Halotolerant as plant growth promoting Rhizobacteria (PGPR)

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Abstract

Extremophile species known as halophiles flourish in conditions when salt concentrations are extremely high. The microbial communities in the soil of the Kutch region are highly salinized and have not been thoroughly studied. Soil bacteria known as plant growth promoting rhizobacteria (PGPR) live in the rhizosphere, rhizoplane and roots of plants where they support plant growth through a number of direct and indirect means. Nineteen halotolerant bacterial strains were obtained from the Kutch region for this study. These strains are capable of growing in medium with 1-20% NaCl. Morphological, physiological and biochemical traits were examined and factors like pH, NaCl and temperature tolerance were found to be important for their proliferation. It was possible for the isolates to release extracellular enzymes such as cellulose, lipase, amylase and protease. The efficiency of nitrogen fixation was discovered but quantitative estimation needs to be done.

Additionally, the isolates demonstrated effective zinc and phosphate solubilization on the appropriate substrate. We examined whether wheat, a crop sensitive to salt, may serve as a suitable host for halotolerant bacteria that have the ability to stimulate plant growth by evaluating their impact on biomass, survival and seed germination. Research on halotolerant bacteria in the presence of heavy metals such as nickel, has benefited plant roots. Nevertheless, before these strains are regarded as biofertilizer, more crops and field testing are required. According to this preliminary study, soil of Kutch exhibits a high concentration of halotolerant microbes. These bacteria may have properties that promote plant growth and can be used as biological fertilizer.

Keywords: Biofertilizer, Nickel, Plant growth promoting rhizobacteria (PGPR), Zinc solubilization.

Introduction

Halophiles are "salt-loving"microorganisms and have the ability to regulate the osmotic pressure of the surrounding medium while fending off the denaturing effects of salts. Halophilic bacteria are categorised into the following groups based on this scheme: extreme halophiles that can thrive in media with 15–30% w/v (2.5–5.2 M) NaCl, extremely borderline halophiles that need at least 12% w/v NaCl, moderate halophiles can grow best in media with 3–15% w/v (0.5–2.5 M) NaCl, while slight halophiles can grow best in media containing 1–3% w/v (0.2–0.5 M) NaCl.

On the other hand, microorganisms classified as non-halophilic are those that grow best in conditions containing less than 1% (0.2 M) NaCl. However, bacteria that are able to thrive both in the presence and absence of salt and that can withstand relatively high concentrations of NaCl are referred to as halotolerant, or highly tolerant, if their tolerance goes above 15% of (2.5 M) NaCl[4,20].

Plants are continuously exposed to a variety of biotic and abiotic stresses in their natural settings which can have an impact on their longevity and yield. Continuous salt deposition from encroaching saltwater interferes with the physicochemical characteristics of soil in agricultural fields, causing plants to permanently lose their ability to absorb water which in turn causes dehydration and osmotic stress[18]. The build-up of ions (Na^+ and Excess Cl^-) negatively impacts transpiration system, photosynthesis, plant metabolic machinery and most crucially, ionic balance and nutrient (N, Ca, K, P, Fe, Zn) uptake; this slows down seed germination, reproductive development and overall crop yield[2,9,12,14,16].

Stress, which can be caused by a variety of natural events, is an unfavourable state or substance that impacts or impedes a plant's development or metabolism. Abiotic and biotic stresses are the two categories of stress. Abiotic stress is caused by things like water logging, drought, heat, cold, wind, intense light and salt of the water while biotic stress is caused by things like human and animal intervention and a range of pathogenic microbes. Therefore, the greatest option that is employed to get over this restriction and stressful situations is PGPR. Bacteria known as PGPR live in the rhizosphere and promote plant growth in a number of direct and indirect ways (fig. 1). Plant-induced systemic resistance and the synthesis of antimicrobial substances that impede pathogen growth, are examples of indirect pathways[19,26].

Using inoculants based on helpful microorganisms, which promote plant development or protect plants from pathogens and abiotic influences, is one of the greatest ways to achieve sustainable and environmentally friendly agriculture. Therefore, the primary goal of this effort is to identify PGPR strains that are halotolerant and have good enzymatic activity, making them suitable for use as fertilizers in high salinity environments.

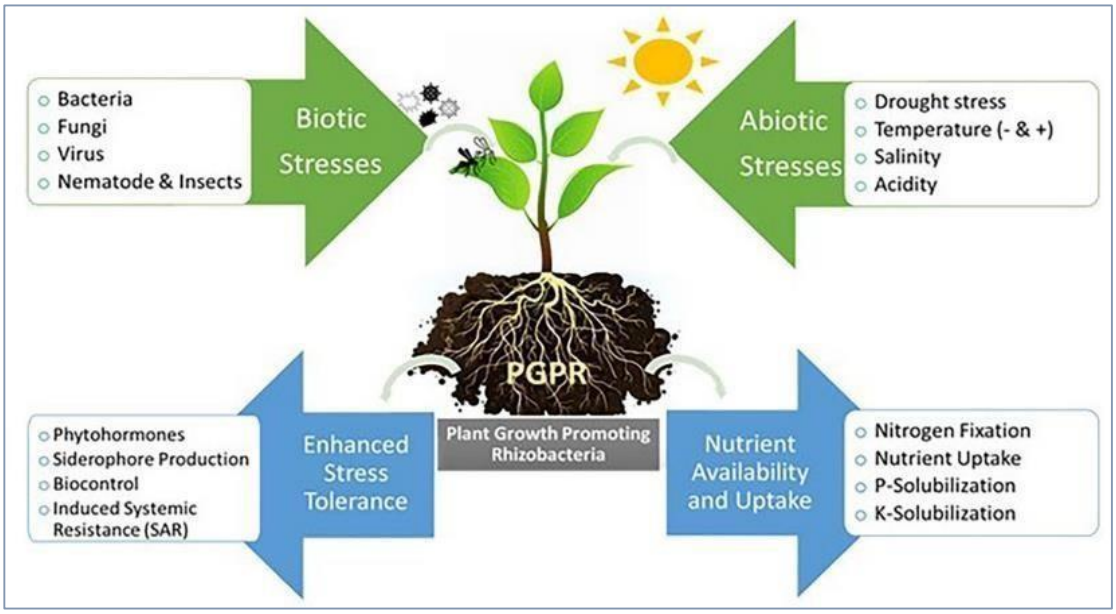


Figure 1: Plant interaction in rhizosphere and plant growth promoting rhizobacteria (PGPR)[30]

Material and Methods

Enrichment and isolation of halotolerant bacteria from soil samples: To enrich halotolerant bacteria, one gram of Kutch soil samples was added to 100ml of nutrient broth having various concentration of salts (1-20% NaCl) and incubated at 100 rpm for six days. For isolation of bacteria, a loopful enriched culture was streaked onto nutrient agar plates that contained varying 1-20% concentration of NaCl. After that the plates were incubated at 37°C[20].

Characterization of isolates: A number of morphological, physiological and biochemical analyses were performed to characterise the isolates. Bergey's Manual of Systematic Bacteriology[5] was used for morphological and physiological characterization.

Native potential of isolates and morphological characterization: The study of the cultural traits of various bacterial isolates involved several growth parameters such as size, shape, elevation, margin, texture, opacity and colour, with respect to colonial features.

