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Anita Tudu
Department of Botany, Ranchi
Women's College, Ranchi
University, Ranchi,
Jharkhand, India

Vinay Oraon
Department of Biotechnology,
University Department of
Botany, Ranchi University,
Ranchi, Jharkhand, India

Latika Sharan
Department of Botany, Ranchi
University, Ranchi,
Jharkhand, India

Isolation, characterization, molecular identification and abiotic stress tolerance profiling of nodule associated bacteria from *Clitoria ternatea* L.

Anita Tudu, Vinay Oraon and Latika Sharan

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Abstract

This study was conducted to identify and characterize the rhizobacteria found in the root nodules of *Clitoria ternatea* L. Seeds of *Clitoria ternatea* L. (blue single petal and blue double petal varieties) were grown in normal soil, and four types of bacteria were isolated. Morphological and biochemical analyses were performed to identify and characterize these bacteria. The ability of each bacterium to tolerate various abiotic stresses (such as pH, salinity, and temperature) was also examined. Their phylogenetic identification was determined based on 16S rRNA analysis. The four isolated bacteria were found to belong to the species *Bacillus velezensis* and *Bacillus zanthoxyli*.

Keywords: *Clitoria ternatea*, rhizobacteria, root nodules, abiotic stresses, 16S rRNA

Introduction

Nitrogen (N_2) is an essential element for all forms of life. It is found in amino acids and proteins, and the process of nitrogen fixation leads to the formation of numerous other organic compounds ^[1]. N_2 is the most abundant gas in Earth's atmosphere; however, it is not highly reactive ^[2]. Biological nitrogen fixation is a vital component of microbial processes ^[3]. The Leguminosae family is the largest plant family on Earth. A key reason for the success of leguminous plants is their ability to form a nitrogen-fixing symbiosis with specialized bacteria known as Rhizobia ^[4, 5].

Nodulation is the most well-known example of a symbiotic relationship between legume plants and Rhizobium bacteria. The host legume plant utilizes the fixed ammonia obtained from Rhizobium as a nitrogen source, while Rhizobium, in turn, receives a source of carbon. Consequently, this symbiotic relationship within root nodules is mutually beneficial and plays a crucial role in both agriculture and the nitrogen cycle ^[6-9]. Recently, there have been increasing reports indicating the presence of non-rhizobial bacteria acting as endophytes alongside the true symbiotic Rhizobium within root nodules. These include bacterial groups such as *Streptomyces*, *Agrobacterium*, *Bacillus*, *Enterobacter*, *Staphylococcus*, etc. were referred as putative endophytes which are found within the nodules either alongside the true symbiont or independently ^[10-15].

The Faboideae (or Papilionoideae) subfamily one of the three subfamilies of the Leguminosae family and the third largest family of terrestrial plants includes the majority of important crop species. Nevertheless, a large number of leguminous plants remain unstudied. *Clitoria ternatea* L., the primary plant in our study, is one such legume whose nodule flora has not yet been identified. *Clitoria ternatea* L. is found in tropical and subtropical regions, including the Caribbean, Central America, Africa, Southeast Asia, and India ^[16, 17]. Its roots, stems, leaves, flowers, and seeds are commonly used in traditional medicine and cuisine; its seeds can also be used as a food coloring agent ^[18-24].

The objective of this research is to characterize the morphological, biochemical, molecular, and stress-tolerance traits of root-nodule bacteria associated with *Clitoria ternatea* L. and to identify effective bacterial isolates. These isolates can be utilized as inoculants to restore soil fertility and enhance crop yields.

Corresponding Author:
Anita Tudu
Department of Botany, Ranchi
Women's College, Ranchi
University, Ranchi,
Jharkhand, India

Material and Methods

Seed collection

Seeds of the blue-flowered, single-petal *Clitoria ternatea* L. were purchased online from 'Jai Shree Radhe' (New Delhi) and blue flowered, double petal was purchased from Banrujjedar Orchid Bagan Private Limited, Kolkata, West Bengal and grown in pots containing normal soil.

Collection and surface sterilization of root nodules

After flowering, nodules were harvested from the roots of freshly uprooted plants. The roots were thoroughly washed with tap water to remove impurities. Healthy nodules were selected for bacterial isolation. Initially, the harvested nodules were washed under running tap water to remove soil particles adhering to their surfaces. Subsequently, the surfaces of nodules were sterilized using 70% alcohol for 3 minutes, followed by 0.1% mercuric chloride for another 3 minutes, and finally rinsed multiple times with sterilized distilled water under aseptic conditions [25, 26, 27, 28, 29, 30].

