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Isolation and characterization of bacteria possessing Osmotolerance activity from phylloplane of *Centella asiatica*

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Abstract

Centella asiatica, commonly known as Asiatic pennywort and Gotukola, harbors a wide variety of phytochemicals. This complex phytochemical composition of the plant makes it suitable for broad medicinal and commercial applications. The plant body provides a habitat for the growth and survival of various microorganisms and has its microbiome. In this study, efforts were made to isolate and identify bacterial inhabitants from the leaves of the plant. The bacterial isolates were further tested for osmotolerance activity which enables them to survive and grow in high salt concentrations. The high salt concentration is one of the important abiotic parameters which affects the growth of living organisms negatively. High salt concentration induces abiotic stress on the plant by producing an excessive amount of reactive oxygen species which leads to damage to biomolecules and osmotic shock. However, certain living organisms especially bacteria come under the group called osmotolerant and have molecular and biochemical machinery which help in the alleviation of this salt stress and enable them to survive in high salt concentrations. During the study, various bacteria were isolated from the phylloplane of *C. asiatica* using specific microbial cultural methods. The isolated bacterial populations were subjected to identification and characterization. Various morphological and biochemical methods were applied to characterize the bacterial isolates. The characterized bacteria were further grown on media plates containing an increasing amount of salts such as sodium chloride, mannitol, and sorbitol which help in the isolation of osmotolerant bacteria. The osmotolerant bacteria were finally identified using advanced molecular methods like 16s rRNA sequencing. The results of this study show that the plant has varieties of bacterial inhabitants in its phylloplane and all three bacterial isolates were known and identified for having osmotolerance activity.

1. INTRODUCTION

Centella asiatica, which belongs to the family Apiaceae, is a herbaceous and perennial plant. The plant has immense commercial and economical significance due to its complex biochemical and phytochemical compositions. The phytochemical composition of the plant consists of pentacyclic triterpenoids, asiaticoside, brahmoside, asiatic acid, brahmic acid centellose, centelloside, and madecassoside [1-3]. These phytochemicals (phytochemicals are a group of bioactive chemical substances synthesized by plants) are of medicinal significance and play an important role to treat various diseases such as headache, drowsiness, wound healing lactation, and anti-aging [4,5] and are known to have anxiolytic (agents or drugs used to decrease anxiety level) properties as well [6]. The plant has significant dermatological applications [7]. The plant also contributes to the agricultural field as weed control and biomonitoring agent of soil for heavy metals.

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The plant body has many aerial or above-ground parts. The most dominant aerial part of the plant is the leaf. The aerial body parts of plants such as stems, buds, flowers, and leaves provide a suitable habitat for the growth and development of a wide variety of microorganisms [8]. Such all the above-ground body parts of plants that are densely populated with microorganisms are altogether termed as phyllosphere whereas the surface area specifically associated with plant leaves is called phylloplane. Bacteria are the most commonly found inhabitants of the phyllosphere [9]. Phyllospheric bacteria can be found as epiphytes and entophytes. Epiphytic bacteria are those who colonize the external surface of the plant whereas endophytic bacteria grow inside the plant body.

These phyllosphere bacteria are closely associated with the host plant and affect the plant's growth and development in varieties of ways [10,11]. These bacteria can affect the host plant positively or negatively. Some of the phyllosphere bacteria are known to play beneficial roles in various biological activities of host plants such as stress alleviation mechanism, carbon, nitrogen cycle, phosphate solubilization, and disease resistance. The study of phyllosphere bacteria helps to evaluate their role as plant growth-promoting bacteria which provides a platform for use of such bacteria for sustainable

agriculture which helps to fulfill the food and commercial needs of society without leaving any harmful impact on the environment.

