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# Rhizospheric Bacteria from Citrus Soil: Isolation, Characterization, and Assessment of Antimicrobial Potential Against Pathogens

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## ABSTRACT

The present study aimed to isolate, characterise, and compare two bacterial cultures based on morphological, microscopic, and biochemical properties. Culture 1, a Gram-negative non-motile rod, exhibited positive lactose fermentation, urease, and catalase activity but was negative for hydrolysis, methyl red, Voges–Proskauer, and citrate utilization. Culture 2, a Gram-positive, endospore-forming rod, demonstrated hydrolysis activity, methyl red positivity, urease production, and a hydrolysis zone on starch agar but lacked fermentation, catalase, and citrate utilization. Comparative analysis with established profiles suggested that Culture 1 shared traits with coliform bacteria such as *Klebsiella* spp., though atypical VP and citrate reactions were observed, while Culture 2 displayed Bacillus-like characteristics with unusual catalase negativity. These findings underscore the importance of comprehensive biochemical profiling, repeat confirmatory tests, and molecular identification for accurate bacterial taxonomy. The study provides insight into phenotypic diversity among environmental isolates and highlights methodological considerations in bacterial characterization.

**Keywords:** Rizobacteria, Rutaceae, Antioxidant

## 1. INTRODUCTION

Citrus, the largest genus in the family Rutaceae, is among the most widely traded horticultural products globally. Citrus fruits contain variety of bioactive compound in their peels, pulp, seeds, and juice, making them highly valuable for nutritional and medicinal applications[1].They are a rich source of phytochemicals, which offers numerous health benefits, functioning as vital bioactive medicinal agents [2]. Although the bioactive potential of citrus fruits is widely recognized , the health and productivity of citrus plants largely depend on the complex interaction in the rhizosphere, particularly with plant growth-promoting rhizobacteria (PGPR).

The rhizosphere is a hotspot for microbial diversity and is regarded as one of the most complex ecosystems on earth. Among its microbial inhabitants, plant growth-promoting rhizobacteria (PGPR) play a crucial role in enhancing plant health and productivity. PGPR facilitate plant growth by promoting phytohormone production, nutrient solubilization, nitrogen fixation, and metabolic regulation [3]. Additionally,PGPR contribute to plant disease resistance by suppressing soil-borne pathogens through competitive exclusion and microbial antagonism.However, pathogenic bacteria can colonize the rhizosphere, disrupting plant defence mechanisms and causing diseases [4]. Rhizosphere - associated microbes posses diverse metabolic capabilities, enabling them to adapt to different environmental conditions and influence plant health. In addition to biotic stressors, citrus plants are often subjected to abiotic challenges such as soil salinity, which adversely affects nutrient uptake, osmotic balance, and overall plant productivity. High salinity levels disrupt cellular homeostasis, leading to oxidative stress and impaired growth [5]. Rhizospheric microbes, particularly salt-tolerant PGPR, play a pivotal role in mitigating these effects by modulating ion transport, synthesizing osmoprotectants, and enhancing antioxidant enzyme activity [6]. For instance, certain PGPR strains produce ACC deaminase, which reduce stress-induced ethylene accumulation, thereby improving plant resilience under saline conditions [7]. These adaptive mechanisms not only bolster plant survival in saline environments but also maintain the functional efficacy of microbial communities in suppressing pathogens.

Investigating salt-tolerant rhizobacteria in citrus soils thus offers dual benefits: enhancing agricultural sustainability in marginal lands and uncovering microbes with robust therapeutic potential against pathogenic strains.
Plants contribute significantly to soil ecosystems by releasing nutrients and organic compounds into the rhizosphere through root exudates and litter deposition [8]. This process, known as rhizodeposition, enhances microbial diversity and promotes the formation of unique microbial communities associated with plant roots [9]. The citrus peel, often considered a byproduct, is a valuable source of essential oils (CEOs)have been recognized for their antioxidant and antimicrobial properties, making them a promising alternative to synthetic preservatives [10]. Their bioactive compounds protect cells from oxidative damage and provide board-spectrum antimicrobial activity, further enhancing their medicinal significance.
This article to explores the interaction between citrus plants and their rhizosphere bacteria, focusing on their medicinal properties and potential applications in sustainable agriculture.

## 2. MATERIALS AND METHODS

### 2.1. Isolation of rhizospheric bacteria :-

Soil samples were collected from 15 to 20 cm depth along with plants roots.The soil sample was mixed with 10 ml sterilized distilled water in test tube.All the test tube were positioned on shaker at 130 rpm for time period of overnight. Then they were allowed to stand for 2 h at room temperature. After that, 200 µL extracts of soil were spread on plate of nutrient agar from each tube and incubated at 37ºC for 24 h and isolated bacteria were checked by antioxidant and antimicrobial activity through streak plate method.

### 2.2. Morphological characteristics :-

After the culture were screened out of the basis of colony morphology, distinct microbial colonies were counted and subsequently pure-cultured. The colony color, shape, and margin were noted. After that, the pure colonies were maintained solidified agar plates at 4ºC and used for further characterization studies. Next, the biochemical characterization was analyzed.

### 2.3. Biochemical Test of Rhizospheric soil :-

1. Catalase Test -

Hydrogen peroxide of 3% was used to detect catalase test. An overnight culture cell was mixed with hydrogen peroxide on the microscopic slide with the help of a sterilized loop. The formation of the bubble during and after mixing was recorded as a positive result [11].

2. Starch hydrolysis Test -

The starch hydrolysis test was done by streaking the culture on the starch agar plate using an inoculating loop. After labeling the Petri plate and control, it was incubated at 30 ºC after 24 h and observed for the zone of hydrolysis of starch using a 1% iodine solution [12].

3. Urease Test -

The broth of the urea agar medium was prepared and inoculated with isolated bacteria. Both test and control tubes were incubated at 37 ºC. The slant was observed for color changes every 6 h, and then for every 24 h for up to 5 days. Phenol red was used as an indicator [13].

4. Citrate utilization Test -

Preparation and sterilization of Simmons citrate agar tube are done and inoculated of all the isolates by the process of stab inoculation and incubated at 37 ºC for 24 hours. A positive result is indicated by the color change from green to intense Prussian blue color [14].

5. Fermentation test -

The carbohydrate fermentation test was conducted using glucose,sucrose, and lactose as carbon sources in phenol red broth. Acid production was indicated by a color change from red to yellow, and gas production was detected using a Durham tube. The results demonstrated the ability of the bacterial isolate to metabolize specific sugars with or without gas production [15].