Bacterial isolation and purification

The nodules were crushed using a sterilized mortar and pestle to obtain a milky suspension of bacteroid. This suspension was spread onto Yeast Extract Mannitol Agar (YEMA) medium. The plates were then incubated at $28 \pm 2^\circ\text{C}$ for 24 hours. After 24 hours, colonies were selected for sub-culturing to obtain pure cultures.

Characterization of cultures

The isolated bacteria were characterized based on their size, form, elevation, margin, color (pigmentation), Gram staining reaction, and appearance on YEMA medium. Biochemical tests such as Casein test, Catalase test, Citrate utilization test, Gelatin Hydrolysis test, Indole test, Methyl red test, Nitrate Reduction test, Starch Hydrolysis test, TSI Test were also performed.

Morphological characterization

Size, form, elevation, margin, pigmentation, and appearance were observed on YEMA medium.

Gram's reaction (Vincent, 1970): This method was used to classify bacteria into Gram-positive and Gram-negative. Using a clean slide and a sterilized wire loop, a loopful of normal saline was placed in the center of the slide, and the loop was re-sterilized. A sample was collected using the wire loop, and a circular smear was prepared. After preparing the smear, the slide was gently heat-fixed by passing it over a Bunsen flame. Crystal violet was applied to the smear for 30 seconds and then washed off with water. Iodine solution was added and left for one minute, followed by washing with water. Subsequently, acetone or ethanol was applied for decolorization and immediately rinsed off with water. Counter-staining was performed using safranin within one minute. After drying, the slide was examined under a compound microscope. Gram-negative cells appear pinkish-red, while Gram-positive cells appear violet.

Biochemical characterization

The bacterial isolates were characterized by various biochemical methods [31, 32, 33, 34, 35].

a) Casein test: Milk agar medium was prepared and inoculated with the isolated strains, then incubated at $28 \pm 2^\circ\text{C}$ for 24 hours. After 24 hours, a clear zone of

hydrolysis was observed around the culture growth, indicating a positive result.

- b) Catalase test:** This test was performed to check for the presence of the catalase enzyme in the test organism. Catalase activity was assessed by mixing a loopful of the organism into a drop of 3% hydrogen peroxide on a clean glass slide. The formation of air bubbles indicates a positive result for the catalase test.
- c) Citrate utilization test:** This test was conducted to determine whether the test organism utilizes citrate as a carbon source. The test was performed by incubating the organism on Simmons citrate agar slants. A distinct colour change from green to blue is considered a positive result for the citrate utilization test.
- d) Gelatin hydrolysis test:** Gelatin medium was prepared and dispensed into test tubes; these were then inoculated with the isolated strains and incubated at $28 \pm 2^\circ\text{C}$ for 24 hours. After 24 hours, the cultures were placed in a refrigerator at 4°C for 30 minutes. The cultures were examined after 30 minutes; those that remained in a liquid state indicate a positive result.
- e) Indole Test:** Nutrient broth medium was prepared and dispensed into a test tube. The isolated strain was inoculated into the broth and incubated at 28°C for 2 days. Then, 1 ml of Kovac's reagent was added; the tube was gently shaken and observed after one minute. If the upper layer of the liquid in the test tube turned red, then it indicates a positive result for Indole test.
- f) Methyl red test:** For the Methyl Red test, nutrient broth medium was prepared and dispensed into a test tube. The isolated strains were inoculated into the broth and incubated at 28°C for 2 days. Then, 5 ml of Methyl Red was added to the test tube. The appearance of a red colour indicates a positive result for the Methyl Red test.
- g) Nitrate reduction test:** Nitrate reduction was assessed using nitrate broth. Nitrate broth was prepared by adding 1% potassium nitrate to nutrient broth; it was inoculated with an actively growing culture and incubated at 28°C for 3 days. After incubation, 5 drops of sulfanilic acid and alpha-naphthylamine (dissolved in acetic acid) were added to the broth. The formation of a red color indicates nitrate reduction activity.
- h) Starch hydrolysis test:** This test was conducted to demonstrate the production of amylase enzymes by the organism. The test was performed by incubating the organism on starch agar medium for 24 hours. After incubation, a drop of iodine solution was added; the formation of a clear zone of hydrolysis indicates a positive result.
- i) TSI test:** Triple Sugar Iron medium was prepared, dispensed into test tubes, and allowed to solidify. The isolated strain was inoculated and incubated at $28 \pm 2^\circ\text{C}$ for 24 hours. The colour of both the butt (bottom section) and the slant (sloped section) was observed.
- j) Congo red test:** The strain culture was plated on CRYEMA medium and incubated at 28°C for 24 hours. After this period, the dye-absorbing capacity of the bacteria was observed.