Native potential of isolates and physiological characterization: 39 halophilic bacterial isolates were subjected to physiological characterization of their native potential like salt tolerance, pH tolerance and temperature tolerance[20].

Determination of pH Tolerance: In the nutrient broth medium, which was adjusted to pH 2.0, 4.0 and 6.0 using 0.1 N HCl and to pH 8.0 and 10.0 with the aid of 1N KOH, the pH tolerance of each isolate was examined. The liquid media were autoclaved for 20 minutes at 121°C and 15 psi of pressure. These tubes were inoculated with 0.1 ml of a pure culture of every isolate, containing 10^7 CFU/ml. For every pH range, one uninoculated tube served as the negative control. The tubes were incubated at 37°C for three days. By comparing it to a negative control, the presence or absence of growth was noted and the strain that can grow at a specific pH, was designated as tolerant[28, 29].

Determination of salt tolerance: Each strain was cultured independently in nutrient broth supplemented with varing concentrations of NaCl (5, 10, 15, 20 and 25%) while an uninoculated tube served as a negative control in order to assess each isolate's salt tolerance and capacity to thrive at various NaCl concentrations. All isolates' bacterial suspension (0.1 ml, 10^7 CFU/ml) was added to the corresponding salt concentrations of broth in tubes for inoculation. For three to five days, the tubes were incubated at 37°C with a control. The strains that cultivated at a given salt concentration were designated as tolerant while the isolates that showed little to no growth were classified as sensitive to the corresponding salt concentrations. The presence or absence of growth in the isolates was recorded by comparing them with the negative control. The next step involved scoring the growth using three levels of positive signs: one positive meant limited growth (took more than 5 days), two positives meant average growth (took 3–4 days) and three positives meant satisfactory growth (took 2 days or less)[28,29].

Determination of temperature tolerance: By cultivating the isolates in nutrient broth, the isolates' capacity to grow at various temperatures was examined. All isolates were inoculated with a 0.1 ml bacterial suspension into the broth tubes, which were then incubated at 37°C, 40°C, 45°C and 60°C respectively. As a control, one uninoculated tube containing the same liquid media was used. By comparing the isolates with the negative control, the presence or absence of growth was noted. The strain that grew at a certain temperature was designated as tolerant whereas the isolates that showed little to no growth were classified as sensitive to that temperature.

Three degrees of positive signs were used to score growth: one positive indicated low growth (took more than five days), two showed average growth (took three to four days) and three indicated satisfactory growth (took 2 days or less)[28,29].

Native potential of isolates; biochemical characterization: The test isolates of halotolerant bacteria were subjected to manual biochemical analysis[22].

Carbohydrate fermentation Test: Three beakers containing 250 ml each of glucose, lactose and sucrose were used for the carbohydrate fermentation test. Each beaker was filled with 5g of the corresponding sugars and 0.0045g of phenol red. Durham's tube was carefully added to two test tubes after 10 ml of each fermentation broth had been filled into test tubes. Following autoclaving, tubes in each of the three prepared sugar fermentation media were marked as test and control. The test organism was only inoculated into the test. For two days, the tubes were incubated at 37ºC.

Methyl red test: A test tube with glucose phosphate broth was inoculated with test organisms and left for 48 hours at 45°C in the incubator. Following incubation, methyl red indicator was added and the color of the test tubes was monitored for changes. Red colour in the tubes indicates positive result.

Voges Proskauer test: Test organisms were inoculated into the glucose phosphate broth. They were cultured for 48 hours at 45°C. Following incubation, Barritt's reagent was added and the colour of the test tubes was monitored. The appearance of rose pink colour indicates a positive result.

Indole production test: To observe the organism's production of indoles, place a Kovac's reagent strip between the tube's plug and inner wall above the inoculated peptone water and incubate it for 18 to 24 hours at 35 to 37°C. Positive response shows that the lowest part of the strip is pink.

Urea hydrolysis test: A loopful of test culture was inoculated into the urea broth. The tubes were incubated at 37 °C for 24 hrs. Observe the change in color of the broth after incubation (pink).

Citrate utilization test: Organisms were streaked on to Simmons citrate agar slant and were incubated for 24 hrs for the colour change. The color change from green to blue was observed. The positive test determines the ability of bacteria to use citrate as a sole of carbon and energy. This ability depends on the presence of a citrate permease that facilitates transport of citrate into the bacterium.

Triple sugar iron test: A loopful of test cultures was streaked onto triple sugar iron slant surface and stab the same culture into the butt of the slant. TSI slant was incubated at 37 °C for 24 hrs. After incubation, the TSI medium was observed for the presence of acid, gas hydrogen sulphide in the media

Catalase test: A tube test can be used to measure catalase activity. The hydrogen peroxide solution was then combined with the test organism-containing test tube. The development of gas bubbles was observed. Positive responses are indicated by effervescence (bubble formation) right away.

Motility test by agar stab method: Using a sterile needle, choose a well-isolated colony and stab the medium within 1 cm of the tube's bottom to test for motility. When inserting and withdrawing the needle from the medium, make sure to maintain the same line. For eighteen hours, or until growth is noticeable, incubate at 37°C. A dispersed cloud of growth that is moving away from the line of inoculation indicates a positive motility test.

Native potential of isolates - extracellular enzyme production test

Starch hydrolysis test: The starch agar plates were inoculated with bacterial isolates and they were then incubated for 48 hours at 37°C. After incubation, the plates were filled with Gram's iodine, they were closely examined for a distinct zone of hydrolysis surrounding the colonies. A positive result is indicated by the presence of a clean zone around the growth[1].

Lipid hydrolysis test: On tributyrine agar plates, bacterial isolates were inoculated and the plates were then incubated for 48 hours at 37°C. A distinct zone of hydrolysis surrounding the colonies was carefully noticed on the plates. A clear zone surrounding the colonies is indicative of a positive result[1,24,25].

Protease enzyme test: The casein-containing nutrient agar plate was used for the qualitative protease enzyme production test. It was incubated at 37°C for 24 to 72 hours at 150 rpm in a shaking incubator. Following incubation, isolates exhibiting a zone of casein hydrolysis were identified as cultures that produced proteases[24,25]

Cellulase enzyme test: In order to further enzyme produced by positive strains, the cellulase enzyme production test was first run on a qualitative basis to screen out isolate strains with positive cellulase activity. The chosen bacterial isolate strains were inoculated into medium containing CMC 1%, $NaNO_3$ 0.2%, $MgSO_4$ 0.05%, K_2HPO_4 0.005%, $FeSO_4$ 0.001%, $CaCl_2$ 0.002% and $MnSO_4$ 0.002%. The plates were then incubated for 24- 72 hrs and 37°C in order to produce cellulase enzymes. After incubation 0.1 % Congo red staining solution was added in CMC plates, discard stain after 5 min and the plates were destained by 1M NaCl solution with continuous stirring for 15-20 min. The clear zone around colonies indicated cellulose hydrolysis; the isolates exhibiting for cellulase were classified as cellulase-producing bacteria[24,25].

Gelatin hydrolysis test: Nutrient gelatin-containing test tubes were inoculated with test organisms. Test organisms were cultured for 48 hours at 37°C. After that, tubes were either chilled with ice or placed in the freezer for 30 minutes to evaluate the hydrolysis of gelatin. The absence of solidification in the tubes indicates a positive outcome[28].

