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Unveiling Bacterial Communities in *Shankapushpi* (*Clitoria ternatea* L.) : Isolation and Biochemical Characterization

Haralakal Keerthi Kumari and K. Nagaraju
Department of Agricultural Microbiology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
e-Mail : keerthihk2283@gmail.com

Authors Contribution

Haralakal Keerthi Kumari :
Carried out experiment, data analysis and draft prepration

K. Nagaraju :
Conceptualization, supervision and draft correction

Corresponding Author :
Haralakal Keerthi Kumari

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Abstract

Shankapushpi (*Clitoria ternatea* L.), a medicinal plant of significant pharmacological importance, harbours diverse bacterial communities that play crucial roles in its growth, nutrient cycling and overall plant health. This study aimed to isolate and biochemically characterize bacterial communities associated with *C. ternatea* to identify their functional diversity and tentative taxonomic placement. A total of 80 bacterial isolates were obtained from the rhizosphere, rhizoplane and root nodules of *C. ternatea* and subjected to morphological and biochemical characterization using standard tests, including gram reaction, catalase, Voges-Proskauer, urease, indole production, citrate utilization, H_2S production, motility, oxidase and starch hydrolysis assays. The results revealed the presence of multiple bacterial genera belonging to both Gram-positive and Gram-negative groups. Nine isolates cut down in to the Bacillus group and were predominantly identified among Gram-positive rod isolates due to their catalase positivity and starch hydrolysis activity. *Staphylococcus* spp. and *Micrococcus* spp. were identified among Gram-positive cocci isolates. Gram-negative rods were primarily represented by *Pseudomonas* spp., characterized by citrate utilization and urease production. Other Gram-negative small rods and cocci were identified as *Acinetobacter* spp. (21 isolates), while *Azotobacter* spp. (9 isolates) were observed among Gram-negative cocci isolates, exhibiting citrate utilization and indole production. Additionally, rare bacterial genera such as *Neisseria, Bradyrhizobium, Enterobacter* and *Klebsiella* spp. were identified based on specific biochemical traits. The study highlights the diversity of bacterial communities in the rhizosphere, rhizoplane and root nodules of *C. ternatea,* indicating their potential roles in plant growth promotion, nitrogen fixation and nutrient cycling. These findings provide valuable insights into the plant-microbe interactions in medicinal plants and emphasize the need for molecular tools such as 16S rRNA sequencing for precise taxonomic confirmation. Exploring such microbial diversity could further aid in the development of bioinoculants for sustainable agriculture and plant health management.

Keywords : Bacterial communities, Biochemical characterization, *Clitoria ternatea* L., Rhizoplane, Rhizosphere, Root nodules

Medicinal plants have long been recognized as a rich source of bioactive compounds, playing a vital role in traditional medicine across the globe. Among these, *Clitoria ternatea* L., commonly known as *Shankapushpi*, holds a revered position in Ayurvedic and traditional medicine systems due to its diverse pharmacological properties. The plant is known for its neuroprotective, anti-inflammatory, anti-cancer and antioxidant activities, making it an essential part of herbal formulations used to treat neurological disorders, stress and anxiety disorders (Mukherjee *et al*., 2007). However, while the bioactive compounds of *Shankapushpi* have been extensively studied, less attention has been paid to its associated microbial communities and their potential contributions to the plant's medicinal properties.

Plant-associated bacteria, particularly those residing in the rhizosphere or within plant tissues, have been shown to promote plant health and growth, as well as contribute to plant secondary metabolism, including the synthesis of pharmacologically active compounds (Compant *et al*., 2010). These bacteria can interact symbiotically with their host plants by facilitating nutrient acquisition, producing phytohormones and protecting against pathogens through the production of antimicrobial compounds (Glick, 2012). In this context, the investigation of bacteria associated with medicinal plants like *Clitoria ternatea* L. could provide insights into novel bacterial strains with potential biotechnological and medicinal applications.

The present study aims to isolate and biochemically characterize bacterial communities associated with *Clitoria ternatea* L., focusing on both rhizosphere, rhizoplane and nodule bacteria. By understanding the microbial diversity linked to this medicinal plant, we hope to uncover bacterial strains that may play a role in enhancing the plant's therapeutic properties. Furthermore, this research could open up new avenues for utilizing these bacterial isolates in developing plant-based bioactive formulations and bioproducts for pharmaceutical or agricultural applications.

Material and Methods

Source of Sample Collection and Isolation of Bacteria

To isolate the bacteria associated with rhizosphere soil, rhizoplane and root nodules of the plant *Shankapushpi,* rhizosphere soil and five plant sample were collected randomly from wildly grown *Shankapuspi* plants (Plate 1) near jasmine field, college of Horticulture, University of Horticulture, Bagalkot.

For isolation from rhizosphere, soil samples were obtained from a depth of 5 to 15 cm and placed in sterile HDPE bags. Samples were kept at ambient temperature during the expedition and at 4°C upon return to the laboratory (Mythra and Krishna naik, 2023). Sample (about 10 g) of air-dried soil was mixed with sterilized distilled water (90 ml) and considered as 10^{-1} dilution. This soil suspension was shaken at 200 rpm for 30 min at 28°C. One ml of soil mixtures was transferred to 9 ml of sterile distilled water to get 10^{-2} dilution and similarly stepwise final dilution of 10^{-4} to 10^{-7} was attained. About 100µl of final dilution was spread with the help of sterile glass spreader on Nutrient agar. These plates were incubated at 28 ± 2°C for 24 hrs. Morphologically different

Plate 1: *Shankapushpi* plants grown under wild conditions

colonies were sub cultured and maintained in refrigerator for further study.

