 and HS7 showing maximum results with PGPR tests hence these two isolates were found to closely resemble *Bacillus subtilis*, *Bacillus licheniformis*, and

*Bacillus cereus*. These can be further formulated as biofertilizers and can be used in agriculture as a better environment-friendly fertilizer.

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