Physiological characterization

- 1. pH Tolerance:** The pH tolerance capacity of all bacterial isolates was tested using Yeast Mannitol (YEM) broth adjusted to various pH levels (e.g., 4, 5, 6,

7, 9, and 10). The pH of the YEM broth was adjusted using HCl and NaOH. Each isolate was inoculated into YEM broth and incubated at 28°C. The effect of pH on the growth of each isolate was recorded at 24, 48, and 96 hours.

2. **Temperature Tolerance:** The effect of temperature on the growth of each bacterial isolate was assessed using YEM broth. Each isolate was inoculated into YEM broth and incubated at various temperatures (e.g., 20, 24, 28, 32, 36, 40, and 45°C), and the growth pattern of each isolate was recorded at 24-hour intervals.
3. **Salinity tolerance:** Tolerance to sodium chloride (NaCl) was tested for each bacterial isolate using YEM broth supplemented with varying concentrations of NaCl (e.g., 0.5%, 1%, 2%, 3%, 4%, and 5%). The effect of salinity on the growth of each isolate was recorded at 20, 48, and 96 hours.

The growth of bacterial isolates were recorded using spectrophotometer by measuring the optical density (OD) at 600 nm.

Molecular identification: All four bacterial isolates were selected for molecular characterization. DNA was extracted from the isolates, and its quality was assessed on a 1.0% agarose gel. Amplification of the rRNA gene was performed using LCO 1490 and HCO 2198 primers. Upon resolution on an agarose gel, a single, distinct PCR amplicon band of 1500 bp was observed. Forward and reverse DNA sequencing reactions of the PCR amplicons were conducted using the BDT v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyzer. The rRNA gene sequences were used to perform BLAST searches against the NCBI GenBank database to identify the bacterial strains. The top ten sequences based on identity scores were selected and aligned using the multiple alignment software program Clustal W. Phylogenetic trees were constructed using the Maximum Likelihood method based on the Kimura 2-parameter model within the MEGA 11 software package [36, 37].

2.9. **Statistical Analysis** - All experiments were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using appropriate statistical software. Differences among treatments were analyzed using one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p \leq 0.05$. The relationship between phytochemical constituents and antioxidant activity was statistically evaluated using correlation analysis. The experimental data were presented graphically using mean values with appropriate error bars.

Result

Isolation and morphological characterization

A total of four strains were isolated from the root nodules of *Clitoria ternatea* L. (two from the blue single-petal plant, designated CTspbNSI and CTspbNSII, and two bacterial isolates from the blue double-petal plant, designated CTdpsbNSI and CTdpsbNSII). Gram staining was performed on all isolates, and microscopic examination revealed that all four studied isolates were Gram-positive. The nitrogen-fixing bacteria were isolated on YEMA selective medium, and all samples showed good growth. The colony morphology of the isolates was observed; all isolates exhibited creamy-white colonies (Table 1).

Cultural and metabolic characterization

Results from qualitative tests on YEMA + Congo Red medium showed that three isolates (CTspbNSII, CTdpsbNSI, and CTdpsbNSII) appeared pale pink, while one isolate (CTspbNSI) appeared pale yellow. This indicated that the isolates did not absorb the red dye from the Congo Red indicator present in the medium.

Biochemical characterization

Several biochemical tests were conducted to identify and characterize the isolates in accordance with Bergey's Manual. The purity of the test isolates was re-confirmed through various biochemical tests on different media. The biochemical behavior of the test strains is presented in the table (Table 2.).

Table 1: Morphological characterization of the bacteria isolated from *Clitoria ternatea* L.