PGPR Characteristics

Phosphate solubilization capacity; qualitative test for phosphate solubilization in solid medium: In Pikovaskys agar medium, a qualitative test for phosphate solubilization in a solid medium was conducted. Using a sterile inoculating needle, 10 µl of the bacterial suspensions (~10^4CFU/ml) were spotted into the medium's centre to test phosphate solubilization capabilities. At 37°C, the infected plates were incubated. Three, five and seven days following the inoculation were used to measure the solubilization zone[1,21,28,29].

Potash solubilization capacity: On Aleksandrow agar plates, the chosen isolates' ability to solubilize potash was qualitatively assessed. From the corresponding fresh isolation culture broth, a loopful of bacterial cells was taken and spotted in the centre of the plates that contained the solidified media. After that, the inoculation plates were incubated for four days at 37°C[28,29].

Zinc solubilization capacity: Using agar plates as the zinc solubilizing medium, the ability of the chosen halophilic bacterial isolates to solubilize zinc was qualitatively assessed. From the corresponding fresh isolation culture broth, a loopful of bacterial cells was taken and spotted in the centre of the plates that contained the solidified media. After that, the inoculation plates were incubated for four days at 37°C with the cover on.

Positive zinc-solubilizers were defined as several bacterial isolates that formed a zone surrounding their colony expansion and showed the ability to solubilize zinc. After three, five and seven days, the diameter of the bacterial colony and the zone of clearing or solubilization (halo) surrounding it were measured[20].

Nitrogen fixation capacity: On a nitrogen-free Jensen's agar medium, the ability of the chosen isolates to fix nitrogen was investigated. HiMedia provided the premade Jensen's agar medium. In order to ensure appropriate solidification, 1.5% agar was added to Jensen's agar medium plates. After that, the plates were streaked with the isolate culture and incubated for five days at 35°C[28,29].

Heavy metal tolerance test: With a few adjustments, the heavy metal tolerance test was carried out using the general procedure[13]. The ability of the chosen isolates to withstand heavy metals was assessed through inoculation in nutrient broth supplemented with different amounts (25, 50 and 100 µg/ ml for Nickel). The inoculation broth was incubated at 37°C for 24 hours and the corresponding isolates' optical density (OD) was measured at 600 nm.

Testing the Selected Strains' for its PGPR Potential on Wheat plant: The impact of a particular wheat PGPR strain's plant growth-promoting activity on seedling growth was done. The purpose of these experiments was to ascertain how inoculating PGPR strains might affect seed germination rates. Wheat (technical name) seeds were utilized as plant components for this. After rinsing the seeds six times with sterile distilled water, healthy seeds were surface sterilized for two minutes using NaOCl. For 24 hours, PGPR strains were cultured in their corresponding broth in a shaking incubator at 120 rpm and 28 ± 2°C. The pot experiment was run in triplicate[1,31,32]. This study examined the impact of heavy metals on wheat plants by introducing nickel into wheat seeds. Graph Pad prism was used for the graphical presentation.

Observation recorded: Wheat germination has been recorded on the tenth day. Every replication is carried out in triplicate.

Seedling shoot length: On randomly chosen seedlings from each replication, the shoot length (in centimetres) was measured using a scale. The result was computed by taking the average and standard deviation of each seedling for each repetition.

Seedling root length: On the tenth day following the commencement of the germination test, a random selection of normal seedlings was made from each replication. Using a measuring scale, the length of the radicle (in centimetres) was determined. The mean and standard deviation root length were then computed.

Seedling fresh weight: At the conclusion of the seed germination test on the tenth day, the seedling fresh weight was noted. Every replication's normal seedlings were selected at random, weighted and their fresh seed weight was calculated using an electronic balance to get the weight in grams.

Seedling dry weight: During the tenth day of the seed germination test, the normal seedlings from each replication were selected at random. For 24 hours, seedlings were dried in an oven set to 80°C. Using an electronic balance, the dried seedlings' weight was calculated and reported in grams.

Results and Discussion

Soil samples were collected from Padana district of Kutch. After enrichment (Fig. 2), a total of 39 halotolerant isolates were obtained by spreading the respective enriched samples on nutrient agar plate with varying salt (NaCl) concentration (1% to 20%). The production of extracellular enzymes (amylase, lipase, protease and cellulose), phosphate, potash, zinc, nitrogen fixation and heavy metal tolerance were among the PGPR traits of the isolates that were examined and positive results were found. A pot trial utilizing wheat

plant was used to establish the isolates' capacity to promote plant development in addition to their PGPR features. The application of PGPR in agriculture is growing and may offer helpful alternatives to synthetic pesticides and fertilizers. Effective microbial competitors can promote plant growth through the production of phytohormones. The synthesis of secondary metabolites can increase the availability of nutrients or the action of biocontrol agents can protect plants against phytopathogen infection.

The phenotypic features of halotolerant isolates

Characteristics of isolated colonies: All the 39 isolates showed different cultural characteristics in terms of size, shape, margin, elevation texture and opacity and pigment production. This isolates showed growth in different salt concentrations after 2-3 days and the following colonial morphology was noted which is depicted in table 1. The colony morphologies of isolates ranged from irregular to circular in shape with a majority being small to medium in size with flat to raised and convex type of elevations while some of them were pigment producing. The morphological characteristics of the isolates in this investigation were inferred from the observed colony characteristics which included size, shape, elevation, margin, texture, opacity, colour and microscopic features. After 24 hours of inoculation, the colonies on media exhibited the typical halophilic like colonies.

Morphological characteristics: Gram staining was used to examine the isolates' morphological features. Under a microscope, the isolates also showed variations in cell morphologies and sizes (Table 2). All isolates were Gram positive, short or big rod, purple color, arranged in single, chain and clusters while few were also found to be filamentous in structure (Fig. 3). In addition to that, some isolates were also found to have sporulation. Motility was also checked by hanging drop method.

Test for determination of salt, pH and temperature tolerance of isolates: The isolates' tolerance to varying NaCl, pH and temperatures is shown in table 3. The isolates showed tolerance to high salt concentrations up to 20% with optimal growth occurring in the range of 10–15% NaCl; optimal pH ranged from 6–8 with increased growth towards alkalinity and tolerance to extreme acidity and maximum temperature tolerance up to 45 °C, with optimal temperature recorded at 35 °C.

Biochemical test: The results pertaining to various biochemical tests conducted are shown in table 4. The biochemical tests were carried out in order to determine the different biochemical characteristics of the isolates to check their capability for carbohydrate utilization of different sugars, as well as some tests like IMVIC and TSI. All the isolates showed different potentiality for sugar utilization as well as no organisms showed methyl red and VP tests positive.

The positive tryptophanase enzyme activity of the isolates having salt concentration form (1, 1.5, 2.5,3, 4, 6.5, 8.5 and 9 %) was confirmed by indole production tests. No urease activity was observed which showed urease test negative. The isolates with salt concentration (2, 4%) showed positive results for citrate utilization. All 39 isolates showed positive catalase test which confirms that the organisms belongs to aerobic or facultative anaerobes.