For isolation of bacteria from rhizoplanes the standard procedure given by Mergeay *et al*., 1985 was followed in this study. As per the protocol adhering dirt was removed from the roots and one gram of root tissue was shaken in 10 ml of sterile 1 per cent sodium hexametaphosphate solution for 30 minutes in an end-over-end shaker in order to isolate rhizoplane bacteria. Suspensions were plated on nutrient agar in duplicate after being diluted ten times over.

To isolate *Shankapushpi* root nodule bacteria, nodules were collected and washed with distilled water followed by surface sterilization of the nodules was carried out by placing them in 70 per cent ethanol for 30 sec and then washing them with distilled water. The nodules were then treated with 3 per cent sodium hypochlorite for one min, followed by 10 washes with distilled water. Each nodule was crushed in 100µl of 15 per cent glycerol and 10µl of the mixture was spread on yeast extract mannitol agar medium (Cole and Elkan, 1973) containing 1.5 per cent agar and incubated at 28°C for 3-4 days.

After isolation, bacteria were purified by four-way streaking of individual bacteria on Nutrient agar plate and stored in slants at 4 °C for further characterization.

Morphological Characterization

The bacterial isolates were identified on the basis of morphological and biochemical characteristics according to the standard methods described in Bergey's manual of systematic bacteriology (Holt *et al*., 1984). For morphological characterization bacterial isolates were streaked in four ways on Nutrient agar medium and incubated at 28°C for colony growth. Further the bacterial colonies were examined for morphological characters given in Table 1.

Biochemical Characterization

Gram's Staining

Homogeneous smears of isolates were prepared on clean glass slides individually, followed by air drying and heat fixing the smears. Smears were stained with crystal violet stain for 2 min followed by draining and covering with Gram's iodine for 30-60 sec. Smears were decolourized by dipping in alcohol and counter stained with Safranine for 30-60 sec to complete gram's reaction. Further the slide was observed under microscope at 40x to confirm the gram reaction and to differentiate isolates based on their gram nature (Bartholomew and Mittwer, 1952).

Table 1
List of morphological characters

Sl. No.	Colony characters
1	Colour
2	Shape
3	Margin
4	Elevation

Catalase Test

To detect soil bacteria, using a sterile wooden stick, several colonies of the test organisms were removed from nutrient agar plate followed by smearing it on to a clean glass slide and 2-3 ml of the hydrogen peroxide solution was poured on to it. Bubbles formation was then observed and recorded as positive (Kandjimi *et al*., 2015).

Voges Proskauer Test

To detect the production of acetyl methyl carbinol acetoin, a natural product formed from pyruvic acid in the course of glucose fermentation. The buffered glucose broth together with the organism was inoculated and incubated at 37 °C for 3 days. Approximately 3 ml of alpha naphthol was added followed by 1 ml of 40 per cent KOH and mixed well for 30 minutes. For the result, pink solution means VP (+) and no change means VP (-) was recorded.

Urease Test

In order to detect soil bacteria, a dense 'milky' suspension of the test organism was prepared in 0.25 ml physiological saline in a small tube. Urea was

added into the tube and incubated at 37 °C for overnight. Colour change of the test organism was taken as positive and recorded.

Indole test

Indole test was done for detecting the soil bacteria. The test organisms were cultured in tryptophan containing medium with 3 ml of sterile tryptone water. The medium was then added with 0.5 ml Kovac's reagent (4p-dimethylamino-benzaldehyde) with gentle shaking the formation of pink colour ring was observed.

Citrate Utilization Test

Citrate utilization test was based on the ability of an organism to use citrate as its only carbon source and ammonia as its only nitrogen source. If citrate utilization test is positive, the media will turn from green to blue. To test ability of bacteria in utilization of citrate, Simon's citrate agar slants were prepared, individual isolates were streaked and observed for color change.

H_2S Production

H_2S generation can be tested using SIM (Sulfide, Indole, Motility) medium. SIM medium is a semisolid medium that contains sodium thiosulfate, peptone and iron salts (such as ferrous ammonium sulfate). Sodium thiosulfate serves as the substrate and iron salts react with H_2S to produce ferrous sulfide, a black precipitate. The SIM medium should be stabbed with a sterile inoculation needle to introduce the test bacteria. The inoculated media should then be incubated for 24 hours at 37°C. After incubation, look for signs of medium blackening. Positive generation of H_2S is shown by a black precipitate extending throughout the medium (Darland and Davis, 1974).

Motility Test

To test for bacterial motility, a semi-solid medium, like motility agar (0.4% agar), can be inoculated by puncturing it with a straight needle. While non-motile bacteria will grow limited to the stab line, motile bacteria will exhibit diffuse growth radiating from the stab line following 24-48 hours of incubation at 35-37°C. By using this technique, bacteria are distinguished according to how well they can pass through the medium (Jones *et al*., 1986).

Oxidase Test

The oxidase test detects the presence of cytochrome C oxidase in bacteria by applying the bacterial sample to an oxidase reagent-impregnated filter paper or disc. A positive result is indicated by the appearance of a dark purple color within 10-30 seconds, while a negative result shows no color change (Jurtshuk and McQuitty, 1976).