S. No.	Isolates	Morphological characterization of bacterial isolates from <i>Clitoria ternatea</i> L						
		Size	Form	Elevation	Margin	Pigmentation	Gram staining	CRYEMA Media
1	CTspbNSI	Small, Moderate	Circular	Raised	Entire	Off white	+ve	Pale yellow
2	CTspbNSI I	Small, Moderate	Rhizoid	Flat	Undulate	White	+ve	Pale pink
3	CTdpsbNS I	Pin point, small	Circular	Raised	Entire	White	+ve	Pale Pink
4	CTdpsbNS II	Small, Moderate	Circular	Flat	Entire	White	+ve	Pale Pink

(CTspbNSI & CTspbNSII = Bacterial strain I & II isolated from root nodule of *Clitoria ternatea* L. of blue coloured single petal grown in normal soil; CTdpsbNSI & CTdpsbNSII

= Bacterial strain I & II isolated from root nodule of *Clitoria ternatea* L of blue coloured double petal grown in normal soil)

Table 2: Biochemical characterization of the bacterial isolates from *Clitoria ternatea* L.

S. No.		1.	2.	3.	4.
Isolates		CTspbNSI	CTspbNSII	CTdpsbNSI	CTdpsbNSII
Biochemical Test	Casein Test	-	-	-	-
	Catalase test	+	+	+	+
	Citrate utilization Test	+	+	+	+
	Gelatin Hydrolysis test	-	-	-	-
	Indole test	-	-	-	-

	Methyl Red Test	+	+	+	+
	Nitrate reduction test	-	-	+	+
	Starch Hydrolysis test	-	+	-	-
	TSI test	-	-	-	-

(- =negative result, + = positive result)



Fig 1: Plant and root nodules of blue coloured single petal *Clitoria ternatea* L.



Fig 2: Plant and root nodules of blue coloured double petal *Clitoria ternatea* L.



a) Cryema

b) Starch Hydrolysis test



C) Gelatin hydrolysis test

(D). Casein test

(E) Methyl red test



(F). Catalase test

(G). TSI test

(H) Citrate utilization test

Fig 3: Biochemical characterization of bacterial isolates using various biochemical tests.

Physiological Characterisation

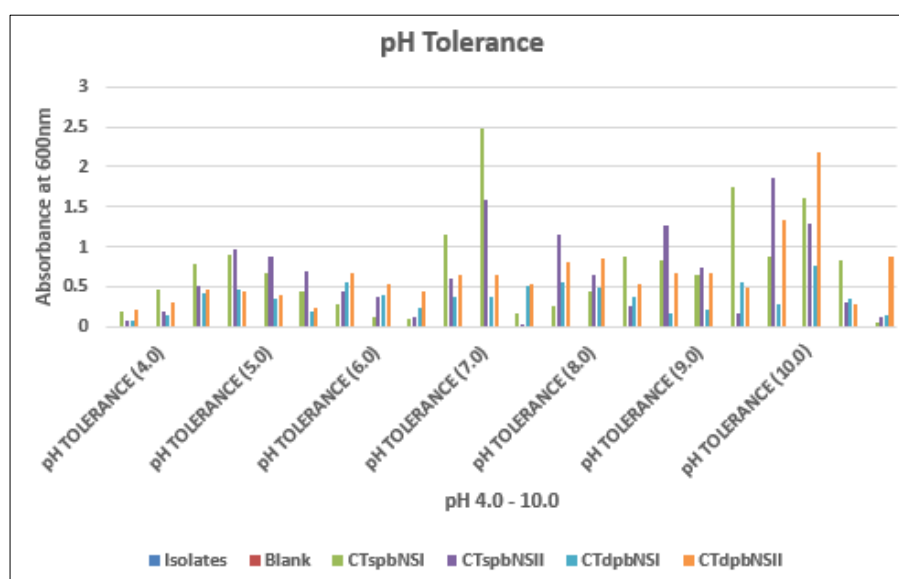
In this study, most isolates were able to tolerate temperatures ranging from 20°C to 44°C, with optimal growth observed at 28°C (Table 3). The results of this test align with previous studies indicating that root nodule bacteria are mesophilic, with an optimal growth temperature of 28°C for most isolates.

All the bacterial isolates tested demonstrated a broad pH tolerance, capable of growing across acidic and alkaline ranges from pH 4 to 10 (Table 4). The optimal pH for the growth of root nodule bacteria typically falls between 6 and 7.

All isolates are capable of tolerating high salt concentrations (up to 5% NaCl) (Table 5).

Table 3: Effect of Different pH Levels and Incubation Periods on the Growth of CTspbNSI, CTspbNSII, CTdphNSI and CTdphNSII at OD 600 nm.