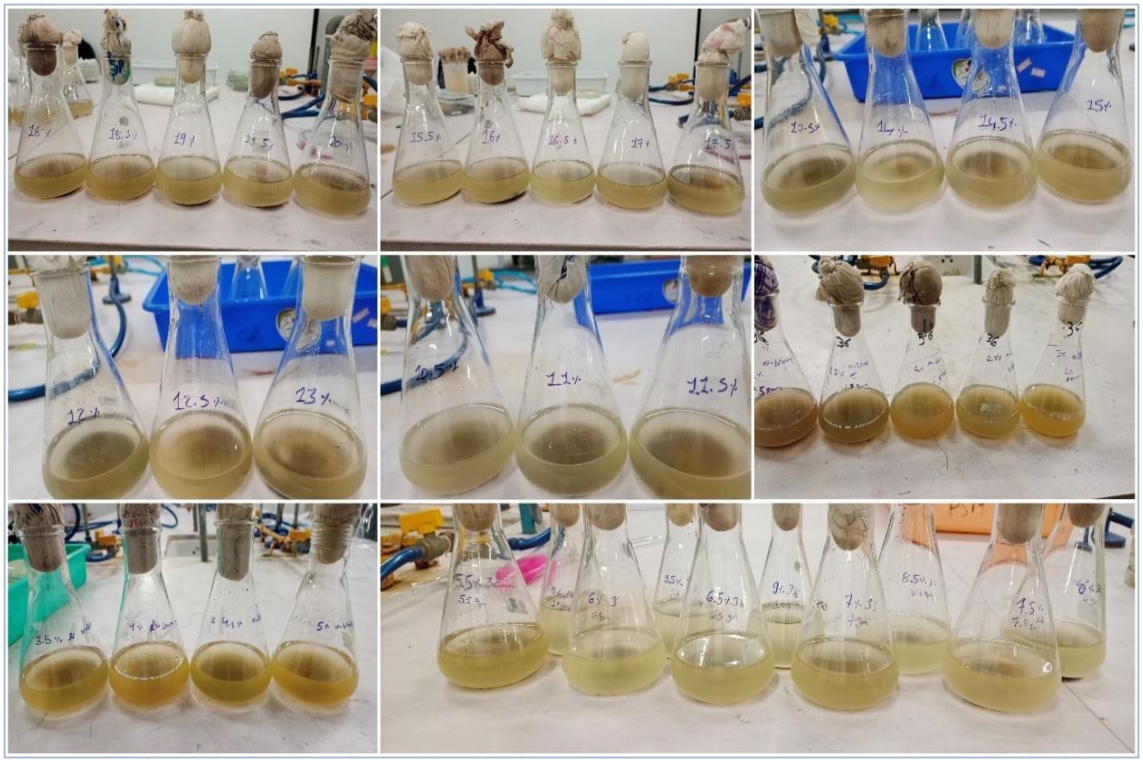


Figure 2: Enrichment of soil samples with varying 1-20% salt concentrations

Catalase test: All 39 isolates showed positive results with rapid appearance and sustained production of gas bubbles or effervescences (fig. 4).

Motility test: All 39 isolates showed the presence of turbid growth across the line of inoculation which indicates that the organisms are motile (fig. 4).

Extracellular enzyme production: Every isolate was examined to determine if it could grow on a medium containing the appropriate substrates and could produce extracellular enzymes such as lipases, proteases, cellulases and amylases etc. Each of the 39 isolates exhibited amylase production as shown by positive enzymatic activity on soluble starch.

Table 1
Colony characteristics

Isolates with percent salt concentration	Size	Shape	Margin	Elevation	Opacity	Texture	Pigment
1	Pin point	Round	Entire	Convex	Translucent	Smooth	No
1.5	Small	Round	Entire	Flat	Opaque	Smooth	No
2	Small	Round	Entire	Flat	Opaque	Smooth	No
2.5	Small	Round	Entire	Flat	Opaque	Smooth	No
3	Large	Round	Entire	Flat	Translucent	Smooth	No
3.5	Small	Round	Entire	Flat	Opaque	Smooth	No
4	Large	Round	Entire	Flat	Opaque	Smooth	No
4.5	Small	Round	Entire	Flat	Opaque	Smooth	No
5	Large	Round	Entire	Flat	Opaque	Smooth	Yellow
5.5	Medium	Irregular	Wavy	Flat	Opaque	Smooth	Yellow
6	Large	Round	Entire	Convex	Opaque	Smooth	Yellow
6.5	Large	Round	Entire	Flat	Transparent	Smooth	No
7	Large	Irregular	Wavy	Flat	Transparent	Smooth	Cream
7.5	Small	Round	Entire	Flat	Transparent	Smooth	No
8	Small	Round	Entire	Flat	Opaque	Smooth	Cream
8.5	Pin point	Round	Entire	Flat	Opaque	Smooth	No
9	Small	Round	Entire	Flat	Opaque	Smooth	No
9.5	Small	Round	Entire	Flat	Opaque	Smooth	No
10	Pin point	Round	Entire	Flat	Transparent	Smooth	No
10.5	Small	Round	Entire	Flat	Transparent	Smooth	No
11	Small	Round	Entire	Flat	Transparent	Smooth	No
11.5	Small	Round	Entire	Flat	Transparent	Smooth	No
12	Small	Round	Entire	Flat	Transparent	Smooth	No
12.5	Small	Round	Entire	Flat	Transparent	Smooth	No
13	Small	Round	Entire	Flat	Transparent	Smooth	No
13.5	Small	Round	Entire	Flat	Transparent	Smooth	No
14	Small	Round	Entire	Flat	Transparent	Smooth	No
14.5	Small	Round	Entire	Flat	Transparent	Smooth	No
15	Small	Round	Entire	Flat	Transparent	Smooth	No
15.5	Small	Round	Entire	Convex	Sebaceous	Gummy	No
16	Small	Round	Entire	Flat	Sebaceous	Gummy	No
16.5	Small	Round	Entire	Flat	Sebaceous	Gummy	No
17	Small	Round	Entire	Convex	Sebaceous	Gummy	No
17.5	Small	Round	Entire	Convex	Sebaceous	Gummy	No
18	Small	Round	Entire	Convex	Sebaceous	Gummy	No
18.5	Small	Round	Entire	Convex	Sebaceous	Gummy	No
19	Small	Round	Entire	Flat	Sebaceous	Gummy	No
19.5	Small	Round	Entire	Flat	Sebaceous	Gummy	No
20	Small	Round	Entire	Flat	Sebaceous	Gummy	No