Starch Hydrolysis

The process of hydrolysing starch is used to measure an enzyme's capacity to generate amylase. Using an inoculating loop, the test organism is injected into a starch agar plate, creating a tiny central line or streak. The plates that have been injected with bacteria will be incubated at 37°C for 48 hours. Following the incubation period, the plates' surface is saturated with iodine solution for roughly 30 seconds and any extra iodine is disposed of. The colour shift will be checked on the plates. A clear area surrounding the microbial colonies indicates that the starch hydrolysis process is going well (Anderson, 1995).

Results and Discussion

Association of bacteria with the plant *Shankapushpi* is least explored, in this study an effort was made to unveil their communities from different parts of plant *Shankapushpi i.e.,* rhizosphere soil, rhizoplane and root nodules (Plate 2).

Isolation of Bacteria Associated with the Plant *Shankapushpi*

In this study, bacteria associated with the medicinal plant *Shankapushpi* (*Clitoria ternatea* L.) were isolated from different parts, *viz*., rhizosphere soil, rhizoplane and root nodules, by following the serial dilution and plating method. Bacteria having different morphology have been selected and the

Plate 2 : Collection of *Shankapushpi* plant rhizosphere soil, roots and root nodules for bacteria isolation

Table 2a

Morphological characteristics of rhizosphere bacteria

Isolate	Colony colour	Colony shape	Colony margin	Elevation	Gram reaction	Cell shape
RSB1	Cream	Circular	Entire	Flat	+	Rod
RSB2	Cream	Circular	Entire	Convex	-	Rod
RSB3	Cream	Irregular	Undulate	Umbonate	+	Cocci
RSB4	Yellow	Round	Entire	Convex	-	Cocci
RSB5	Cream	Irregular	Filiform	Flat	+	Cocci
RSB6	Cream	Filamentous	Lobate	Flat	-	Small rods
RSB7	White	Convex	Entire	Raised	-	Rod
RSB8	Creamy white	Round	Entire	Flat	+	Rod
RSB9	Creamy white	Filamentous	Lobate	Flat	-	Cocci
RSB10	Creamy white	Circular	Entire	Flat	-	Cocci
RSB11	Creamy white	Irregular	Curled	Flat	-	Cocci
RSB12	Dirty white	Irregular	Undulate	Flat	+	Cocci
RSB13	Yellow	Round	Entire	Crateriform	-	Rod
RSB14	Cream	Round	Entire	Convex	-	Cocci
RSB15	Cream	Round	Entire	Convex	-	Small rod
RSB16	White	Circular	Entire	Raised	-	Cocci
RSB17	White	Irregular	Undulate	Flat	-	Cocci
RSB18	Lite pink	Irregular	Undulate	Umbonate	+	Rod
RSB19	Creamy white	Irregular	Undulate	Umbonate	+	Cocci
RSB20	Creamy white	Irregular	Undulate	Flat	-	Rod
RSB21	Cream	Irregular	Filliform	Flat	+	Cocci
RSB22	Pale yellow	Round	Entire	Flat	-	Cocci
RSB23	Pale yellow	Irregular	Undulate	Flat	-	Cocci
RSB24	Creamy white	Round	Entire	Convex	+	Cocci

Continued....

Table 2a Continued....

Isolate	Colony colour	Colony shape	Colony margin	Elevation	Gram reaction	Cell shape
RSB25	White	Round	Entire	Umbonate	+	Cocci
RSB26	Cream	Round	Entire	Raised	-	Cocci
RSB27	Yellow	Irregular	Undulate	Raised	-	Cocci
RSB28	White	Round	Entire	Convex	-	Rod
RSB29	Cream	Irregular	Undulate	Raised	-	Rod
RSB30	Cream	Round	Entire	Convex	-	Cocci
RSB31	Translucent	Spindle	Entire	Convex	-	Cocci
RSB32	Cream	Round	Entire	Raised	-	Cocci
RSB33	Cream	Round	Entire	Flat	-	Cocci
RSB34	Yellow	Round	Undulate	Convex	-	Cocci
RSB35	Dirty white	Irregular	Undulate	Flat	-	Rod

Note : RSB - Rhizosphere Bacteria

results revealed that in total, eighty bacterial isolates were isolated. From the rhizosphere soil, thirty-five bacterial isolates were isolated, which is the highest number of isolates among all the isolates from different parts, *i.e.,* rhizosphere soil, rhizoplane and root nodules of the plant *Shankapushpi*. A total of twenty-five bacteria were isolated from the rhizoplane and twenty bacteria from root nodules. A greater number of isolates was isolated from the rhizosphere since the communication between the plant and microorganisms (bacteria) will be very high in rhizosphere soil, while only plant-selected microorganisms (bacteria) can be isolated using rhizoplane and root nodules; hence there are fewer isolates found (Kennedy and De Luna, 2005).