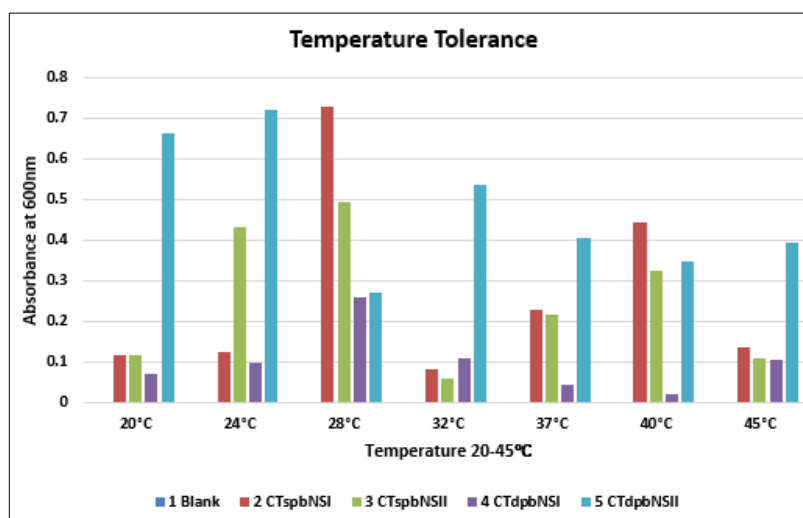
pH	Time	CTspbNSI	CTspbNSII	CTdphNSI	CTdphNSII
4	24 h	0.202	0.078	0.086	0.216
	48 h	0.476	0.198	0.156	0.299
	96 h	0.79	0.519	0.421	0.475
5	24 h	0.898	0.978	0.476	0.445
	48 h	0.674	0.874	0.349	0.399
	96 h	0.449	0.699	0.199	0.232
6	24 h	0.275	0.443	0.564	0.682
	48 h	0.132	0.384	0.394	0.537
	96 h	0.094	0.121	0.234	0.433
7	24 h	1.159	0.611	0.368	0.656
	48 h	2.484	1.577	0.377	0.655
	96 h	0.161	0.035	0.504	0.543
8	24 h	0.252	1.161	0.554	0.818
	48 h	0.447	0.652	0.488	0.85
	96 h	0.876	0.258	0.377	0.54
9	24 h	0.842	1.264	0.18	0.664
	48 h	0.657	0.75	0.206	0.673
	96 h	1.737	0.174	0.549	0.491
10	24 h	0.887	1.872	0.273	1.34
	48 h	1.6	1.281	0.768	2.178
	96 h	0.83	0.311	0.353	0.285



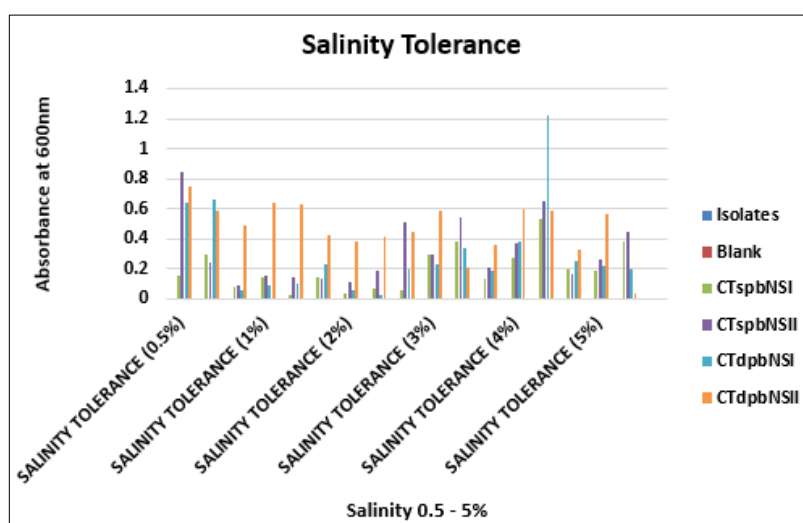
Graph 1: Effect of pH on the Growth of CTspbNSI, CTspbNSII, CTdphNSI and CTdphNSII

Table 4: Effect of Different Temperature Levels on the Growth of Bacterial Isolates at OD 600 nm.