Table 2
Physiological Features of Isolates

Organism with salt content % percentage	Grams reaction	Shape	Arrangement
1	Gram positive	Sporulated	Chain
1.5	Gram positive	Short rod	Single,pair
2	Gram positive	Short rod	Single,pair
2.5	Gram positive	Sporulated	Single,pair
3	Gram positive	Short rod	Single
3.5	Gram positive	Bacillus	Single,pair
4	Gram positive	Filamentous; Actinomycetes	Cluster
4.5	Gram positive	Bacillus	Single,pair,chain
5	Gram positive	Big rods	Single,pair,chain
5.5	Gram positive	Bacillus	Single,cluster
6	Gram positive	Bacillus	Single,chain,cluster
6.5	Gram positive	Bacillus	Single,pair
7	Gram positive	Big bacillus	Chain,single
7.5	Gram positive	Big bacillus	Single,chain
8	Gram positive	Bacillus	Cluster
8.5	Gram positive	Big bacillus	Single,pair
9	Gram positive	Big bacillus	Single,chain
9.5	Gram positive	Filamentous; Actinomycetes	Single,cluster
10	Gram positive	Big bacillus	Chain,cluster
10.5	Gram positive	Bacillus	Single,cluster
11	Gram positive	Bacillus	Single,pair,cluster
11.5	Gram positive	Big bacillus	Chain,cluster
12	Gram positive	Big bacillus	Chain,cluster
12.5	Gram positive	Big bacillus	Single,pair
13	Gram positive	Big bacillus	Single,cluster
13.5	Gram positive	Big bacillus	Single,chain,cluster
14	Gram positive	Filamentous; Actinomycetes	Cluster,single
14.5	Gram positive	Big bacillus	Single,pair
15	Gram positive	Big bacillus	Single,chain
15.5	Gram positive	Big bacillus	Single,cluster
16	Gram positive	Bacillus	Single,pair
16.5	Gram positive	Bacillus	Single,pair
17	Gram positive	Big bacillus	Single,cluster
17.5	Gram positive	Big bacillus	Single,cluster,chain
18	Gram positive	Bacillus	Single,chain,cluster
18.5	Gram positive	Bacillus	Single,pair
19	Gram positive	Big bacillus	Single,pair
19.5	Gram positive	Bacillus	Single,pair
20	Gram positive	Bacillus	Single,pair,cluster

For every isolate, there was a clearance zone surrounding the colony on casein agar indicating positive protease activity. The clear zone of lipid hydrolysis indicated positive lipase activity as well. It was noted that only one isolate produced cellulase, however none of the isolates produced gelatinase. By weakening the structural integrity of plant pathogens' cell walls, PGPR generates hydrolytic enzymes such as glucanases, cellulases, proteinases and chitinases that directly inhibit plant pathogen growth[7]. According to reports, hydrolytic enzymes break down elements of fungi's and oomycetes' cell walls[23]. It has been shown that PGPR strains of *Pseudomonas* and *Bacillus* release hydrolytic enzymes that inhibit the growth and development of filamentous fungus both *in vivo* and *in vitro*. According to reports, bacterial enzymes demolish oospores, interfere with spore germination and lengthen the germ tube of phytopathogenic fungus[10]. According to reports, bacteria that produce hydrolytic enzymes are employed as biocontrol agents in the fight against phytopathogenic fungi[3,6,33,34].

Starch hydrolysis test for amylase production: After iodine was added, all 39 isolates displayed a distinct zone of starch hydrolysis as a result of amylase synthesis. This suggests that the extracellular enzyme amylase can be produced by all

isolates (Table 5).

Lipid hydrolysis test for lipase production: On a tributyrene agar plate, each of the 39 isolates displayed a distinct zone of lipid hydrolysis. The synthesis of lipase by the isolates is shown by these positive results (Table 5).

Protein hydrolysis test for protease production: Protease is responsible for hydrolyzing casein, as evidenced by the formation of a clear zone around the growth. All 39 isolates produced favourable results (Table 5).

Cellulase enzyme assay: A positive result was found in 3% of isolates. By doing quantitative cellulase production estimation, this can be verified (Table 5).

Table 3
Determination of salt, pH and temperature tolerance of isolates.

Organism with a salt content in percentages	37°C	40°C	45°C	60°C	1 pH	2 pH	3 pH	4 pH	5 pH	6 pH	7 pH	8 pH	9 pH	10 pH
1	++	++	++	++	-	-	-	-	-	-	+++	+++	+++	+++
1.5	++	++	++	++	-	-	-	-	-	++	++	+++	+++	++
2	++	++	++	++	-	-	-	-	-	-	++	+++	+++	+++
2.5	+++	+++	+++	+++	-	-	-	-	-	++	+++	++	++	++
3	++	++	++	++	-	-	-	-	++	++	++	+	++	++
3.5	++	++	++	++	-	-	-	-	-	++	++	++	+++	+++
4	++	++	++	++	-	-	-	-	-	++	++	+	+++	++
4.5	++	++	++	++	-	-	-	-	-	++	++	+	++	++
5	++	++	++	++	-	-	-	-	-	++	++	++	++	++
5.5	++	++	++	++	-	-	-	-	-	++	++	+	+++	++
6	++	+++	+++	++	-	-	-	-	++	+++	+++	+++	+++	++
6.5	+++	+++	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++
7	++	+++	+++	+++	-	-	-	-	++	++	+++	+++	+++	+++
7.5	++	+++	+++	++	-	-	-	-	+++	+++	+++	+++	+++	+++
8	+	++	++	+	-	-	-	-	+++	+++	+++	+++	+++	+++
8.5	+++	+++	++	+	-	-	-	-	+++	+++	+++	+++	+++	+++
9	+	+	+	+	-	-	-	-	++	++	+++	+++	+++	+++
9.5	++	+++	+++	+	-	-	-	-	+++	+++	+++	+++	+++	+++
10	++	+++	+++	++	-	-	-	-	+++	+++	+++	+++	+++	+++
10.5	+++	+++	+++	+++	-	-	-	-	-	-	++	++	++	++
11	++	+++	+++	+++	-	-	-	-	-	++	+++	+++	+++	+++
11.5	+++	+++	+++	+++	-	-	-	-	-	++	+++	+++	+++	+++
12	+++	+++	+++	+++	-	-	-	-	-	-	+++	+++	++	++
12.5	++	+++	+++	++	-	-	-	-	-	-	++	++	++	++
13	++	+++	+++	+++	-	-	-	-	-	-	++	+++	+++	+++
13.5	+++	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	+++
14	++	+++	+++	++	-	-	-	-	-	-	+++	+++	+++	+++
14.5	++	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	+++
15	++	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	+++
15.5	++	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	+++
16	++	+++	++	+++	-	-	-	-	-	-	+++	+++	+++	+++
16.5	++	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	+++
17	++	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	+++
17.5	++	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	+++
18	++	+++	+++	+++	-	-	-	-	-	-	+	+	+	+
18.5	++	+++	+++	+++	-	-	-	-	-	-	++	++	+	+
19	+++	+++	+++	+++	-	-	-	-	-	-	++	++	+++	+
19.5	++	+++	+++	+++	-	-	-	-	-	-	++	++	++	+
20	++	++	+++	+++	-	-	-	-	-	-	++	+	++	++
+ indicate positive results and – indicate negative results														

Table 4
Biochemical test of the isolates

Organism with a salt content in percentages	Glucose		Lactose		Sucrose		MR	VP	Indole	Urea	Citrate	TSI	
	G	g	G	g	G	g						But	Slant
1	+	+	+	+	+	+	-	-	+	-	-	yellow	yellow
1.5	+	+	+	+	+	+	-	-	+	-	-	yellow	yellow
2	+	+	+	+	+	+	-	-	-	-	+	yellow	yellow
2.5	+	+	+	+	+	+	-	-	+	-	-	yellow	pink
3	+	+	+	+	+	+	-	-	+	-	-	yellow	pink
3.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
4	+	+	+	+	+	+	-	-	+	-	+	yellow	pink
4.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
5	+	+	+	+	+	+	-	-	-	-	-	yellow	pink
5.5	+	+	+	+	+	+	-	-	-	-	-	yellow	yellow
6	+	-	+	-	+	-	-	-	-	-	-	pink	pink
6.5	+	+	+	+	+	+	-	-	+	-	-	yellow	pink
7	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
7.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
8	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
8.5	+	+	+	+	+	+	-	-	+	-	-	yellow	pink
9	+	+	+	+	+	+	-	-	+	-	-	yellow	pink
9.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
10	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
10.5	+	+	+	+	+	+	-	-	-	-	-	yellow	pink
11	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
11.5	+	+	+	+	+	+	-	-	-	-	-	yellow	yellow
12	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
12.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
13	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
13.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
14	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
14.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
15	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
15.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
16	+	-	+	-	+	-	-	-	-	-	-	pink	pink
16.5	+	-	+	-	+	-	-	-	-	-	-	pink	pink
17	+	-	+	-	+	-	-	-	-	-	-	pink	pink
17.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
18	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
18.5	+	-	+	-	+	-	-	-	-	-	-	pink	pink
19	+	-	+	-	+	-	-	-	-	-	-	pink	pink
19.5	+	-	+	-	+	-	-	-	-	-	-	yellow	pink
20	+	-	+	-	+	-	-	-	-	-	-	yellow	pink