Morphological Characterization of Bacterial Isolates

All isolates were subjected to morphological characterization, focusing on aspects such as colony color, shape, margin and elevation. Tables 2a, 2b and 2c detail the results. Rhizosphere and rhizoplane isolates exhibited colony colors ranging from cream, creamy white, dirty white and yellow, while nodule isolates displayed translucent, white and light pink colonies. The majority of isolates exhibited cream to white colony color, although they differed in other morphological characteristics (Plate 3). Among the eighty isolates, 64 per cent displayed round colonies, followed by irregular, circular, regular, filamentous,

Table 2b

Morphological characteristics of rhizoplane bacteria

Isolate	Colony colour	Colony shape	Colony margin	Elevation	Gram reaction	Cell shape
RPB1	Cream	Round	Entire	Convex	-	Rod
RPB2	Yellow	Round	Entire	Convex	-	Rod
RPB3	Cream	Round	Entire	Umbonate	+	Cocci
RPB4	Cream	Round	Entire	Flat	-	Cocci
RPB5	Creamy white	Irregular	Filliform	Raised	+	Cocci
RPB6	Lite pink	Round	Entire	Raised	-	Small rods
RPB7	Cream	Irregular	Undulate	Flat	-	Rod

Continued....

TABLE 2b Continued....

Isolate	Colony colour	Colony shape	Colony margin	Elevation	Gram reaction	Cell shape
RPB8	Yellow	Round	Entire	Convex	+	Rod
RPB9	Brown	Round	Entire	Raised	-	Cocci
RPB10	Cream	Round	Entire	Convex	-	Cocci
RPB11	Cream	Round	Entire	Raised	-	Cocci
RPB12	Cream	Round	Entire	Raised	+	Cocci
RPB13	Orange	Round	Entire	Convex	-	Rod
RPB14	White	Round	Entire	Raised	-	Cocci
RPB15	Yellowish cream	Round	Entire	Raised	-	Small rod
RPB16	Yellow	Round	Entire	Convex	-	Cocci
RPB17	Lite yellow	Round	Entire	Convex	-	Cocci
RPB18	Cream	Round	Entire	Raised	+	Rod
RPB19	Brown	Round	Entire	Raised	+	Cocci
RPB20	Cream	Irregular	Undulate	Flat	-	Rod
RPB21	Yellow	Round	Entire	Raised	+	Cocci
RPB22	Cream	Round	Entire	Flat	-	Cocci
RPB23	Cream	Round	Entire	Flat	-	Cocci
RPB24	Cream	Irregular	Undulate	Flat	+	Cocci
RPB25	Cream	Irregular	Undulate	Flat	+	Cocci

Note : RSB - Rhizosphere Bacteria

TABLE 2c

Morphological characteristics of bacteria isolated from root nodules of plant *Shankapushpi*

Isolate	Colony colour	Colony shape	Colony margin	Elevation	Gram reaction	Cell shape
SNB1	Translucent	Irregular	Erose	Flat	-	Rod
SNB2	Translucent	Regular	Entire	Raised	-	Rod
SNB3	Creamy white	Round	Entire	Umbonate	-	Cocci
SNB4	Lite pink	Round	Entire	Raised	-	Cocci
SNB5	Translucent	Regular	Entire	Raised	-	Cocci
SNB6	Cream	Circular	Entire	Raised	-	Rod
SNB7	Cream	Round	Entire	Flat	-	Cocci
SNB8	Translucent	Round	Entire	Raised	-	Cocci
SNB9	Lite pink	Round	Entire	Convex	-	Cocci
SNB10	Translucent	Round	Entire	Convex	+	Rod
SNB11	Lite pink	Round	Entire	Convex	-	Rod
SNB12	Lite pink	Round	Entire	Convex	-	Rod
SNB13	Creamy white	Round	Entire	Convex	-	Rod
SNB14	Brown	Round	Entire	Convex	-	Rod

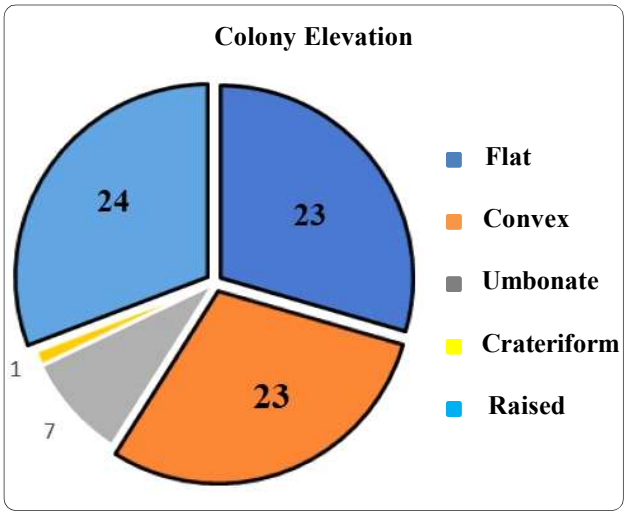
Continued....

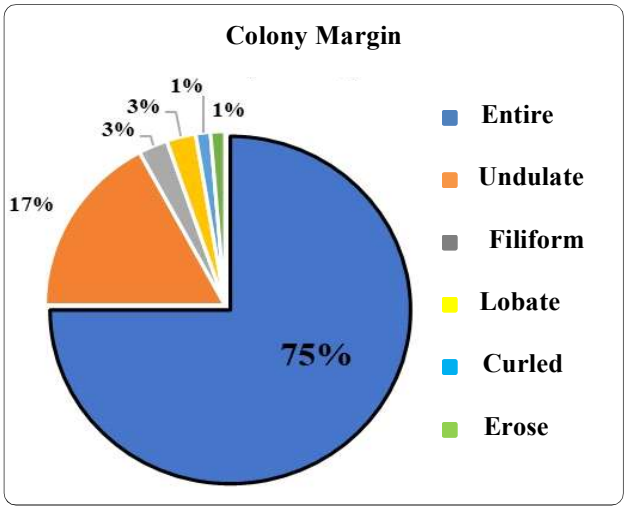
Table 2c Continued....