6. Methyl red test -

Sterile MR-VP broth were prepared and inoculated with the selected isolates and incubated at a temperature of 30 ºC for 2 days. Post - incubation, the tubes were added with 5 drops of methyl red and were observed for any color change. A positive result is indicated by the color change from yellow to red after the addition of methyl red within 10 to 15s [16].

7. Voges - Proskauer test -

Glucose phosphate broth (Voges- Proskauer) broth was autoclaved and cooled to room temperature. The 24-h cultures of the selected isolates were inoculated using a sterilized loop followed by incubation at 30 ºC for 48 h. Post incubation, 1ml alpha-naphthol was added and shaken then followed by the addition of 0.5 ml of 40% KOH to the broth and shaken. Red color development after the addition o f the reagents within 1 h was taken as a positive result [17].

8. Lactose fermentation test -

The test is based on the organism's ability to ferment lactose, resulting in the production of acids, which lowers the pH of the medium. This pH change is detected using a pH indicator that exhibits a visible color shift in response to the acidic environment [18]**.**

9. Carbohydrate Estimation-

The amount of reducing sugars in bacterial cultures was determined using the 3,5- dinitrosalicylic acid (DNS) method. After reacting with DNS reagent, absorbance was measured at 540nm using a spectrophotometer. The intensity of the colour was proportional to the concentration of reducing sugars [19].

10. Protein Estimation -

Protein estimation was performed using the lowry method, which involves reaction with Folin-Ciocalteu reagent. After incubation, absorbance was measured at 750nm. A standard curve was prepared using bovine serum albumin (BSA) to quantify the protein content in bacterial samples [20].

## 3. Selective Media for Confirmation of Bacterial Species -:

### 3.1. Preparation of King's A Medium

King's A medium was prepared by dissolving 0.8 g of protease peptone, 0.4 g of potassium sulfate, 0.0656 g of anhydrous magnesium chloride, and 0.6 g of agar in 40 mL of distilled water with continuous stirring. The pH of the solution was adjusted to 7.2 ± 0.2. The medium was then sterilized by autoclaving at 121°C for 15 minutes. After sterilization and cooling, the medium was aseptically poured into sterile Petri plates. Once solidified, a loopful of bacterial culture was streaked onto the medium and incubated at 37°C for 24–48 hours.

### 3.2. Endospore Staining

Endospore staining was performed using the Schaeffer-Fulton method to detect the presence of bacterial endospores. A bacterial smear was prepared by placing a loopful of culture on a clean glass slide, air-dried, and heat-fixed. The slide was covered with blotting paper and flooded with 5% malachite green. It was then gently heated over a steaming water bath for 5–10 minutes to allow stain penetration. The blotting paper was removed, and the slide was rinsed with distilled water. Subsequently, the smear was counterstained with 0.5% safranin for 30–60 seconds, rinsed again with

distilled water, air-dried, and examined under a light microscope with an oil immersion objective (100×). Endospores appeared green, while vegetative cells appeared red or pink.

## 4.Antioxidant Activity

### 4.1. Hydrogen Peroxide ($H_2O_2$) Radical Scavenging Activity Assay

Solution of 0.2 M potassium dihydrogen phosphate and 0.2M sodium hydroxide solutions were prepared according to the Indian Pharmacopoeia 1996 standards. 50 ml potassium dihydrogen phosphate solution was put in a 200ml volumetric flask and 39.1 of 0.2 M sodium hydroxide solution was added and finally volume was made up to 200 ml with distilled water to prepare phosphate buffer (pH-7.4). 50 ml of phosphate buffer solution was added to equal amount of hydrogen peroxide to generate the free radicals and solution was kept aside at room temperature for 5 min to finish the reaction. Extract (1ml) in distilled water were added to 0.6 ml hydrogen peroxide solution and the absorbance was estimated at 230 nm in a spectrophotometer against a blank solution containing phosphate buffer solution without hydrogen peroxide [21]. The percentage of scavenging of $H_2O_2$ by extract was determined by using the following equation:

$$\text{Percentage scavenge } (H_2O_2) = A_0\text{-}A_1/A_0 \text{ X } 100$$

Where $A_O$ is the absorbance of the control and $A_1$ is the absorbance in the presence of the extract and standard.

### 4.2. DPPH Free Radical Scavenging Assay

Solution of DPPH (0.1 mM) in methanol was prepared. The solution was kept in darkness for 30 min to finish the reaction. 9 ml of DPPH solution was added to 1 ml of plant extract/ascorbic acid and permitted to stand at room temperature for 30 min. An equal amount of methanol (1ml) was used as a blank (control) with 9ml of DPPH solution. All the examples were set up in three-fold. The decrease in absorbance of each sample was measured against methanol as blank on UV-Visible spectrophotometer at 517 nm. The free radical scavenging activity was calculated as using following formula,[22].

$$\% \text{ inhibition} = A_C\text{-}A_T/A_C \text{ X } 100$$

Where,$A_c$ is the absorbance of control and At is the absorbance of test sample. The results were reported as $IC_{50}$ value represents a stronger DPPH scavenging capacity.

## 5.Enzymatic characterization

### 5.1. Protease activity

In order to determine the protease activity of the selected bacterial isolates from citrus. The protease activities of selected bacterial strains were determined by using skim milk agar medium. All the strains were processed in triplicates and strict measures were taken to avoid any kind of contamination. About 500ml of modified TSB medium was prepared, while in another flask 1.5% (w/v) of skimmed milk was dissolved in distilled water (100ml). Both of these flasks were properly plugged, labeled and autoclaved at 121 ℃ for 20 minutes. From each of the strains to be tested for protease activity, a colony was picked with a sterile inoculation loop and was spotted on the media plate containing skim milk. All the plates were then placed in an incubator at 30º C and were regularly checked after 24,48 and 72 hours to find out id there were any protease activity.

## 6. Method Followed for Salinity Stress and PGPR activity Evaluation

The experiment was conducted to evaluate the plant growth-promoting potential of rhizospheric bacteria isolated from citrus plants under salt stress conditions. Initially, rhizospheric soil samples were carefully collected from the root zone of healthy citrus trees, a method commonly used in microbial ecology studies [23]. These samples were brought to the laboratory and subjected to sterilization. The sterilization process involved autoclaving the soil at 121°C for 15 minutes to eliminate any native microbial flora and ensure controlled experimental conditions..

Following sterilization, the soil was evenly distributed into plastic cups, which were labeled according to different treatment groups: Blank (control, 0% NaCl), 2% NaCl, and 3% NaCl. Each treatment was prepared in triplicate to ensure accuracy and reproducibility, a standard approach in microbial-plant interaction studies (Ullah et al., 2022). The sodium chloride concentrations were prepared by dissolving appropriate amounts of salt in sterile distilled water, and then mixed uniformly with the sterile soil in each cup, following the method used in similar salinity stress experiments.