Gelatin hydrolysis test for gelatinase: Every isolate yielded a negative result. The gelatin agar tube showed no signs of liquefaction based on table 5 and fig. 4. It can be concluded that every isolate is gelatinase negative and cannot synthesise the enzyme.

PGPR characteristics

Test for phosphate solubilization of isolates: Fig. 5 displays the findings of the isolates' qualitative phosphate solubilization capacity. A portion of the isolates had positive test results, whilst the remaining isolates had negative results and no zone of solubilization occurs on the Pikovaskys agar medium. Containing (concentrations of 1 to 10% NaCl) the isolates had a distinct hallo zone surrounding the growth, signifying the presence of positive phosphatase activity and the solubilization of phosphate in its insoluble form before

its coversion into soluble form. But there was no zone of solubilization in the isolates with NaCl concentrations ranged from 10.5 to 20%.

Test for potash solubilization of isolates: The ability to solubilize potash was tested. The ability of any isolate to solubilize potassium in Aleksandrow's medium was absent. As a result, every isolate produced negative results.

Test for zinc solubilization of isolates: Fig. 5 shows the outcomes of zinc solubilization. It was possible for the isolates with salt concentrations of 1 to 10% to change insoluble zinc into soluble form. With a larger zone of zinc solubilization, these isolates demonstrated good zinc solubilization activity. However, zinc solubilization activity reduced as the salt concentration rise. Furthermore, isolates with salt concentrations ranging from 10.5% to 20% did not exhibit any zinc solubilization zones.

Table 5
Production of extracellular enzymes by isolates

Organism with a salt content in percentages	Amylase	Lipase	Proteases	Cellulases	Gelatinase
1	+	+	+	-	-
1.5	+	+	+	-	-
2	+	+	+	-	-
2.5	+	+	+	-	-
3	+	+	+	+	-
3.5	+	+	+	-	-
4	+	+	+	-	-
4.5	+	+	+	-	-
5	+	+	+	-	-
5.5	+	+	+	-	-
6	+	+	+	-	-
6.5	+	+	+	-	-
7	+	+	+	-	-
7.5	+	+	+	-	-
8	+	+	+	-	-
8.5	+	+	+	-	-
9	+	+	+	-	-
9.5	+	+	+	-	-
10	+	+	+	-	-
10.5	+	+	+	-	-
11	+	+	+	-	-
11.5	+	+	+	-	-
12	+	+	+	-	-
12.5	+	+	+	-	-
13	+	+	+	-	-
13.5	+	+	+	-	-
14	+	+	+	-	-
14.5	+	+	+	-	-
15	+	+	+	-	-
15.5	+	+	+	-	-
16	+	+	+	-	-
16.5	+	+	+	-	-
17	+	+	+	-	-
17.5	+	+	+	-	-
18	+	+	+	-	-
18.5	+	+	+	-	-
19	+	+	+	-	-
19.5	+	+	+	-	-
20	+	+	+	-	-
+ indicate positive results and – indicate negative results					

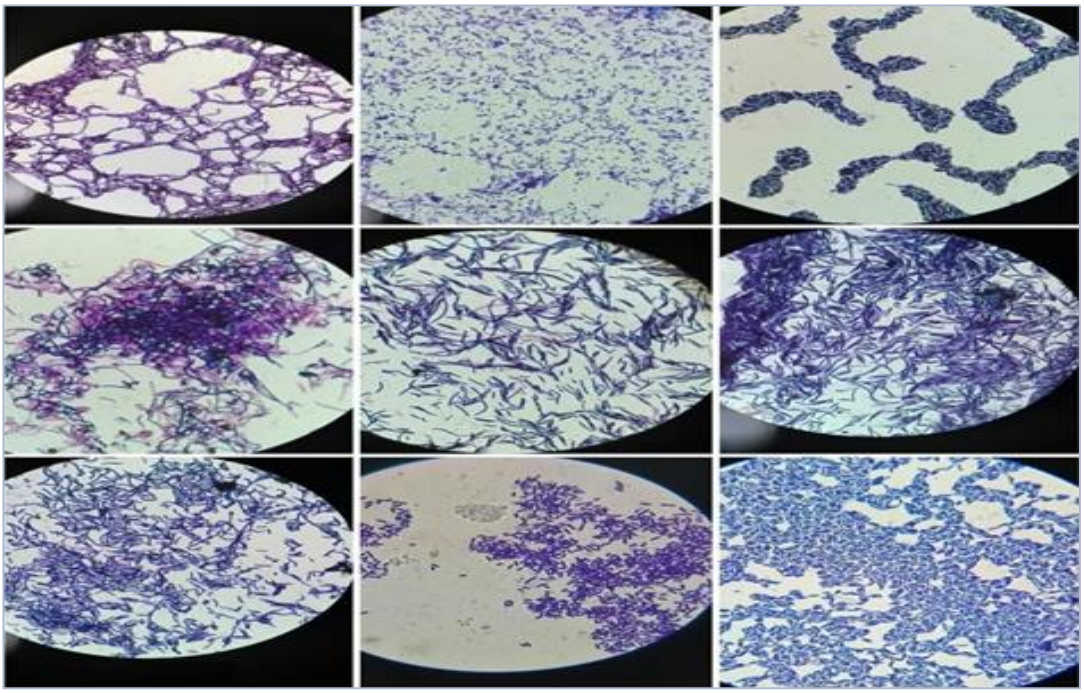
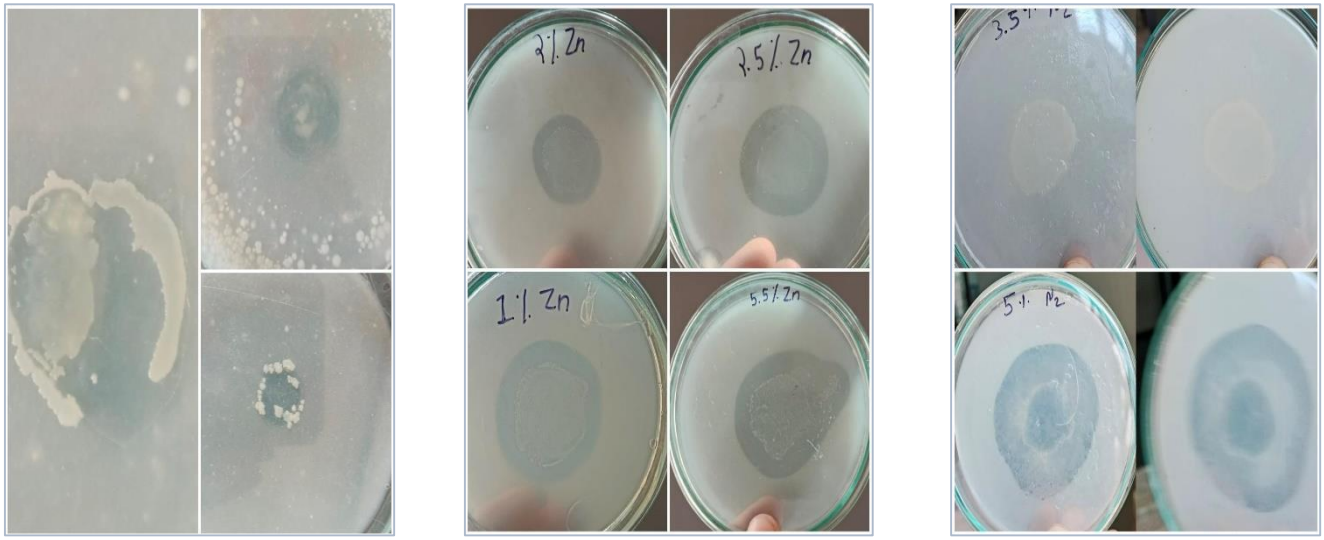