Isolate	Colony colour	Colony shape	Colony margin	Elevation	Gram reaction	Cell shape
SNB15	Cream	Round	Entire	Flat	-	Cocci
SNB16	White	Round	Entire	Convex	+	Coccobaccili
SNB17	Dull white	Round	Entire	Convex	-	Rod
SNB18	Cream	Round	Entire	Raised	-	Coccobaccili
SNB19	Lite pink	Round	Entire	Umbonate	-	Rod
SNB20	Cream	Round	Entire	Raised	-	Coccobaccili

Note : SNB -*Shankapushpi* Nodule Bacteria

and spindle shapes. Regarding elevation, the majority of bacteria exhibited raised, flat and convex elevations, with only 1 per cent bacteria exhibiting crateriform elevation. Seventy-five percent of the bacterial colonies displayed an entire margin (Fig. 1). The aforementioned findings are comparable to the study by Srikanth *et al*., 2023, on the isolation and screening of bacterial isolates, which also covers morphological characterization of isolates. 62 bacterial endophytes were isolated by the research team and the majority of these endophytes had round to irregular colony shapes and cream-white to white colony colors.





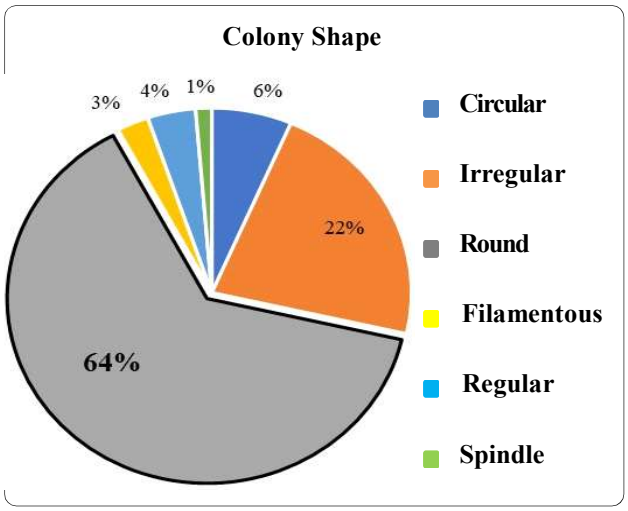


Fig. 1 : Morphological characters of bacterial isolates

Biochemical Characterization of Bacterial Isolates

After confirming through morphological characterization, the bacterial isolates were further characterized biochemically. A detailed biochemical characterization was performed by conduction of various tests (Plate 4) *viz.,* Gram staining, Catalase test, Voges Proskauer test, Urease Indole, Citrate utilization, H_2S production, Motility test, Oxidase test, Starch hydrolysis and the detailed results are

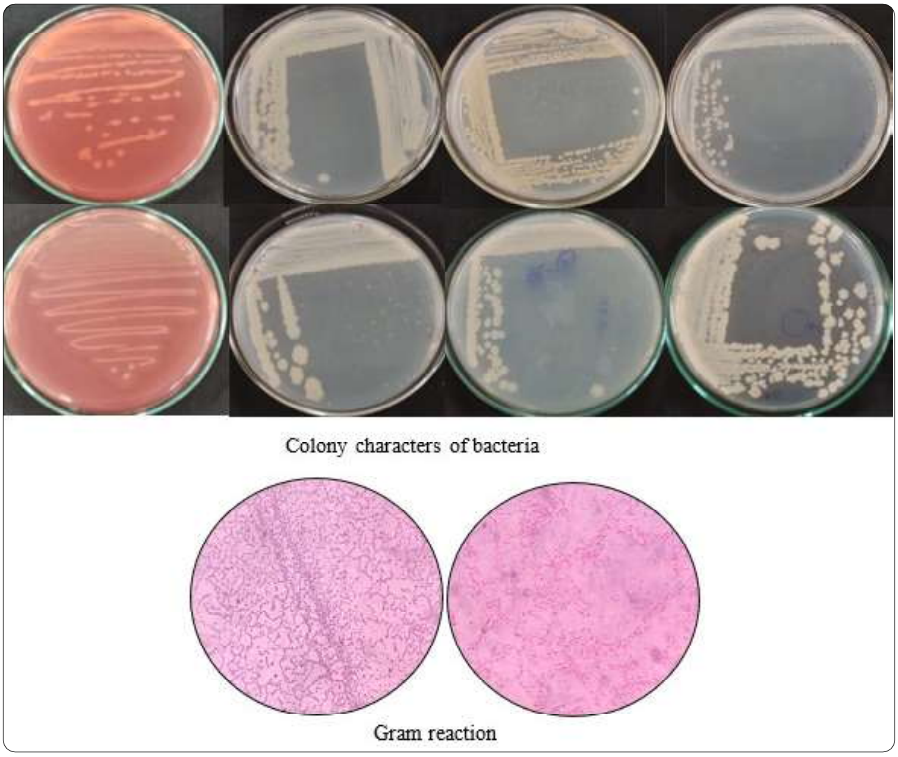
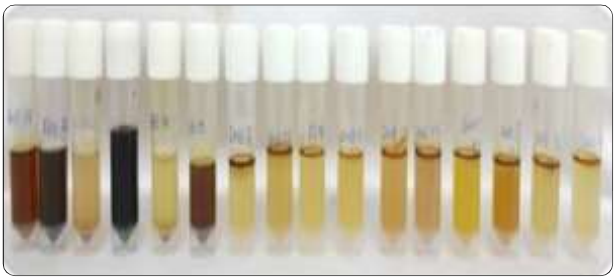