Like all living systems, bacterial growth and development are also affected by various abiotic parameters (it includes various non-living components such as physical and chemical factors of an ecosystem) of its surrounding. High salt concentration or salinity is one of the important abiotic parameters which induces abiotic stress and adversely affects the growth and survival of bacteria. High salt concentration induces osmotic shock (lysis of the cell induced by rapid alteration in salt concentrations in the medium surrounding the cell) which alters the osmotic potential gradient. This altered osmotic potential gradient decreases water and mineral uptake by living cells which leads death of the cell. Some bacteria have an inbuilt biological mechanism that helps them to resist osmotic shock and survive in a high-salt environment. Such bacteria are known as osmotolerant (salt-tolerant bacteria).

In today's anthropogenic era (any change in the environment or natural resources caused by human interventions), most agricultural lands are degraded and destroyed by the continuous increase of various abiotic factors such as salinity, temperature, and soil erosion. Agricultural productivity is greatly threatened by salinity stress. This salinity stress can be alleviated by increasing the salt tolerance potential of the plants. Various microorganisms, which inhabited the plant body as a shelter, help to increase the stress tolerance mechanism of the host plant [12,13]. Certain bacteria have the potential to contribute to the stress tolerance mechanism by regulating the intracellular concentrations of various secondary metabolites in the host plants [14,15]. This ability of such plant commensal microorganisms makes them significant for sustainable agriculture and crop production.

2. MATERIALS AND METHODS

The methodology used for the present study included many steps. These steps were as follows:

2.1. Sample Collection and Preparation

The plant sample, *C. asiatica,* was collected and authenticated from the Botanical Garden of Indian Republic, NOIDA, UP. The fresh leaves of the plant were collected and washed with tap water to remove all dust particles from the leaf surface. Then these leaves were dried at room temperature. Dried leaves were crushed to make a homogenous powder. 1 g of this powder was homogenized into 1000 ml of distilled water. This leaf suspension was used as the sample for bacterial isolation [16].

2.2. Isolation of Bacteria

For the isolation of phyllosphere bacteria, 0.5 ml of the above leaf suspension was spread on the surface of plates containing sterile and solidified nutrient agar media. The inoculated plates were kept inside the incubator overnight. After incubation, bacterial colonies were grown and observed on the plates. Morphologically distinct bacterial colonies were further purified on similar media. The pure cultures of different bacterial isolates were used to perform various tests for their characterization [17].

2.3. Isolation of Osmotolerant Bacteria

The bacterial isolates were examined for their osmotic tolerance potential by inoculating the bacterial culture on the plates containing nutrient media with gradually increased concentrations of salts such as sodium chloride, D-mannitol, and sorbitol. The control plate was prepared by inoculating the same bacterial culture on the plate containing nutrient media without any salt concentrations. All the inoculated plates were kept in the incubator at 37°C for 24–48 h. After incubation, every plate was observed for bacterial growth. The bacteria that possessed osmotolerant capacity were able to grow on the media containing high salt concentrations and were considered osmotolerant bacteria [18].

2.4. Characterization of the Osmotolerant Bacterial Isolates

Isolated osmotolerant bacteria were identified and characterized using various methods of analysis described below.

2.4.1. Morphological analysis

The morphological characteristics of the isolated bacteria were tested by Gram's staining. It was done using the standard protocol. A thin bacterial smear was fixed on a clean glass slide. The smear was stained with crystal violet for 60 s, then wash with water. After this, the smear was treated with Lugol's iodine for 30 s, then washed with water, further with decolorization agent 70% of alcohol for 15 s, and washed with water. Finally, the smear was stained with safranin (counterstain), then washed with water and air-dried. The Gram's-stained smear was observed under a microscope. The bacterial cells appeared purple, called Gram's positive, and the bacterial cells appeared pink, referred to as Gram's negative [19].

2.4.2. Motility test

The motility of the bacterial isolates was tested by inoculating 24 h old culture of the test bacteria into a stab of nutrient agar media in the test tube. The tubes were incubated for 24–48 h at 37°C. The non-motile bacteria had growth restricted to the inoculation line while motile bacteria had diffused growth spreading to the surrounding area from the inoculation line [20].