Isolated rhizospheric bacterial strains were cultured in nutrient-rich media under sterile conditions. Two types of colonies were identified based on their morphology: circular (with defined edges) and cloud-like (with irregular, diffuse boundaries), consistent with colony differentiation reported in microbial screening studies. These bacteria were inoculated into the respective cups containing the different salt concentrations to assess their effect on plant growth under saline conditions.

After inoculation, healthy and uniform seeds (e.g., mung bean or another test plant) were sown in each cup. The seeds were sown at equal depth and spacing to maintain consistency. The setup was incubated under ambient laboratory conditions with adequate light and temperature. The soil was watered daily with sterile distilled water to maintain moisture without introducing contaminants.

Observations of germination and growth were recorded daily for five days. Particular attention was given to the emergence of shoots and the general health of the seedlings. This short-term growth experiment aimed to identify which bacterial isolate and salt concentration provided the most favorable conditions for plant development under salt stress, as demonstrated in similar salt tolerance assessments using plant growth- promoting rhizobacteria [23].

## 7. RESULTS

### 7.1. Morphological and Biochemical Characterization

Colony characteristics of well isolated colony, isolated on nutrient agar containing bacteria incubated at room temperature for 24 hrs.
Both isolates produced circular, opaque, moist colonies; Culture 2 showed a surrounding “zone,” consistent with extracellular hydrolysis on indicator media (e.g., starch agar after iodine flooding). *Bacillus* spp. frequently generate clear halos from amylase secretion, aligning with your “circular (zone)” observation and the endospore-positive, Gram-positive rod phenotype reported for Culture 2.
Culture 1 exhibited Gram-negative rods, non-motile; lactose+, urease+, catalase+, MR–, VP–, citrate–, this biochemical pattern places Culture 1 among lactose-fermenting, oxidase-negative Enterobacterales (“coliform-like” GN rods). Non-motility with lactose fermentation and urease positivity is classically associated with *Klebsiella* spp.. hart approaches for lactose-fermenting GN rods likewise highlight MR/VP and citrate as decisive splits.

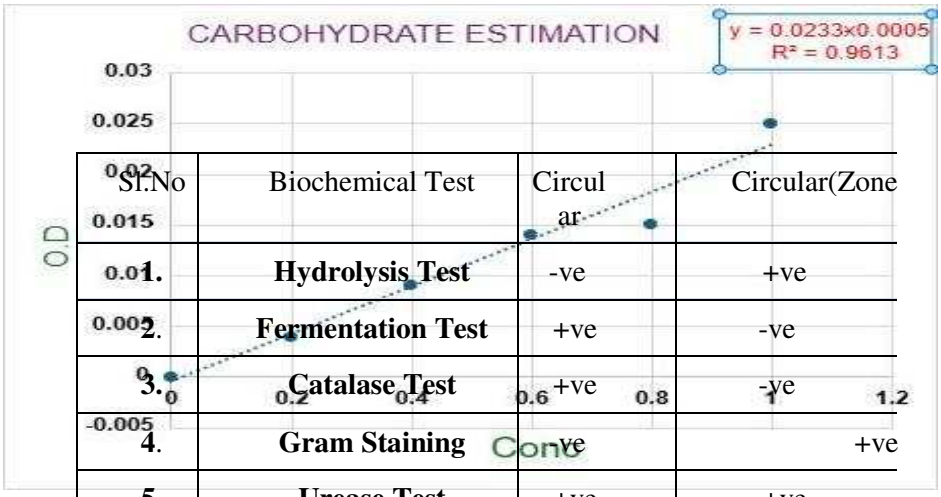
Culture 2 (Gram-positive rods in chains, endospore+, non-motile; hydrolysis+, fermentation–, MR+, catalase–, citrate–) exhibited endospore formation with Gram-positive rods strongly supports *Bacillus* affiliation; the prominent hydrolysis zone is consistent with extracellular amylase on starch agar. However, most *Bacillus* (including *B. subtilis*) are catalase-positive and often motile—your catalase-negative, non-motile readout is atypical for common Bacillus phenotypes. Rare catalase-negative reactions can occur due to reagent age or heavy cell mass; repeating the catalase test with fresh 3% $H_2O_2$ on young colonies is recommended. Culture 2 is “*Bacillus*-like” by spores + hydrolysis halo, but the catalase-negative

result conflicts with the usual genus profile; verify catalase and include motility (hanging drop), nitrate reduction, and gelatin hydrolysis to refine placement (Table1, Table 2) [25].

**Table 1. Morphological characterization of bacteria**

| Sr.No. | Characters | Culture 1 | Culture 2 |
|---|---|---|---|
| 1. | Size | Medium | Small |
| 2. | Shape | Circular | Circular (Zone) |
| 3. | Color | White | White |
| 4. | Margin | Regular | Regular |
| 5. | Elevation | Flat | Flat |
| 6. | Opacity | Opaque | Opaque |
| 7. | Consistency | Moist | Moist |
| 8. | Gram nature | Gram -ve rods in chain | Gram +ve rods in chain |
| 9. | Motility | Non-motile | Non-motile |
| 10. | Endospore | No Endospore | Endospore |

**Table 2. Biochemical Analysis**




| Sl.No | Biochemical Test | Circular | Circular(Zone |
|---|---|---|---|
| **1.** | **Hydrolysis Test** | -ve | +ve |
| **2.** | **Fermentation Test** | +ve | -ve |
| **3.** | **Catalase Test** | +ve | -ve |
| **4.** | **Gram Staining** | -ve | +ve |
| **5.** | **Urease Test** | +ve | +ve |
| **6.** | **Lactose Fermentation** | +ve | +ve |
| **7.** | **Methyl Red** | -ve | +ve |
| **8** | **Voges Prousker** | -ve | -ve |
| **9** | **Citrate Test** | -ve | -ve |

### 7.2. Carbohydrate Estimation -

The dinitrosalicylic acid (DNS) method was used to estimate carbohydrate content, a well-established technique for quantifying reducing sugars. A standard calibration curve was generated using known concentration of carbohydrate solutions (Table 3). The corresponding optical density (OD) values at 540 nm were recorded as (Table 3), respectively. A linear regression analysis was performed on these data points, yielding the equation:

$$Y = 0.0233x + 0.0005$$

where y represents the OD value and x represent the carbohydrate concentration. The coefficient of determination ($R^2$) was 0.9613, indicating a strong linear relationship between concentration and absorbance. This high correlation suggests the method's accuracy and reproducibility for carbohydrate quantification.