S. No.	Isolates	20°C	24°C	28°C	32°C	37°C	40°C	45°C
1	CTspbNSI	0.116	0.127	0.727	0.084	0.227	0.443	0.138
2	CTspbNSII	0.117	0.434	0.495	0.061	0.216	0.324	0.11
3	CTdpbNSI	0.072	0.099	0.261	0.11	0.046	0.023	0.106
4	CTdpbNSII	0.662	0.719	0.271	0.538	0.404	0.348	0.394

**Graph 2:** Effect of Temperature on the Growth of CTspbNSI, CTspbNSII, CTdpbNSI and CTdpbNSII**Table 5:** Effect of Different Salinity Levels and Incubation Periods on the Growth of Bacterial isolates at OD 600nm.

Salinity (%)	Time	CTspbNSI	CTspbNSII	CTdpbNSI	CTdpbNSII
0.5	24 h	0.153	0.845	0.637	0.75
	48 h	0.29	0.244	0.666	0.587
	96 h	0.075	0.094	0.058	0.492
1	24 h	0.144	0.151	0.092	0.639
	48 h	0.023	0.147	0.099	0.633
	96 h	0.147	0.13	0.234	0.422
2	24 h	0.033	0.115	0.059	0.384
	48 h	0.066	0.184	0.029	0.413
	96 h	0.061	0.511	0.201	0.445
3	24 h	0.299	0.295	0.225	0.587
	48 h	0.377	0.543	0.339	0.21
	96 h	0.128	0.211	0.19	0.363
4	24 h	0.277	0.373	0.382	0.6
	48 h	0.533	0.655	1.223	0.588
	96 h	0.198	0.163	0.248	0.331
5	24 h	0.184	0.265	0.22	0.565
	48 h	0.381	0.443	0.193	0.04

**Graph 3:** Effect of Salinity on the Growth of CTspbNSI, CTspbNSII, CTdpbNSI and CTdpbNSII

Statistical analysis

One-way ANOVA revealed that the tested conditions had a significant effect on the four strains of *Clitoria ternatea* L. For Table 6, the effect of pH was significant ($F = 2.752$, $p = 0.048$), while Table 7 showed a highly significant effect of temperature ($F = 89.275$, $p < 0.001$). Similarly, Table 8 showed a significant difference among the strains ($F = 4.692$, $p = 0.005$). Overall, the results indicate that pH, temperature, and the tested conditions significantly influenced the growth/response of the strains.

Table 6: One-way analysis of variance (ANOVA) showing the effect of pH on four strains of *Clitoria ternatea* L.

Anova: Single Factor				
Groups	Count	Sum	Average	Variance
CTspbNSI	21	15.922	0.75819	0.357367
CTspbNSII	21	14.24	0.678095	0.267974
CTdpbNSI	21	7.876	0.375048	0.027756
CTdpbNSII	21	13.411	0.638619	0.187102

Table 7: One-way analysis of variance (ANOVA) showing the effect of pH on four strains of *Clitoria ternatea* L.

Anova						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.73448	3	0.57816	2.752491	0.047973	2.718785
Within Groups	16.80398	80	0.21005			
Total	18.53846	83				

Anova: Single Factor				
Groups	Count	Sum	Average	Variance
Temperature	7	226	32.28571	80.2381
CTspbNSI	7	1.862	0.266	0.056117
CTspbNSII	7	1.757	0.251	0.028919
CTdpbNSI	7	0.717	0.102429	0.005941
CTdpbNSII	7	3.336	0.476571	0.027967

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	5739.097	4	1434.774	89.27495	0.000000000000000324	2.689628
Within Groups	482.1422	30	16.07141			
Total	6221.239	34				

Table 8: One-way analysis of variance (ANOVA) showing the effect of pH on four strains of *Clitoria ternatea* L.

Anova: Single Factor				
Groups	Count	Sum	Average	Variance
CTspbNSI	17	3.369	0.198176	0.020279
CTspbNSII	17	5.369	0.315824	0.04649
CTdpbNSI	17	5.095	0.299706	0.089618
CTdpbNSII	17	8.049	0.473471	0.031118

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.65978	3	0.219927	4.691649	0.005044	2.748191
Within Groups	3.000079	64	0.046876			
Total	3.659859	67				