Figure 3: Typical characteristics of isolates using Gram staining method

(a) (b) (c)

Figure 4: A) Catalase test of isolates B) Motility test by agar stab method C) Gelatine hydrolysis



(a) (b) (c)

Figure 5: A) Solubilization of phosphate on Pikosvaskys Medium B) Zinc solubilization on zinc solubilization agar medium C) Nitrogen fixtation on Jensons agar medium

Test for nitrogen fixation of isolates: On nitrogen-free medium (Jenson agar media), the isolates with salt concentrations of 1 to 20% were able to grow (Fig. 5). However, growth on this medium declined with increasing halotolerant capability and no growth was seen in certain isolates. Nevertheless, using the appropriate procedure, the amount of fixed nitrogen still needs to be determined.

Test for determination of heavy metal tolerance of isolates: Table 6 shows the isolates' tolerance to heavy metals such as nickel (Ni).

The isolates could withstand concentrations of nickel up to 25µg, 50µg and 100µg with 25µg being the optimal growth range. In halotolerant isolates no growth was seen as the concentration of salt rises.

The development of clear zones confirms the solubilization of phosphates. These findings suggested that P-solubilization might be caused by certain chemicals secreted into the environment during growth, which have the ability to solubilize organophosphate or phosphate.

Table 6
O.D. AT 600 nm by spectrophotometer method for heavy metal tolerant

Organism with a salt content in percentages	O.D. at 600 nm 25µg	O.D. at 600 nm 50µg	O.D. at 600 nm 100µg
1	0.075	0.060	0.019
1.5	0.019	0.02	0.001
2	0.038	0.026	0.026
2.5	0.128	0.027	0.043
3	0.050	0.029	0.039
3.5	0.162	0.046	0.016
4	0.095	0.040	0.022
4.5	0.080	0.021	0.027
5	0.085	0.025	0.004
5.5	0.080	0.020	0.016
6	0.130	0.044	0.007
6.5	0.116	0.024	0.008
7	0.005	0.002	0.009
7.5	0.053	0.009	0.012
8	0.042	0.009	0.015
8.5	0.044	0.015	0.008
9	0.041	0.003	0.007
9.5	0.024	0.005	0.006
10	0.035	0.016	0.005
10.5	0.021	0.020	0.012
11	0.104	0.055	0.010
11.5	0.020	0.015	0.016
12	0.035	0.031	0.020
12.5	0.027	0.009	0.201
13	0.056	0.005	0.033
13.5	0.031	0.017	0.014
14	0.009	0.001	0.015
14.5	0.024	0.003	0.022
15	0.022	0.002	0.006
15.5	0.181	0.196	0.296
16	0.150	0.080	0.006
16.5	0.004	0.007	0.004
17	0.004	0.051	0.017
17.5	0.011	0.002	0.078
18	0.119	0.121	0.073
18.5	0.346	0.176	0.011
19	0.004	0.002	0.019
19.5	0.04	0.015	0.021
20	0.003	0.02	0.135

Results of phosphate solubilization are said to differ based on the kind of metabolism, the rate of release and the extent of the material's dispersion. The growth of the ZOS diameter of the isolates in the current investigation was reported to be between 1 and 3 cm; this was found to be significantly less than the 25 to 30 mm reported by Zhu et al[35].

Zinc is vital for development and growth. Soil bacteria are said to have a positive impact on plant growth and make a great alternative to chemical fertilisers. An attempt was made to isolate bacteria that solubilize zinc in the current investigation. To test these bacterial strains' capacity to solubilize zinc, they were screened on media that were already enriched with insoluble zinc salts. 1 to 10% isolates demonstrated varying solubilization efficiencies with zinc oxides, according to the results.

The formation of halo zones surrounding the bacterial colonies on solid medium served as a sign of the ZnO solubilization potential which varied from 1 to 5.4 cm. Zinc was most soluble in the isolate at a concentration of 5.5 NaCl. This suggests that zinc salts could be dissolved by isolated bacteria. Using different zinc sources, isolated bacteria were evaluated in multiple investigations for their ability to solubilize zinc[15]. For, free-living organisms to fix nitrogen, large volumes of organic matter are necessary for the process to be successful. It is stated that the free-living organisms can fix 12–30 g N kg^{-1} of carbon source. On Jensen agar medium, all 39 isolates were able to grow; however, the outcomes of nitrogen fixation remain unclear as no quantitative estimate was carried out.

Pot study

Seed germination experiment: The PGPR isolate treatments had an impact on all growth parameters of the plant throughout the pot study experiment. The case with 9.5% NaCl treatment resulted in the highest plant height ever measured. Every procedure was carried out in triplicate (Table 7 and Fig. 7).

According to reported research, *Bacillus* sp. was found to be tolerant of salinity and heavy metals[27]. It also produced the catalase enzyme, which protected plant cells from oxidative damage. Furthermore, the application of halotolerant bacteria had an impact on the plant's growth and yield.

Because of the salt stress circumstances that were able to slow down plant growth, the control treatment had the lowest average leaf area index. Reduced water and nutrient availability, as well as an excess of Na^+ and Cl^- in plant tissue, resulted in a reduction in leaf area and number in maize plants under salinity stress. Cell differentiation was suppressed during the increasing point.