Plate 3 : Morphological characters of bacterial isolates

Catalase activity

Citrate utilization test

Voges Proskauer test



Indole production test

H_2S Production

Plate 4 : Biochemical tests on bacterial isolates

illustrated in table 3a, 3b and 3c. The bacterial isolates were tentatively identified based on their morphological and biochemical characteristics as per the guidance mentioned in the Bergey's manual of determinative microbiology by Bergey and Breed (1957).

In gram staining, twenty-one isolates (RSB1, 3, 5, 8, 12, 18, 19, 21, 24, 25, RPB3, 5, 8, 12, 18, 19, 21, 24, 25, SNB10, 16) confirmed for gram positive cell wall whereas remaining has gram negative, maximum bacteria were gram negative bacteria. Isolates showing Gram-positive rods with catalase activity and starch hydrolysis, such as RSB1 and RSB18, were tentatively identified as *Bacillus* spp. The RPB isolates followed a similar trend. *Bacillus* spp. was identified in RPB1, RPB8, RPB18 and RPB25 based on their Gram-positive rod or cocci morphology and catalase activity. For the SNB isolates, Gram-positive rods such as SNB10 and SNB16 were identified as *Bacillus* spp. The dominance of *Bacillus* spp. in several isolates is consistent with the well-documented characteristics of this genus. *Bacillus* species are Gram-positive, rod-shaped bacteria with catalase activity and the ability to hydrolyse starch. These traits make them prevalent in soil and nutrient-rich environments (Priest, 1993). *Bacillus* species are known for their roles in bioremediation, enzyme production and plant growth promotion, further emphasizing their ecological importance (Vishwakarma *et al.,* 2019).

Similarly, Gram-positive cocci isolate with catalase positivity were primarily classified as *Staphylococcus* spp. (e.g., RSB5, RSB12, RSB19 and RSB24). *Micrococcus* spp. was identified in RSB3, RPB19, RPB21 and RPB24 by showing Gram-positive cocci morphology and negative or positive for catalase activity. *Staphylococcus* spp. and *Micrococcus* spp. were predominantly identified among Gram-positive cocci isolates. *Staphylococcus* species are catalase-positive and are commonly found in soil, water and human-associated environments, where they can act as both commensals and opportunistic pathogens. *Micrococcus* spp., on the other hand, are generally non-pathogenic and are known for their resilience in nutrient-limited environments (Becker *et al.,* 2014).

Fifteen isolates *viz.,* RSB7, RSB20, RSB28, RSB35, RPB2, RPB7, RPB13, RPB15, RPB20, SNB2, SNB6, SNB11, SNB12, SNB14 and SNB17 out of eighty were identified as *Pseudomonas* spp., given their Gram-negative rod morphology, catalase activity and positive biochemical traits like citrate utilization and urease production. *Pseudomonas* spp. was also frequently identified and characterized by their Gram-negative rod shape, catalase positivity, citrate utilization and urease production. *Pseudomonads* are ubiquitous in soil, water and plant-associated environments and are known for their metabolic versatility and ability to degrade complex organic compounds (Silby *et al.,* 2011). Their role as biocontrol agents and plant growth promoters has also been widely studied, which may explain their abundance in diverse environmental samples.

The isolates RSB6, RSB10, RSB11, RSB15, RSB30, RSB31, RSB32, RSB33, RPB4, RPB6, RPB9, RPB10, RPB11, RPB14, RPB16, RPB23, SNB4, SNB5, SNB7, SNB18 and SNB20 exhibited characteristics of *Acinetobacter* spp. based on their Gram-negative small rod or cocci shape, catalase variability and citrate utilization. The presence of *Acinetobacter* spp. across multiple isolates is noteworthy. *Acinetobacter* species are Gram-negative, non-motile bacteria that exhibit variable biochemical properties such as citrate utilization and indole production. These organisms are typically found in soil and aquatic ecosystems and are known for their involvement in nitrogen fixation, hydrocarbon degradation and antibiotic resistance mechanisms (Doughari *et al.,* 2011). Their identification in this study highlights their adaptability to various environmental niches.