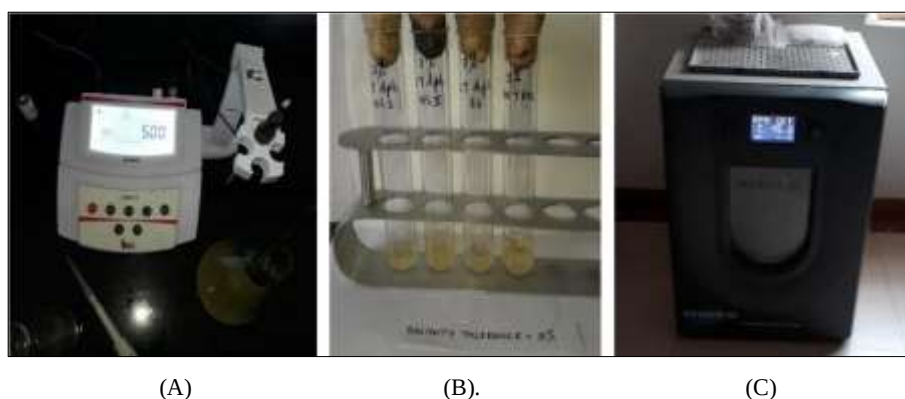


Fig 4: Stress tolerance analysis - (A) pH tolerance; (B) salinity tolerance; (C) temperature tolerance.

Identification and phylogenetic analysis of isolates

Four rhizobacteria were identified using 16S rRNA sequencing. BLAST and phylogenetic tree analysis revealed that three strains (CTspbNSI, CTdpbNSI, and CTdpbNSII)

shared 97.03% to 98.79% similarity with *Bacillus velezensis*, while one strain (CTspbNSII) showed 90.96% similarity with *Bacillus zanthoxyli*.

The nucleotide sequences of all four isolates (i.e., CTspbNSI, CTspbNSII, CTdpbNSI, and CTdpbNSII) were submitted to NCBI, and accession numbers PZ879549,

PZ879550, PZ879549, and PZ879549 respectively were obtained.

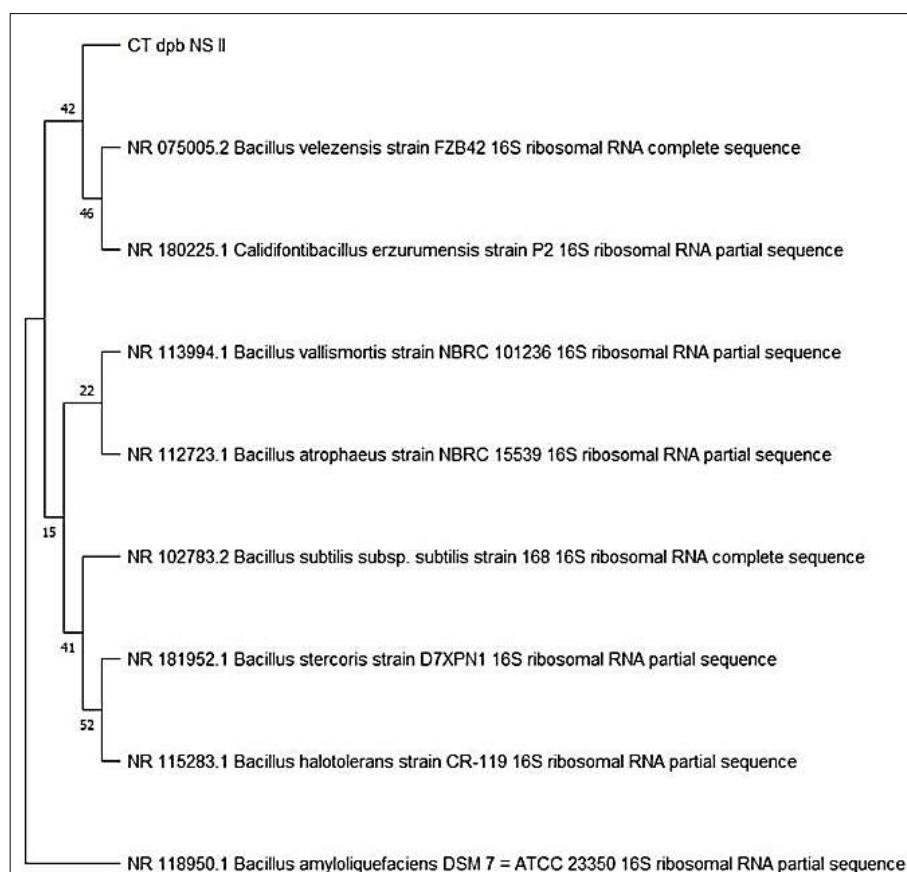


Fig 5: Phylogenetic tree based on 16S rRNA gene sequences of strain CTdpbNSII from the root nodule of *Clitoria ternatea* L. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA 11