2.4.3. Biochemical analysis

According to the classification system published in Bergey's Manual of Systematic Bacteriology, a series of biochemical tests were conducted to identify the biochemical profile of the isolated osmotolerant bacteria. These biochemical tests were –

2.4.3.1. Sugar fermentation test

This test was carried out to test the sugar fermentation ability of the test bacteria for various sugars such as glucose, lactose, and sucrose [21]. Certain bacteria can ferment a carbohydrate component such as sugar and produce acid and gas as products. For this test, a sugar indicator broth was prepared. The media was dispensed into test tubes, a durham tube was placed inside the tube in an inverted position and autoclaved all the tubes. The tubes were inoculated with a loopful culture of the test bacteria and incubated at 37°C for 2–7 days. The tubes were observed daily for any change in the color of the broth (that indicates acid production) and displacement of the broth in the durham tube (that indicates gas production). The test was performed for three sugars which were as follows –

2.4.3.1.1. Glucose utilization test

To test the glucose fermentation ability the test bacteria were inoculated in glucose indicator broth which contained peptone broth, 1% glucose, and 0.01% phenol red. After incubation, the yellow coloration of the broth indicates acid production, and displacement of broth in the durham tube indicates gas production.

2.4.3.1.2. Lactose utilization test

This test was performed using a similar protocol as the glucose fermentation test except that the broth used was the lactose indicator broth.

2.4.3.1.3. Sucrose utilization test

For this test, the media used was the sucrose indicator broth, rest protocol was similar to other sugar fermentation tests.

2.4.3.2. Catalase test

Catalase activity was tested by taking a small quantity of 24 h old bacterial culture on a clean glass slide, then a drop of hydrogen peroxide was added to the culture. The release of gas bubbles as white forth shows positive catalase activity [22].

2.4.3.3. Starch hydrolysis test

This test is used to test the ability of bacteria that can produce an enzyme called amylase that degrades starch. For this test, the plates were prepared by pouring the sterilized nutrient media containing 1% of soluble starch. The solidified plates were inoculated by streaking the bacterial culture across the media surface, the inoculated plates were kept in an incubator for 24 h. The starch-hydrolyzing bacteria were produced colonies that surrounded by the clear zone of starch hydrolysis due to the amylase enzyme released by the bacterial culture [21].

2.4.3.4. Urease test

The urease enzyme catalyzes the breakdown of urea into ammonia and carbon dioxide. The urease activity of the bacterial isolates was tested by inoculating the bacterial culture on the nutrient media containing urea and an indicator dye phenol red and then incubating at 37°C for 24 h. After incubation, the urease-producing bacteria changed the color of the media from yellow to reddish pink [23].

2.4.3.5. Casein digestion test

Caseinase is an enzyme that degrades milk protein into smaller peptides and free amino acids. The casein activity of the bacterial isolates was done by streaking the bacterial culture on the surface of the plates containing autoclaved casein media. After incubation, the plates were observed for the clear zone around the bacterial growth [24].

2.4.3.6. Indole test

The pure culture of the test organisms was mixed in the tube containing tryptone broth medium and then the tube was incubated at 37°C for 24–48 h. A few drops of Kovac's indole reagent were added to the tube. The formation of a pink to cherry red color ring on the top of the tube indicated a positive test [25].

2.4.3.7. Methyl red and vogas proskauer (MR-VP)

5 ml of MR-VP broth was transferred into test tubes and autoclaved. The sterilized tubes were inoculated with test bacteria and incubated at 37°C for 48 h. After incubation, half of the media was transferred into a new sterile tube, and then a few drops of MR indicator were added to the tubes. The tubes were observed for any color change. A formation of red color shows a positive test [26]. The remaining quantity of the MR-VP broth was mixed with Berritt's reagents 1 and 2, then the tube was shaken and allowed to rest for 30 min to 1 h. The development of red coloration on the top of the tube showed a positive test for VP [25].

2.4.3.8. Citrate utilization test

This test checks the ability of the bacteria to use citrate as a carbon source. It was performed by preparing slants in test tubes containing autoclaved citrate agar media. The media contains an indicator dye that indicates a change in P^H by changing its color from green to blue. The tubes were inoculated with test bacteria and then incubated at 37°C. A change in media color from green to blue shows a positive test [21].