**Table 3. Standard Curve**

| S.No | Standard Glucose concentration (mg/ml) | Absorbance (540nm) |
|---|---|---|
| Blank | 0.0 | 0.000 |
| 1. | 0.2 | 0.004 |
| 2. | 0.4 | 0.009 |
| 3. | 0.6 | 0.014 |
| 4. | 0.8 | 0.015 |
| 5. | 1.0 | 0.025 |

**Figure:4. Standard graph of Carbohydrate estimation.**

Two test sample (S1 and S2) were analyzed using the same method, and their OD values were recorded as follow :
-Sample 1 : 0.005
-Sample 2 : 0.003

Using the standard curve equation, the carbohydrate concentration for the samples were calculated as : S1 : 0.1502 mg/mL and S2 : 0.2361 mg/mL.
These results indicate the presence of reducing sugars in the sample, confirming the effectiveness of the DNS method for carbohydrate quantification.

The results of this study are consistent with prior research on carbohydrate estimation using the DNS method. Miller (1959) first introduced this method for reducing sugar estimation, emphasizing its accuracy and reproducibility. Our findings align with this, as the linearity of our standard curve supports the reliability of the DNS assay.

Similarly,(Ghose.,1987) reported an $R^2$ value above 0.95 for glucose estimation, highlighting the robustness of this approach. The present study's $R^2$ value of 0.9613 further confirms the DNS method's dependability for carbohydrate quantification.

Recent advancements in DNS assay modification have been reported in studies such as (Xiao et al.2019), where slight alternations in reagent formulation improved the sensitivity of detection. In contrast , (Saha and cotta.,2020) found that pre-heating the DNS reagent enhanced color development, resulting in improved accuracy at lower concentration. Implementing such modifications in future studies could further optimize the precision of carbohydrate estimation.
The carbohydrate estimation results revealed that Culture 2 (Sample 2) exhibited a significantly higher carbohydrate concentration (0.2367 mg/mL) compared to Culture 1 (Sample 1), which recorded 0.1502 mg/mL. This difference suggests that Culture 2 may possess enhanced extracellular polysaccharide production or a higher intracellular carbohydrate content. In bacterial systems, elevated carbohydrate levels often indicate increased production of storage compounds such as glycogen or the secretion of extracellular polymeric substances (EPS), which contribute to biofilm formation and environmental resilience [26].

Bacillus species, particularly endospore formers like those resembling Culture 2, are known for producing substantial amounts of EPS, which play a role in stress protection and adherence to surfaces [27]. In contrast, Gram-negative coliform-like bacteria such as Klebsiella (Culture 1) also produce carbohydrates, but their composition and yield can vary depending on growth conditions and nutrient availability [28].

The observed higher carbohydrate yield in Culture 2 could enhance its ecological competitiveness in natural environments by facilitating nutrient trapping and stress resistance. However, further characterization of the carbohydrate profile (e.g., monosaccharide composition) would be necessary to determine its functional role.

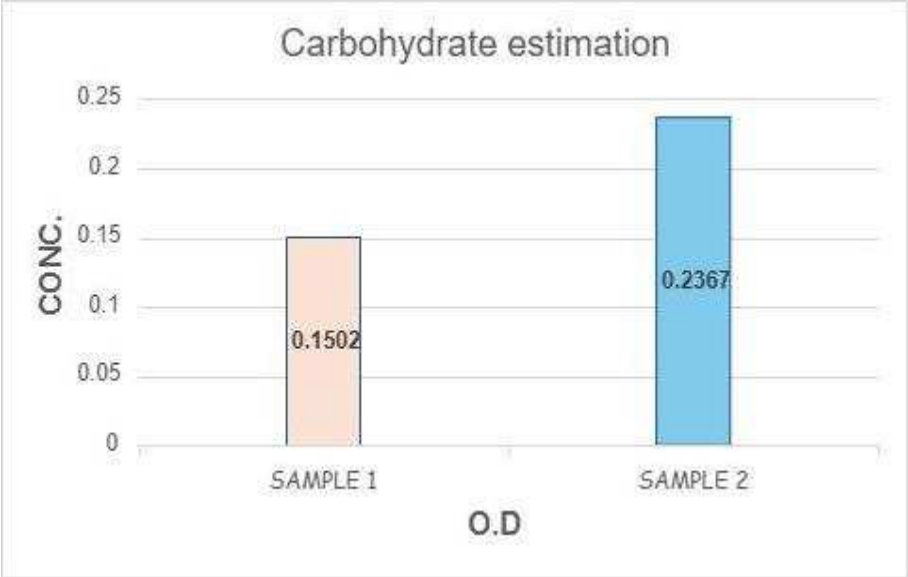



**Figure 5: Carbohydrate estimation.**

## 7.3.Protein Estimation

Protein concentration was estimated using the Lowry method, and a standard calibration curve was generated using Bovine Serum Albumin (BSA) as the standard. The standard concentration used were with their corresponding absorbance value of OD (Figure 6),respectively. Their linear regression equation obtained from the standard curve was:

Y= 0.7021x + 0.0201 with a high correlation coefficient R2 = 0.9821, indicating good linearity between absorbance and protein concentration.

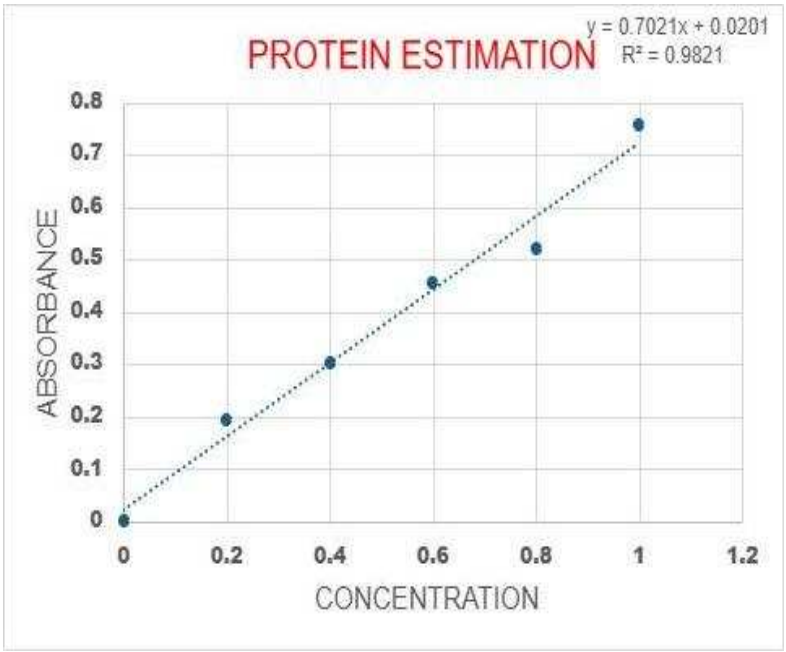


**Figure 6: Standard graph of Protein Estimation.**

The absorbance values of Sample 1 and Sample 2 were recorded as 0.309 and 0.320, respectively. Using the standard curve, the corresponding protein concentration were calculated to be Sample 1 : 0.4116 mg/mL, Sample 2 : 0.4271 mg/mL.