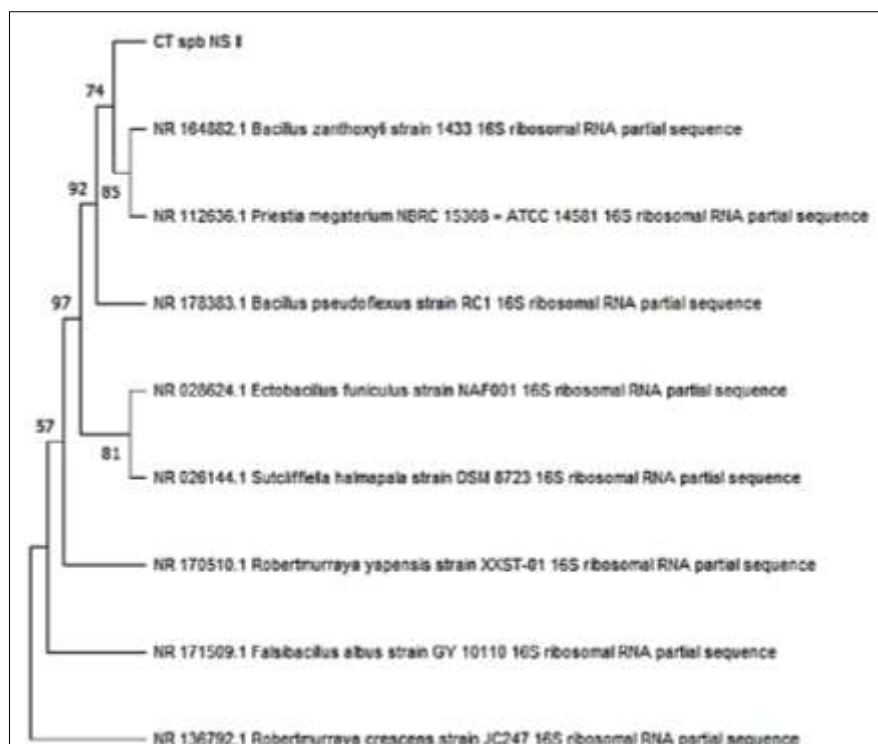


Fig 6: Phylogenetic tree based on 16S rRNA gene sequences of strain CTspbNSII from the root nodule of *Clitoria ternatea* L. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA 11

Discussion

This study is expected to reveal the diversity of certain rare native bacterial strains. Isolating native bacterial populations and understanding their characteristics could provide a valuable biochemical resource for utilizing new bacteria to enhance agricultural productivity. The initial phase involved isolating and purifying bacterial strains from *Clitoria ternatea* L. plants grown in ordinary soil.

The test strains were identified and classified based on their cultural characteristics, biochemical properties, and other factors. The bacterial strains exhibited variations across several characteristics. The growth rates of the strains and their behavior on different media were recorded.

A total of four isolates were obtained from the root nodules of *Clitoria ternatea* L. (two each from the single-petal blue and double-petal blue varieties). The morphological characteristics of all four isolates were observed on YEMA media plates. Bacterial colony sizes ranged from pin-point to small and medium. The isolates appeared white to off-white and translucent, with round and rhizoidal shapes; colony diameters ranged from 2 to 3 mm, featuring entire margins, convex or flat profiles, and a mucoid (sticky) texture. All four isolates were found to be Gram-positive.

The isolates exhibited light pink and light yellow colors on Congo Red YEMA medium; the pink coloration resulted from the poor absorption of the Congo Red dye present in the medium. The results of the biochemical characterization of the isolates are presented in Tables 1 and 2. All isolates tested positive for the catalase test, citrate utilization test, and methyl red test, while testing negative for the casein test, gelatin hydrolysis test, indole test, and TSI test. The nitrate reduction test was positive for two isolates, and the starch hydrolysis test was positive for one isolate.

Based on the aforementioned morphological and biochemical characteristics and 16S rRNA sequencing, the isolates were identified as *Bacillus velezensis* and *Bacillus zanthoxyli*.

Conclusion

This study revealed that bacterial isolates obtained from the root nodules of *Clitoria ternatea* L. are genetically and physiologically diverse. These isolates exhibited varying degrees of tolerance to different stress factors (pH, salinity, and temperature). Phenotypic analysis enabled the selection of strains capable of growing under diverse stress conditions. Consequently, this research facilitates the selection of superior strains that can enhance nitrogen fixation in legumes and contribute to food and nutritional security in a highly sustainable manner.

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