2.4.3.9. Nitrate reduction test

Some bacteria produce nitrate reductase enzyme that reduces nitrate into nitrite. For this test, a peptone broth containing potassium nitrate was prepared and its small quantity was dispensed in test tubes. The test tubes were autoclaved and inoculated with the culture of test bacteria. Incubate the test tubes at 37°C for 24 h. After incubation, a few drops of sulfanilic acid and alpha naphthylamine were added. Finally, zinc dust was added to the tubes, then tubes were observed for any change in the coloration of the broth [27].

2.4.4. Molecular Characterization of the Osmotolerant Bacteria

The osmotolerant bacterial isolates were characterized by 16s rRNA sequencing. The procedure includes standard protocols of genomic DNA isolation, gel electrophoresis, PCR amplification, DNA sequencing, and phylogenetic analysis. The genomic DNA was isolated by homogenizing the bacterial culture in extraction or lytic buffer. The resultant cell lysate was further treated by a series of solvent extraction and centrifugation. The genomic DNA was precipitated by adding a solution of 0.1 volume of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol. The precipitated DNA pellet was washed with 70% ethanol and air-dried. Then, the genomic DNA was dissolved in TE buffer which was further subjected to RNAase treatment for the removal of RNA impurities. After a quality check, PCR amplification of the genomic DNA was carried out by using 16S rRNA forward primer (GGATGAGCCCGCGGCCTA) and reverse primer (CGGTGTGTACAAGGCCCGG). Thermos cycler was used for PCR amplification using 30 cycles at 94°C for 1 min (denaturation step), 50°C for 1 min (annealing step), and 72°C for 2 min (extension step) with a final extension of 7 min at 72°C. The amplified genomic DNA was tested by using agarose gel electrophoresis [Figures 1a and 2a]. The sequences of PCR products were analyzed using the ABI 3130xl platform. The resultant nucleotide sequences were further analyzed for homology and then the phylogenetic relationship was studied using the BLAST platform. Finally, the nucleotide sequences were submitted to NCBI (National Center for Biotechnological Information) for accession number [28,29].

3. RESULTS

The results of the study were also recorded and studied in the same cascade-like methodology. The results were as follows –

3.1. Isolation of Bacteria from *C. asiatica*

In the present study, a total of 12 bacterial strains (B-1 to B-12) were isolated from the leaf extract of the *C. asiatica* plant. All the isolated bacterial strains were maintained as pure culture forms using pure culture techniques. These bacterial isolates were isolated using nutrient agar media plates [Figure 1].

3.2. The Osmotolerant Potential of the Bacterial Isolates

The data of the Osmotolerance study revealed that two out of the 12 bacterial isolates were found salt tolerant at varying concentrations

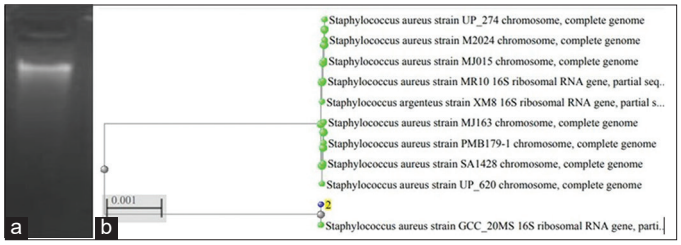

Figure 1: (a) Identification of Isolated DNA of the bacterial isolate B-6 using gel electrophoresis and (b) Phylogenetic analysis of 16s rRNA sequence of the isolate B-6.