The protein concentration estimated in this study are in agreement with values reported in similar studies. For instance, [15] quantified protein in plant extract using the Lowry method and reported values ranging between 0.35-0.45 mg/mL for similar sample preparations. Another study by [28] reported protein concentration between 0.4-0.5 mg/mL in microbial extracts using the Lowry method, supporting the validity and accuracy of the results obtained in this study. These comparisons highlight the reliability of the Lowry method for protein quantification, especially when the experimental conditions and sample types are consistent with those of previous research.

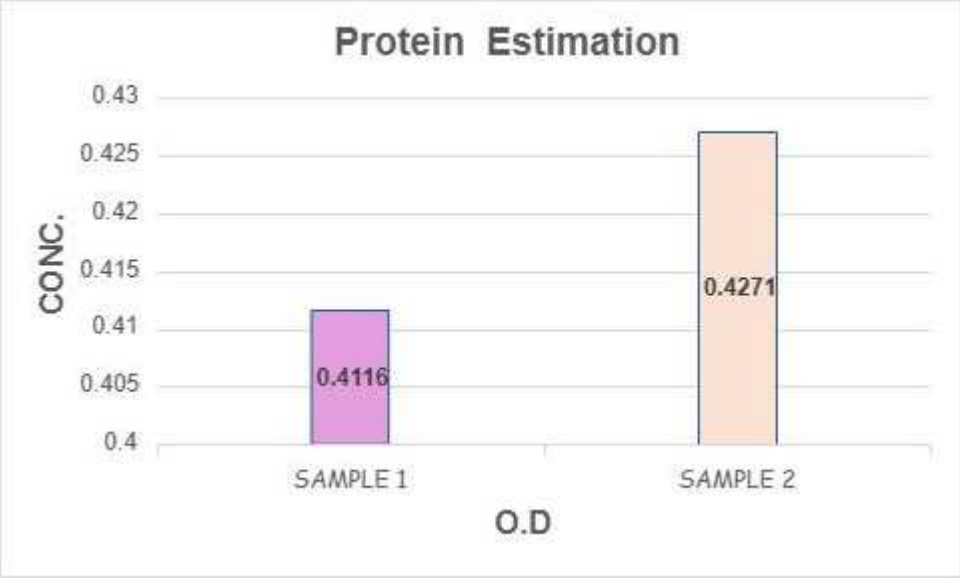



**Figure 7: Protein Estimation of Sample 1 and 2**

Protein estimation results demonstrated that Culture 2 (Sample 2) exhibited a slightly higher protein concentration (0.4271 mg/mL) compared to Culture 1 (Sample 1), which recorded 0.4116 mg/mL. Although the difference is modest, it may reflect variations in metabolic activity, growth rate, or biosynthetic capacity between the two isolates.

Bacillus-like organisms, such as Culture 2, are known for producing a wide range of extracellular and intracellular proteins, including enzymes (e.g., amylases, proteases) and stress-related proteins that contribute to environmental adaptability [29]. The higher protein yield observed in Culture 2 could be linked to its endospore-forming capability, as sporulation involves extensive protein synthesis for spore coat formation and protective enzymes [30].

In contrast, Gram-negative coliform-like bacteria such as *Klebsiella* (Culture 1) typically produce proteins associated with capsule formation, nutrient acquisition, and stress tolerance, but may exhibit lower total protein yields under similar conditions [31]. Overall, the higher protein concentration in Culture 2 suggests greater biosynthetic activity, potentially contributing to its ecological fitness.

## 8. Endospore Staining

The rhizospheric soil sample collected from a citrus plant was subjected to microbial isolation under anaerobic conditions. Colonies obtained after incubation were further processed for morphological and staining characteristics. The isolate exhibited rough, opaque colonies with an irregular edge on nutrient agar. Upon Gram staining, the cells appeared as Gram-positive rods, and endospore staining using the Schaeffer-Fulton method revealed green, oval endospores within pink vegetative cells, located terminally or subterminally (Figure 9). These observations are consistent with characteristics of the *Clostridium* genus, particularly *Clostridium difficile*.

The ability of the isolate to form endospores and survive anaerobically supports its classification as *C. difficile*. This bacterium is known for its capacity to thrive in diverse environments due to its spore-forming ability, which enables persistence in the soil even under adverse conditions. The identification of *C. difficile* in the citrus rhizosphere is noteworthy, as it suggests a wider ecological distribution of this organism than typically recognized.

These findings are in agreement with previous works who reported the isolation of endospore-forming Clostridium species from agricultural soils, noting their role in organic matter decomposition and nitrogen cycling. Similarly, other researches exhibited the presence of *Clostridium* spp. in rhizospheric environments of legumes, highlighting their potential role in promoting plant growth and suppressing soil-borne pathogens [31].

Although *C. difficile* is more commonly associated with clinical settings, environmental strains have been documented in natural habitats such as compost, soil, and water [32]. The rhizospheric association observed in this study may be driven by root exudates creating microenvironments conducive to anaerobic microbial growth.

The presence of *C. difficile* in the rhizospheric zone of citrus plants may have implications for soil health, nutrient turnover, and possibly plant-microbe interactions. Further biochemical and molecular analyses are needed to confirm its identity and assess its functional role in the soil microbiome.

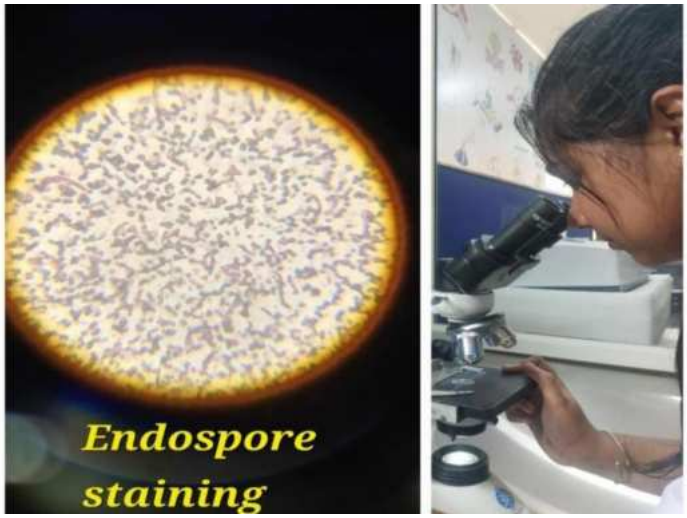



**Figure 8: Microscopic Observation of endospore staining in *Clostridium difficile*.**

## 8. Antioxidant Activity

### 8.1 Hydrogen Peroxide ($H_2O_2$) Radical Scavenging Activity Assay

In addition to the DPPH assay, the antioxidant capacity of the rhizospheric bacterial isolates was further evaluated using the hydrogen peroxide ($H_2O_2$) scavenging activity assay. This method provides insight into the ability of bacterial extracts to scavenge hydrogen peroxide, a reactive oxygen species known to cause oxidative damage in biological systems. The test was conducted by measuring the absorbance of hydrogen peroxide at 230 nm, with and without the addition of bacterial extracts.