Table 7
Treatment combination of inoculum for seed germination

Treatment 1 (soil+wheat)	**Control**
Treatment 2 (soil+wheat+organisms)	Nutrient broth + Organism
Treatment 3 (soil+wheat+organism + heavy metal)	Nutrient broth + Organism + Heavy Metal

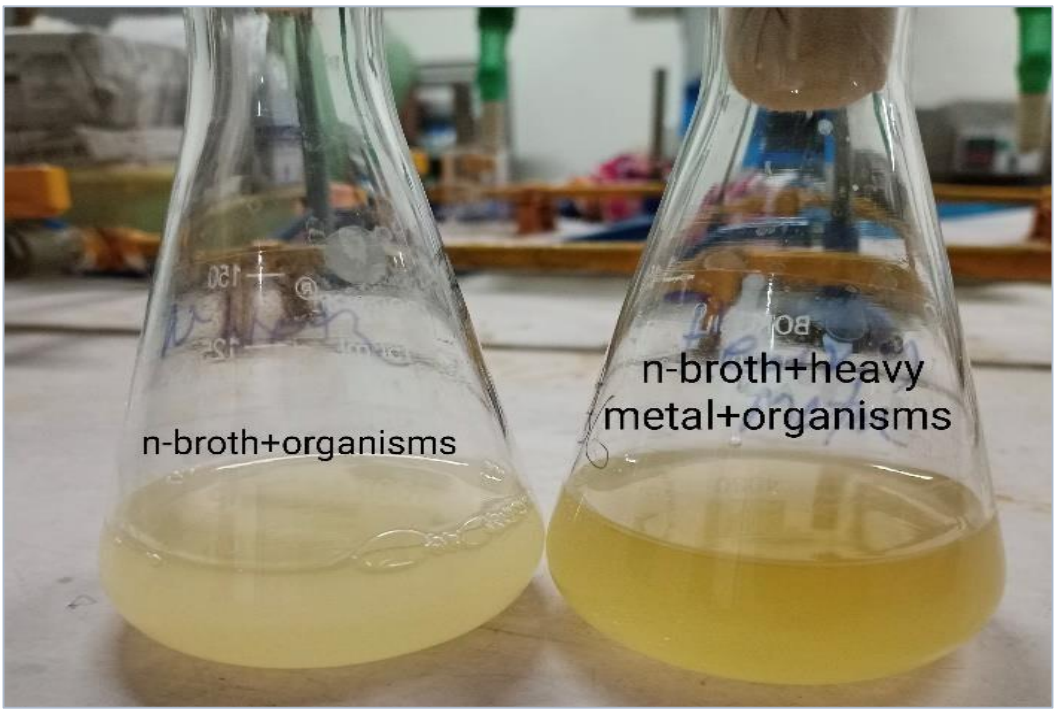


Figure 6: 9.5% NaCl enriched culture use as a inoculum for treatment of wheat seed

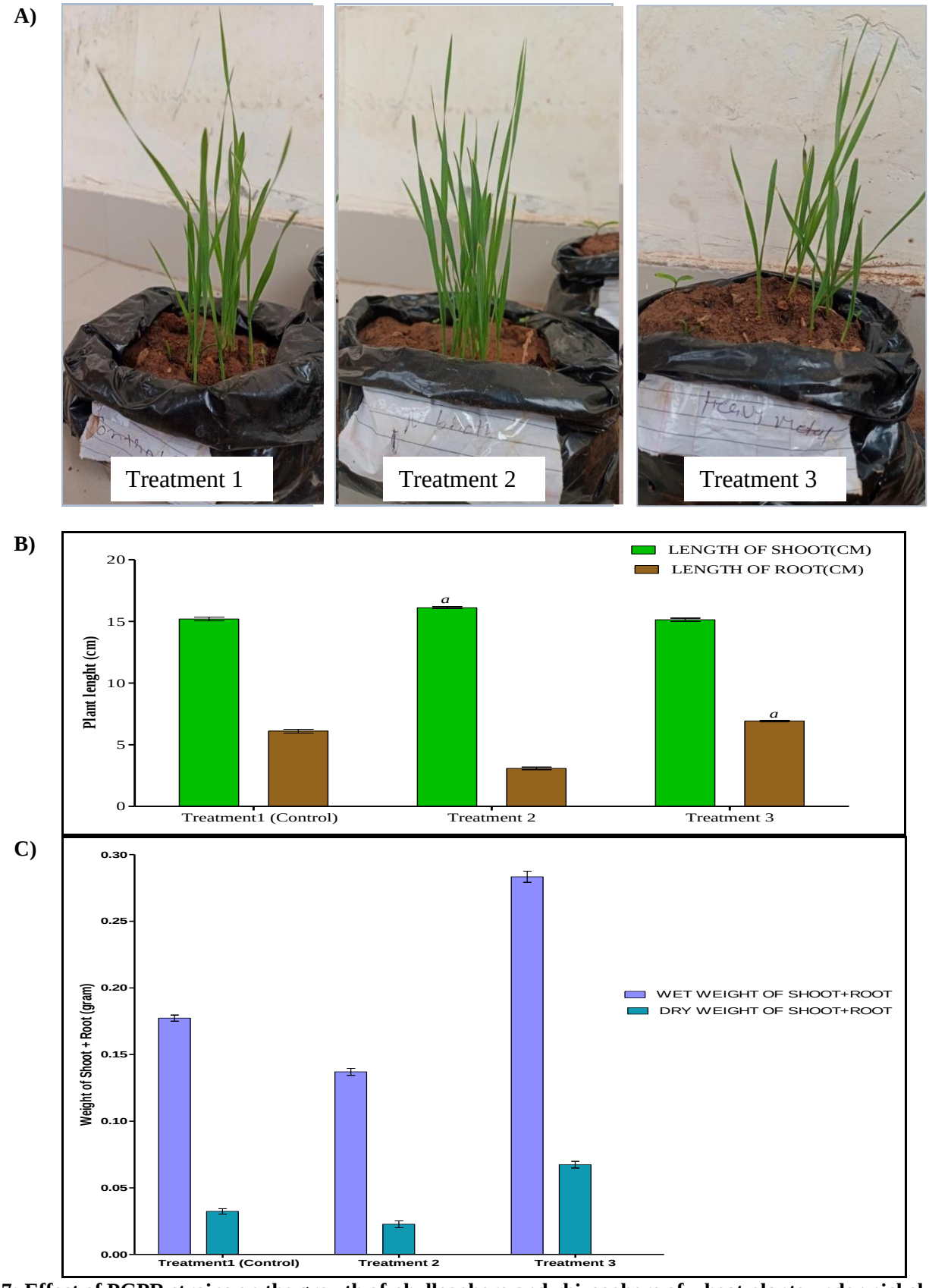


Figure 7: Effect of PGPR strains on the growth of phyllosphere and rhizosphere of wheat plants under nickel induced heavy metal stress. A) Photographs are representative of wheat plants each treatment (1: soil+wheat as control, 2: soil + wheat + PGPR strain, 3: soil + wheat + PGPR strain + heavy metal). B) Graph shows length of shoots and root of wheat plant after each treatment c) Graph shows Wet weight and dry weight of shoots and root of wheat plant after each treatment. ANOVA: The p value 0.0001 and [a] value are highly significant between wheat plant are treated for a period of 10 days

When halotolerant bacteria isolates were applied, the average leaf area index rose by 132.17% as compared to the control group. The halotolerant isolates in this investigation were able to withstand a 200 microgram concentration of the heavy metal nickel and an increase in plant growth as measured by morphometric traits was seen.

Conclusion

The soil samples of the Kutch of Gujarat were used for isolation of halotolerant bacteria. Characterization of several halotolerant PGPR isolated was performed. Such microbes can also be used as a source of plant growth promoter through pot study of wheat plant. However, field testing and studying their efficiency in promoting growth under natural conditions should be considered.

Future studies are needed to investigate the mechanisms of induced salinity tolerance by the identified PGPR strains at molecular levels. In conclusion, PGPR can be considered as sustainable alternative to biofertilizers to promote seed germination, biomass and wheat yield.

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