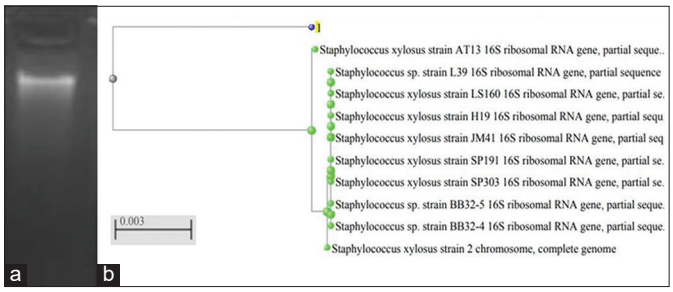

Figure 2: (a) Identification of Isolated DNA of the bacterial isolate B-7 using gel electrophoresis and (b) Phylogenetic analysis of 16s rRNA sequence of the isolate B-7.

(200mM, 400mM, 600mM,800mM, and 1000mM) of salts such as NaCl, mannitol, and sorbitol.

For NaCl salt, all the bacterial isolates (B-1 to B-12, except the B-9 isolate) showed good growth up to 200mM NaCl concentration but B-9 showed very slight growth at 200mM NaCl. The growth of all the bacterial isolates, except B-6, B-7, and B-8, were adversely affected by an increase in NaCl concentrations from 400 to 1000 mM. B-8 isolate showed good growth up to 600mM NaCl and the growth decreased up to slight and very slight at 800, and 1000 mM NaCl concentrations, respectively. The growth of B-9 was decreased with an increase in NaCl concentrations and showed no growth at 400mM to 1000mM NaCl concentration. The B-10 isolate was also adversely affected by increased salt concentration and showed no growth at higher concentrations of NaCl (800mM and 1000mM). the growth of B-11 and B-12 isolates was decreased with NaCl stress and showed no growth at 600 mM and above NaCl concentrations. The only two bacterial isolates, B-6 and B-7, showed good growth at the highest NaCl concentration (1000 mM), Table 1.

The data of the osmotolerance study of the bacterial isolates using varied concentrations (200 mM to 100 mM) of mannitol are shown in Table 2. The data revealed that the bacterial isolates B-1 to B-8 showed good growth up to 600 mM concentration of mannitol, except the B-5 isolate showed slight growth at 600mM mannitol, then the growth of bacterial isolates B-1 to B-5 was decreased at higher concentrations of mannitol (800 mM, 1000 mM). the B-5 isolate showed no growth at a 1000mM concentration of mannitol. The growth of B-9 isolate was adversely affected by the gradual increase in mannitol concentration from 200 mM to 1000 mM. The bacteria isolate B-10 showed good growth at 400 mM mannitol concentration, then the growth continuously decreased at 600mM, and 800 mM and showed no growth at the highest mannitol concentration (1000 mM). The growth of the B-11 and B-12 isolates was also decreased with the gradual increase in mannitol concentrations and showed no growth at higher concentrations of mannitol (800 mM and 1000 mM).

The Osmotolerance potential (using sorbitol) of all the bacterial isolates (B-1 to B-12) is presented in Table 3. The data revealed that all the bacterial isolates except B-5, B-8, B-9, and B-10 showed good growth at initial concentrations of sorbitol (200 mM and 400 mM). The B-5 isolate showed decreased growth after 200 mM sorbitol concentration and showed no growth at 600 mM, 800mM, and 1000mM sorbitol concentrations. The B-8 isolates showed decreased growth with an increase in sorbitol concentrations from 200 mM to 800 mM and had no growth at 1000 mM sorbitol concentrations. The B-9 isolates showed no growth at all concentrations of sorbitol stress. The growth of B-10 also decreased with sorbitol stress from 400mM to 800mM and showed no growth at the highest sorbitol concentration (100 mM). The B-1 and B-2 isolated showed very slight growth at 600–1000 mM sorbitol concentrations. The growth of the B-3 and B-4 bacterial isolates was decreased at higher ranges of sorbitol stress. The B-3 had no growth at 100 mM sorbitol concentration. The B-4 showed no growth at 800 mM and 1000 mM sorbitol concentrations. The B-11 and B-12 isolates were also negatively affected by sorbitol stress and showed no growth at the highest sorbitol concentrations (800 mM and 1000 mM). two of the total 12 bacterial isolates (B-6 and B-7) were able to grow at all the ranges of the sorbitol stress and showed good growth at the highest sorbitol concentrations (800 mM and 1000 mM).