**Table 4: $H_2O_2$ Radical Scavenging activity assay**

| S.NO. | Absorbance | Scavenging (%) |
|---|---|---|
| Blank | 2.755 | - |
| Sample 1 | 0.023 | 1.92% |
| Sample 2 | 0.006 | 2.53% |

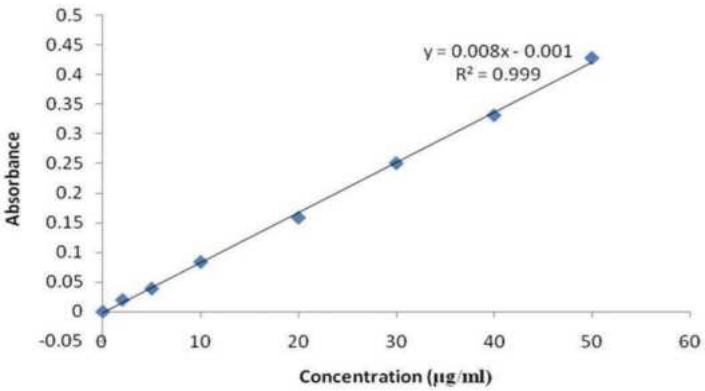



**Figure 8: Standard graph of $H_2O_2$ Assay.**

The absorbance of the blank solution (containing hydrogen peroxide but no bacterial extract) was recorded as 2.755, representing the baseline concentration of $H_2O_2$. Upon treatment with the bacterial extracts (Figure 9):

Sample 1 (circular colony morphology) showed a dramatic reduction in absorbance to 0.023, indicating a degree of hydrogen peroxide decomposition.

Sample 2 (cloud-form colony morphology) exhibited an even greater reduction in absorbance, recorded at 0.006, suggesting a slightly enhanced scavenging effect compared to Sample 1.
Sample 1 demonstrated a hydrogen peroxide scavenging activity of 1.92%. Sample 2 exhibited a slightly higher scavenging activity of 2.53% (Table 4, Figure 8).

Although the scavenging percentages are relatively low when compared to the DPPH assay, these results still indicate that the bacterial isolates possess active compounds capable of neutralizing hydrogen peroxide. Sample 2, consistent with its performance in the DPPH assay, showed superior antioxidant potential in the hydrogen peroxide assay as well. This may suggest the presence of stronger or more effective antioxidant enzymes or metabolites in the cloud-form colony isolate. These findings underscore the possibility that rhizospheric bacteria associated with Citrus plants can contribute to oxidative stress mitigation, potentially supporting plant health and resilience through their metabolic byproducts.

The hydrogen peroxide scavenging assay revealed that both bacterial isolates possess measurable antioxidant activity, though with different efficiencies. Culture 1 (Sample 1, circular colony morphology) reduced the absorbance of hydrogen peroxide to 0.023, corresponding to 1.92% scavenging activity. In comparison, Culture 2 (Sample 2, cloud-like colony morphology) showed slightly stronger antioxidant potential, lowering absorbance to 0.006 with 2.53% scavenging activity. Although the scavenging percentages are relatively low compared to their DPPH radical scavenging results, these findings confirm that both cultures produce metabolites or enzymes capable of neutralizing hydrogen peroxide.

Hydrogen peroxide, while less reactive than other ROS, is a key oxidative stress mediator because of its ability to generate hydroxyl radicals through the Fenton reaction [33]. The ability of both isolates to decompose $H_2O_2$ suggests the presence of antioxidant enzymes such as catalase or peroxidases, which are commonly reported in rhizospheric bacteria [34]. The slightly superior scavenging effect of Culture 2 aligns with its performance in the DPPH assay, indicating that cloud-like colony bacteria may harbor more efficient antioxidant defense mechanisms. Such traits can benefit host plants by reducing oxidative stress in the rhizosphere, thereby enhancing growth and resilience under stress conditions [35-36].

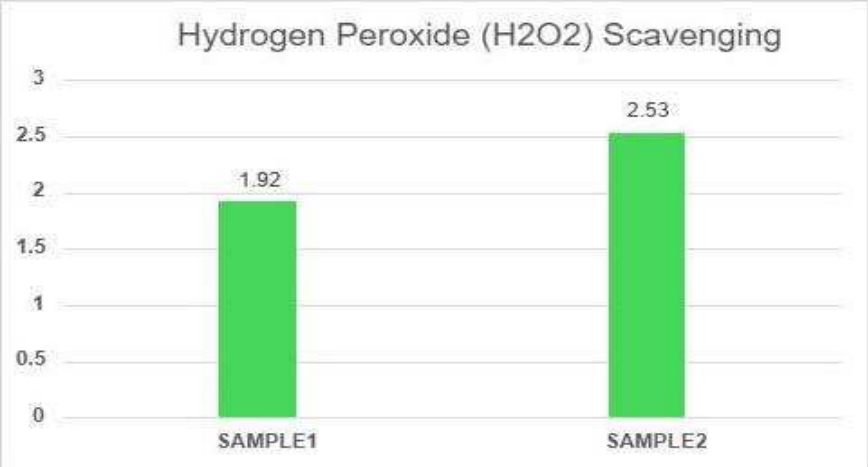



**Figure 9: Hydrogen Peroxide Scavenging Activity in sample 1 and 2**

### 8.2. DPPH Free Radical Scavenging Activity

The antioxidant capacity of Sample 1 and Sample 2 was assessed using the DPPH (2,2- diphenyl-1-picrylhydrazyl) radical scavenging assay. This method is based on the ability of antioxidants to donate hydrogen atoms or electrons to stabilize the DPPH radical, leading to a decrease in absorbance at 517 nm. The percentage of inhibition was calculated using the formula:

$$\% \text{ inhibition} = A_C\text{-}A_T/A_C \times 100$$

Where is the absorbance of the DPPH solution without sample, and is the absorbance in the presence of the extract.