Rhizosphere isolates *viz.,* RSB4, RSB9, RSB14, RSB16, RSB17, RSB22, RSB23 and RSB26, were tentatively identified as *Azotobacter* spp. due to their Gram-negative cocci morphology and positive biochemical reactions, particularly citrate utilization and indole production. Isolates identified as Azotobacter spp. reflect the importance of this genus in nitrogen fixation. *Azotobacter* species are free-living nitrogen-fixing bacteria that contribute significantly to soil fertility and plant nutrition by

TABLE 3a

Biochemical characteristics of bacteria isolated from rhizosphere soil

Isolates	Biochemical characteristics									Tentative identification
	Catalase	Vp test	Urease	Indole	Citrate utilization	H_2S production	Motility test	Oxidase test	Starch hydrolysis	
RSB 1	++	-	+	+	+	-	-	-	+	*Bacillus* spp.
RSB 2	++	+	+	+	+	-	+	+	-	*Aeromonas* spp.
RSB 3	-	-	-	+	-	-	+	+	-	*Micrococcus* spp.
RSB 4	+	+	-	+	+	-	-	-	-	*Azotobacter* spp.
RSB 5	+	-	-	-	+	-	-	-	-	*Staphylococcus* spp.
RSB 6	-	-	+	+	+	-	-	-	-	*Acinetobacter* spp.
RSB 7	+	-	+	+	+	-	-	-	-	*Pseudomonas* spp.
RSB 8	+	-	+	+	+	-	+	-	+	*Streptomyces* spp.
RSB 9	+	-	-	+	+	-	+	-	-	*Azotobacter* spp.
RSB 10	-	-	-	+	+	-	-	-	-	*Acinetobacter* spp.
RSB 11	-	-	-	+	+	-	-	-	-	*Acinetobacter* spp.
RSB 12	+	-	-	+	+	-	-	-	-	*Staphylococcus*
RSB 13	-	-	-	-	+	-	-	-	+	*Pseudomonas* spp.
RSB 14	-	-	-	+	+	-	-	-	-	*Azotobacter* spp.
RSB 15	+	-	-	+	+	-	-	-	+	*Acinetobacter* spp.
RSB 16	-	-	+	+	+	-	-	-	-	*Azotobacter* spp.
RSB 17	-	-	-	+	+	-	-	-	-	*Azotobacter* spp.
RSB 18	-	-	+	+	+	-	-	-	-	*Bacillus* spp.
RSB 19	-	-	-	+	+	-	-	-	-	*Staphylococcus* spp.
RSB 20	-	-	+	+	+	-	-	-	-	*Pseudomonas* spp.
RSB 21	-	-	-	+	+	-	-	-	-	*Staphylococcus* spp.
RSB 22	+	-	-	+	+	-	-	-	-	*Azotobacter* spp.
RSB 23	-	-	-	+	+	-	-	-	-	*Azotobacter* spp.
RSB 24	+	-	+	+	+	-	-	-	-	*Staphylococcus* spp
RSB 25	+++	-	-	+	+	-	-	-	-	*Bacillus* spp.
RSB 26	+	-	+	+	+	-	-	-	+	*Azotobacter* spp.
RSB 27	-	-	-	+	+	-	-	-	-	*Azotobacter* spp.
RSB 28	+	-	-	-	+	-	-	-	-	*Pseudomonas* spp.
RSB 29	+	+	-	+	+	-	-	-	-	*Pseudomonas* spp.
RSB 30	+	+	-	+	+	-	-	-	+	*Acinetobacter* spp.
RSB 31	+	+	+	+	+	-	+	-	+	*Acinetobacter* spp.
RSB 32	+	+	-	+	+	-	+	-	-	*Acinetobacter* spp.
RSB33	+	-	-	+	+	-	+	-	-	*Acinetobacter* spp.
RSB34	+	-	-	+	-	-	-	-	-	*Bacillus* spp.
RSB35	+	-	+	+	-	-	-	-	+	*Pseudomonas* spp.

Note : RPB – Rhizoplane Bacteria, + (Low), ++ (Medium), +++ (Strong)

TABLE 3b

Biochemical characteristics of bacteria isolated from rhizoplane of plant *Clitoria ternatea* L. (*Shankapushpi*)

Isolates	Biochemical characteristics									Tentative identification
	Catalase	Vp test	Urease	Indole	Citrate utilization	H_2S production	Motility test	Oxidase test	Starch hydrolysis	
RPB1	++	+	-	+	-	-	+	-	+	*Pseudomonas* spp.
RPB2	++	+	-	+	+	-	+	+	-	*Pseudomonas* spp.
RPB3	+	_	-	+	-	-	_	_	-	*Staphylococcus* spp.
RPB4	+	_	_	+	+	-	_	_	-	*Acinetobacter* spp.
RPB5	+	_	_	+	-	-	_	_	-	*Staphylococcus* spp.
RPB6	-	_	_	+	-	-	+	_	+	*Acinetobacter* spp.
RPB7	+	_	_	+	+	-	+	_	-	*Pseudomonas* spp.
RPB8	+	_	_	+	-	-	_	_	-	*Bacillus* spp.
RPB9	+	_	_	+	-	-	_	_	-	*Acinetobacter* spp.
RPB10	-	_	_	+	-	-	_	+	-	*Acinetobacter* spp.
RPB11	-	_	_	+	-	-	_	+	-	*Acinetobacter* spp.
RPB12	+	_	_	-	-	-	_	_	-	*Staphylococcus* spp.
RPB13	+	_	_	+	+	-	_	_	+	*Pseudomonas* spp.
RPB14	+	_	_	+	-	-	_	_	-	*Acinetobacter* spp.
RPB15	+	_	_	+	+	-	_	_	-	*Pseudomonas* spp.
RPB16	+	_	+	+	-	-	+	_	-	*Acinetobacter* spp.
RPB17	-	_	_	-	-	-	_	+	-	*Neisseria* spp.
RPB18	+	_	+	+	-	-	_	_	-	*Bacillus* spp.
RPB19	-	_	_	-	-	-	_	+	-	*Micrococcus* spp.
RPB20	+	_	_	+	-	-	+	_	-	*Pseudomonas* spp.
RPB21	-	_	_	-	-	-	_	+	-	*Micrococcus* spp.
RPB22	-	_	_	-	-	-	_	+	+	*Neisseria* spp.
RPB23	+	_	_	+	-	-	_	_	-	*Acinetobacter* spp.
RPB24	-	_	_	-	-	-	_	+	-	*Micrococcus* spp.
RPB25	+++	_	_	-	-	-	_	_	-	*Bacillus* spp.