3.3. Morphological and Biochemical Characterization of the Osmotolerant Bacterial Isolates

The results of morphological and biochemical characterization are shown in Table 4. The morphological (microscopic) test indicated that both the isolated osmotolerant bacterial isolates (B-6 and B-7) were Gram-positive, cocci (round in shape), and often arranged in grape-like clusters. The motility test showed that both the isolates B-6 and B-7 were non-motile. Both of these isolates showed positive tests for MR, VP, catalase, urease, citrate utilization, and sugar fermentation tests for glucose, lactose, and sucrose. The isolates (B-6 and B-7) were negative for amylase (starch hydrolysis test) and indole test.

3.4. Molecular Characterization of the Osmotolerant Bacterial Isolates

Molecular identification of the isolated osmotolerant bacteria (B-6 and B-7) was performed by PCR amplification of 16SrRNA sequences. For this 16 S rRNA gene of both the isolates was amplified by PCR successfully, then the amplified products were checked with agarose gel electrophoresis [Figures 1a and 2a]. The results of the gel electrophoresis revealed that the size of the PCR-amplified products of both isolates was approximately 2500bp (2.5 kb). The amplified products were sequenced and the sequences were generated in FASTA format. These resultant sequences were compared with the NCBI nucleotide database using BLAST-N comparison. The BLAST-N comparison study revealed 100% similarity of the isolate B-6 with Staphylococcus aureus strain GCC_20MS (NCBI accession number MZ305087.1) and 97.85% similarity of the isolate B-7 with Staphylococcus xylosus strain AT13 (NCBI accession number MW175729.1). The determined 16S rRNA sequences of both the isolates (B-6 and B-7) were submitted to the database of NCBI, GeneBank under the accession numbers (OM993290) (OM860203), respectively. The phylogenetic tree study of the isolated osmotolerant bacteria (B-6 and B-7) was performed and the data are shown in [Figures 1b and 2b].

Table 1: The study of the osmotolerance potential of the bacterial isolates (B-1 to B-12) using various concentrations of NaCl (200 mM, 400 mM, 600 mM, 800 mM, and 1000 mM).

S No.	Salt Conc → Bacterial isolates ↓	0 mM	200 mM	400 mM	600 mM	800 mM	1000 mM
1.	B-1	+++	+++	+	+	+	-
2.	B-2	+++	+++	++	+	+	-
3.	B-3	+++	+++	++	+	+	-
4.	B-4	+++	+++	++	-	-	-
5.	B-5	+++	+++	+	-	-	-
6.	B-6	+++	+++	+++	+++	+++	+++
7.	B-7	+++	+++	+++	+++	+++	+++
8.	B-8	+++	+++	+++	+++	++	+
9.	B-9	+++	+	-	-	-	-
10.	B-10	+++	+++	+	++	-	-
11.	B-11	+++	+++	+++	-	-	-
12.	B-12	+++	+++	+	-	-	-

+++ = Good growth, ++ = Slight growth, + = Very slight growth, − = No growth.

Table 2: The study of the osmotolerance potential of the bacterial isolates (B-1 to B-12) using various concentrations of mannitol (200 mM, 400 mM, 600 mM, 800 mM, and 1000 mM).

S. No.	Salt Conc → Bacterial isolates ↓	0 mm	200 mM	400 mM	600 mM	800 mM	100 mM
1.	B-1	+++	+++	+++	+++	++	+
2.	B-2	+++	+++	+++	+++	++	+
3.	B-3	+++	+++	+++	+++	++	+
4.	B-4	+++	+++	+++	+++	++	+
5.	B-5	+++	+++	+++	++	+	-
6.	B-6	+++	+++	+++	+++	+++	+++
7.	B-7	+++	+++	+++	+++	+++	++
8.	B-8	+++	+++	+++	+++	++	-
9.	B-9	+++	+	+	-	-	-
10.	B-10	+++	+++	+++	++	+	-
11.	b-11	+++	++	++	+	-	-
12	B-12	+++	++	++	+	-	-

+++ = Good growth, ++ = Slight growth, + = Very slight growth, − = No growth.