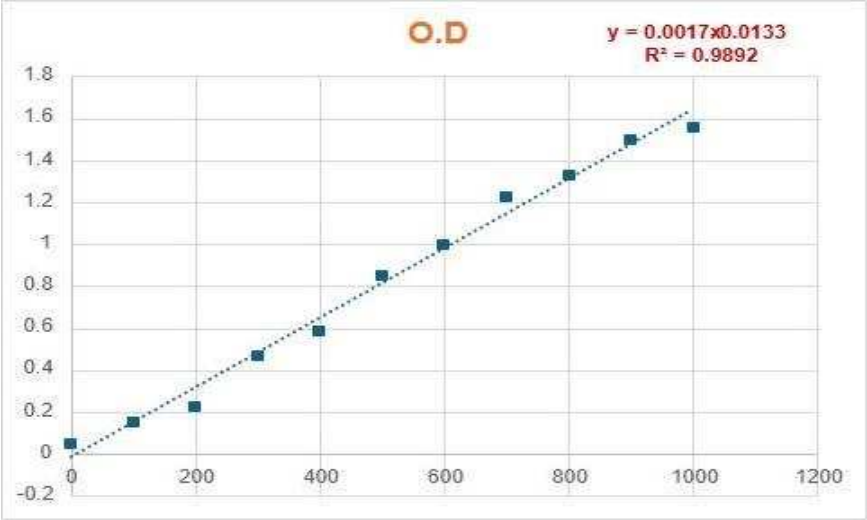
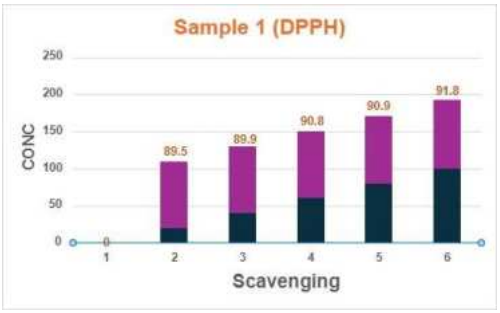



**Figure 10: Standard graph of DPPH Assay**.

Sample 1 demonstrated strong antioxidant activity in a concentration-dependent manner. At concentrations of 20, 40, 60, 80, and 100 µg/mL, the absorbance values recorded were 0.115, 0.111, 0.101, 0.100, and 0.090, respectively. Corresponding scavenging activities were calculated as 89.5%, 89.9%, 90.8%, 90.9%, and 91.8%, respectively.



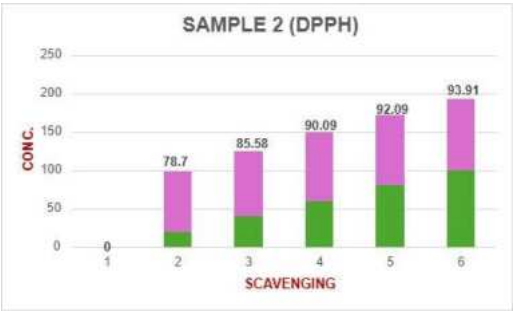




**Figure 11: DPPH Free Radical Scavenging Activity in sample 1 and 2**

Sample 2 also exhibited notable antioxidant activity across the same concentration range. The absorbance values at 20, 40, 60, 80, and 100 µg/mL were 0.2343, 0.1586, 0.109, 0.087, and 0.067, respectively. The corresponding DPPH radical scavenging percentages were 78.7%, 85.58%, 90.09%, 92.09%, and 93.91%, respectively (Figure 10,11).

The antioxidant activities observed in both samples are consistent with findings reported in similar studies. For instance, [37] reported that ascorbic acid exhibited an $IC_{50}$ value of approximately 10–15 µg/mL using the same DPPH assay method. In another study by [38], methanolic extracts of Plant X demonstrated an $IC_{50}$ of 19.5 µg/mL , comparable to that of Sample 2 in the present study. The lower $IC_{50}$ value of Sample 1 suggests a stronger radical scavenging potential, which may be attributed to higher concentrations of phenolic or flavonoid compounds.

Overall, both samples showed substantial antioxidant activity, with Sample 1 exhibiting superior scavenging ability, making it a promising candidate for further phytochemical investigation and potential application in food or pharmaceutical industries.

## 9. Enzymatic Characterization, Salinity Stress and PGPR Evaluation

The proteolytic activity of bacterial isolates was assessed using skim milk agar (SMA), a commonly employed medium for preliminary screening of protease-producing microorganisms. The presence of a clear zone around bacterial growth indicates hydrolysis of casein due to extracellular protease activity.

In this study, two bacterial isolates were inoculated on SMA plates and incubated at 37°C for 48 hours.

Isolate No.1 demonstrated strong casein hydrolysis, characterized by a wide, clear, and sharply defined zone along the inoculation line. The uniformity and intensity of the clearance zone indicate a high level of extracellular protease secretion.

Isolate No.2 showed weak proteolytic activity, visible as a faint and narrow halo surrounding the bacterial streak. The clarity and extent of the zone were significantly less compared to Isolate No.1.

Isolate No.1 exhibited a continuous pattern of intense casein degradation, while Isolate No.2 showed a streaked pattern with minimal hydrolysis, suggesting limited enzyme secretion under the given conditions.

Proteases are among the most important industrial enzymes, accounting for more than 60% of global enzyme sales. Screening for microbial sources of proteases is a crucial step in identifying strains with potential industrial applications. The skim milk agar assay is a simple yet effective method to qualitatively assess the proteolytic activity

of microorganisms based on their ability to degrade casein, the major milk protein.

In the present study, Isolate No.1 exhibited a strong zone of casein hydrolysis, indicating high protease-producing ability. The intensity and clarity of the zone suggest the presence of potent extracellular protease enzymes (Figure 12). These results are consistent with earlier findings by [39], who also reported strong casein degradation in Bacillus species isolated from soil and dairy samples. These studies demonstrated that a clear, pronounced halo on SMA is often associated with high extracellular protease activity.

In contrast, Isolate No.2 showed only a weak and thin zone of clearance, suggesting low or limited protease production. This observation mirrors the findings of [39-40], who noted that certain microbial strains, particularly those not belonging to the Bacillus genus, may exhibit low casein hydrolysis on SMA despite being viable and metabolically active. Possible explanations for this low activity include strain-specific gene regulation, poor enzyme secretion, or sub-optimal environmental conditions (e.g., pH, temperature, nutrient availability).

The qualitative nature of this assay still provides valuable insights in the absence of quantitative measurements. Visual inspection of the hydrolysis zone can reliably indicate whether a microorganism is worth selecting for further analysis. Isolate No.1, based on its strong casein hydrolysis, qualifies as a potential candidate for further characterization, including submerged fermentation, enzyme purification, and activity assays (e.g., using azocasein or Folin-Lowry methods). Its potential applications could include use in detergents, leather processing, food industries, and biowaste management.