Note : RPB – Rhizoplane Bacteria, + (Low), ++ (Medium), +++ (Strong)

converting atmospheric nitrogen into bioavailable forms (Bhardwaj *et al.,* 2014). Their detection underscores the role of microbial communities in supporting agricultural productivity and ecosystem stability.

Additionally, RPB17, RPB22, SNB3 and SNB15 were tentatively classified as *Neisseria* spp. based on their Gram-negative cocci morphology and biochemical results. This highlights their role in microbial communities. *Neisseria* species are Gram-negative cocci, often associated with aquatic and host-associated environments. Their presence in this study may reflect specific environmental niches conducive to their growth (Harrison *et al.,* 2008).

Meanwhile, SNB13 was tentatively identified as *Enterobacter* spp. and SNB19 was identified as

Table 3c

Biochemical characteristics of bacteria isolated from root nodules of *Clitoria ternatea* L. (*Shankapushpi*)

Isolates	Biochemical characteristics									Tentative identification
	Catalase	Vp test	Urease	Indole	Citrate utilization	H_2S production	Motility test	Oxidase test	Starch hydrolysis	
SNB1	+	+	+	+	+	+	+	-	+	*Bradyrhizobium* spp.
SNB2	-	+	-	+	+	-	+	+	-	*Pseudomonas* spp.
SNB3	+	-	-	+	+	-	-	+	-	*Neisseria* spp.
SNB4	+	-	-	+	+	-	+	-	-	*Acinetobacter* spp.
SNB5	++	-	-	-	+	-	-	-	-	*Acinetobacter* spp.
SNB6	++	-	-	-	+	-	+	-	-	*Pseudomonas* spp.
SNB7	++	-	-	-	+	-	-	-	-	*Acinetobacter* spp.
SNB8	++	-	+	+	+	-	-	-	-	*Bacillus* spp.
SNB9	+	+	+	+	+	-	-	-	-	*Pseudomonas* spp.
SNB10	+++	-	+	+	+	-	+	-	-	*Bacillus* spp.
SNB11	+	-	-	+	-	-	-	-	-	*Pseudomonas* spp.
SNB12	+++	-	-	+	+	-	-	-	-	*Pseudomonas* spp.
SNB13	+	+	-	+	+	-	+	-	-	*Enterobacter* spp.
SNB14	+++	+	+	+	-	-	+	-	-	*Pseudomonas* spp.
SNB15	+	-	+	+	-	-	+	-	-	*Neisseria spp.*
SNB16	+++	-	-	+	-	-	+	-	-	*Bacillus* spp.
SNB17	+	-	-	-	+	-	-	-	-	*Pseudomonas spp.*
SNB18	+	-	+	+	+	-	+	-	-	*Acinetobacter* spp.
SNB19	+	+	+	+	+	-	-	-	-	*Klebsiella* spp.
SNB20	+	-	+	-	+	-	-	-	-	*Acinetobacter* spp.

Note : SNB - Shankapushpi Nodule Bacteria, + (Low), ++ (Medium), +++ (Strong)

Klebsiella spp., given their Gram-negative rod morphology and biochemical traits. These genera are part of the Enterobacteriaceae family and are known for their roles in nitrogen cycling and plant growth promotion, particularly in agricultural ecosystems (Ahemad and Kibret, 2014).

Nodule bacteria SNB1 was identified as *Bradyrhizobium* spp., given its unique biochemical profile with a Gram-negative rod shape, able to produce H_2S, citrate positive, and starch hydrolysis activity. Interestingly, SNB1 was tentatively classified as *Bradyrhizobium* spp., a genus renowned for its nitrogen-fixing abilities in symbiotic relationships with leguminous plants. Similar results were found in the study conducted by Dhanraj *et al.,* 2020, who tried identification of nodule bacteria associated with the plant *Clitorea ternatea* L. The present results are also on par with the results of the study by Deshmukh *et al.,* 2013, on biochemical studies of *Bradyrhizobium* isolates. The identification of *Bradyrhizobium* spp. emphasizes the significance of nitrogen-fixing bacteria in maintaining soil fertility and enhancing crop yields (Graham, 2008).

The present study successfully characterized bacterial isolates from various samples based on their morphological and biochemical characteristics. These findings align with standard methods outlined in Bergey's Manual of Determinative Bacteriology

(Bergey & Breed, 1957), where morphological traits, such as Gram staining and cellular shape, were combined with biochemical assays to classify the isolates at the genus level. The results emphasize that most isolates were tentatively classified into genera such as *Bacillus, Staphylococcus, Pseudomonas, Acinetobacter, Azotobacter, Micrococcus* and *Bradyrhizobium* based on biochemical characterization. Further molecular identification methods, such as 16S rRNA sequencing, are recommended to confirm these findings and provide precise taxonomic placement. The diversity of bacterial genera identified highlights their ecological significance and functional roles in the studied environments.

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