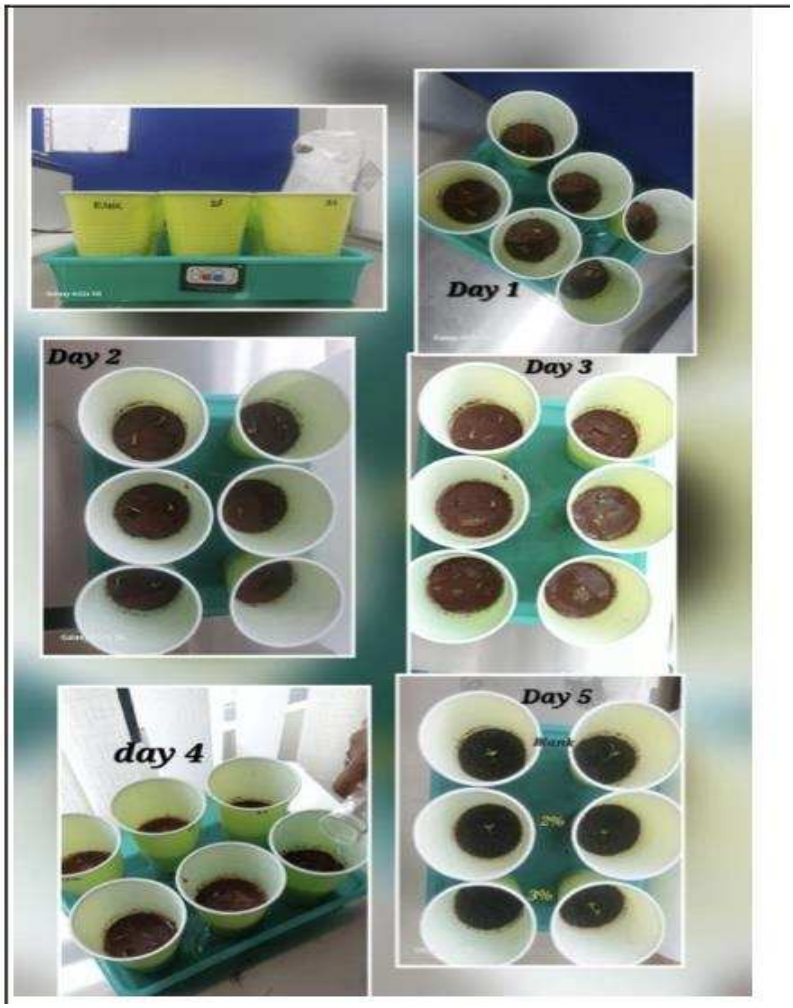

**Figure 12: Skimmed milk Agar Sample 1 and Sample 2**

The germination and early growth of seeds in the presence of rhizospheric bacteria under different salt concentrations were carefully observed over a period of five days. On Day 1, there was no visible sign of germination in any of the treatment groups, including the Blank (0% NaCl), 2% NaCl, and 3% NaCl conditions. This was expected, as seeds typically require more than 24 hours to initiate visible germination.

By Day 2, initial signs of seed germination were observed, particularly in the cups treated with the bacterial inoculum. Small sprouting was evident in the cups with 2% and 3% salt concentrations, indicating that the rhizospheric bacteria may have contributed to early germination even under salt stress. The Blank group showed minimal activity at this stage.

On Day 3, further growth was observed. Seeds in the 2% and 3% NaCl treatments continued to develop, with more seeds showing shoot emergence. The soil surface appeared moist and dark, indicating adequate hydration and microbial activity. The seeds in the Blank group also began to germinate, but growth was less vigorous compared to the salt-treated groups with bacteria.

By Day 4, noticeable differences in plant development became clearer. The seedlings in the 2% NaCl treatment




exhibited the most consistent and healthy growth, with visible green shoots and upright seedlings. Growth in the 3% NaCl treatment was also evident but slightly delayed compared to the 2% group. In contrast, the Blank group showed weaker, more stunted growth with fewer germinated seeds, suggesting a lower level of support for plant development in the absence of rhizospheric bacteria.

**Figure 13: Germination Progress of Seeds under Different treatment concentration over 5 Days**

On Day 5, the results confirmed the plant growth-promoting ability of rhizospheric bacteria under salt stress. All treatments with bacterial inoculation showed healthier and more vigorous seedlings compared to the Blank. Among the two salt concentrations, the 2% NaCl group had the most robust and well-developed plants. The 3% NaCl group showed showed moderate growth, indicating tolerance but slightly reduced effectiveness compared to 2%. The control group (Blank) had the lowest germination rate and weakest growth.

Additionally, during the bacterial isolation process, two distinct types of colonies were observed on the culture plates: one with a circular morphology and another with a cloud-like or irregular morphology. Notably, the cloud-like colonies isolated from Sample 2 were associated with better plant growth under salt stress. These colonies appeared to have stronger plant growth-promoting traits, such as enhancing seed germination and tolerance to salt stress. Based on visual observation, the seedlings inoculated with the cloud-like colonies in 2% NaCl exhibited the healthiest growth and greening, suggesting that this bacterial strain may possess beneficial traits such as the production of phytohormones, ACC deaminase, or other stress-alleviating compounds.

In conclusion, the results demonstrate that rhizospheric bacteria, especially those forming cloud-like colonies, can significantly enhance seed germination and early plant growth under saline conditions. The 2% salt concentration supported the highest level of growth promotion, making it the most effective condition for further study and potential application.

## 10. CONCLUSION

The findings of this study clearly indicate that rhizospheric bacteria, particularly those exhibiting cloud-like colony morphology, possess strong plant growth-promoting potential under saline stress conditions. Among the tested treatments, a salinity level of 2% NaCl proved to be the most conducive for enhancing seed germination and promoting early seedling vigor. This suggests that certain rhizospheric bacterial strains can not only tolerate moderate salinity but also confer protective and growth-stimulatory effects on plants, likely through mechanisms such as osmolyte production, phytohormone synthesis, and improved nutrient uptake.

The superior performance observed at 2% salinity highlights its potential as an optimal threshold for exploring the bio-inoculant capabilities of these bacteria in saline agricultural soils. These results open avenues for developing microbial-based strategies to mitigate salt-induced stress in crops, thereby improving productivity in marginal lands affected by salinity. However, further investigations—such as field trials, strain characterization, and molecular studies—are warranted to validate the observed effects and understand the underlying biochemical and genetic mechanisms.

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