4. DISCUSSION

Microorganisms are found in all the biomes of the blue planet. A huge and diverse microbiome is also associated with plants. Scientists have a long-lasting interest to study the rhizosphere (below-ground region) of plants and its associated microbiome; hence, plenty of reported work is available on the rhizospheric microflora of the plants. As the Phyllosphere covers approximately 60% of the total biomass of the biosphere, it provides a huge shelter for the growth of a diverse microbial community [30]. In this study, the leaves of *C. asiatica* were studied for the presence of inhabitant bacterial communities. A total of 12 bacterial isolates were recovered and maintained as pure culture from the leaves of the *C. asiatica* plant. Numerous studies were performed earlier on microbial diversity of the Phyllosphere of several plants such as maize [31], rice [32], oat [33], and sugarcane [34]. Bhore *et al*., 2010 cultivated the bacterial endophytes from the leaves of Gaynura procumbent plants [35]. Rakotoniriana *et al*. also reported the presence of bacterial communities in the leaves of the *C. asiatica* plant, in their study [36]. Xie *et al*. revealed a phyllospheric bacterial community in macrophytes (aquatic plants) found in paddy soil in the study [37].

In the present study, these 12 bacterial isolates were assessed for salt tolerance using varied concentrations (200 mM, 400 mM, 6000 mM, 800 mM, and 1000 mM) of NaCl, mannitol, and sorbitol. The previous studies reported the occurrence of salt-tolerant microflora in plant-associated habitats [38]. Kamalraj *et al*., 2008 showed the impact of NaCl on the growth rate of endophytic fungus isolated from the leaves of mangrove *C. roxburghiana* [39]. Another work was also reported by Gayathri *et al*., 2010 which revealed that the bacteria isolated from mangrove and salt marsh leaves were found to tolerate salt up to 10%

Table 3: The study of the osmotolerance potential of the bacterial isolates (B-1 to B-12) by using various concentrations of sorbitol (200 mM, 400 mM, 600 mM, 800 mM, and 1000 mM).

S. No.	Salt Conc → / Bacterial isolates ↓	0 mM	200 mM	400 mM	600 mM	800 mM	1000 mM
1.	B-1	+++	+++	+++	+	+	+
2.	B-2	+++	+++	+++	+	+	+
3.	B-3	+++	+++	+++	+	+	-
4.	B-4	+++	+++	+++	+	-	-
5.	B-5	+++	+++	+	-	-	-
6.	B-6	+++	+++	+++	+++	+++	+++
7.	B-7	+++	+++	+++	+++	+++	++
8.	B-8	+++	++	++	+	+	-
9.	B-9	+++	-	-	-	-	-
10.	B-10	+++	+++	++	+	+	-
11.	B-11	+++	+++	+++	++	-	-
12.	B-12	+++	+++	+++	++	-	-

+++ = Good growth, ++ = Slight growth, + = Very slight growth, − = No growth.

Table 4: Morphological and biochemical characteristics of the isolated osmotolerant bacteria.

S. No.	Bacterial isolates → / Biochemical Tests ↓	B-6	B-7
1.	Gram's staining	+ (Cocci in the cluster)	+ (Cocci in the cluster)
2.	Motility test	-	-
3.	Starch hydrolysis test	-	-
4.	Catalase test	+	+
5.	Urease test	+	+
6.	Indole test	-	-
7.	Methyl red test	+	+
8.	Vogas Proskeur test	+	+
9.	Citrate test	+	+
10.	Glucose fermentation test	+	+
11.	Lactose fermentation test	+	+
12.	Sucrose fermentation test	+	+

+ = Positive test, − = Negative test.

of NaCl [40]. The result of the present study showed that only two bacterial isolates (B-6 and B-7) were found to maintain their growth at higher concentrations of NaCl, mannitol, and sorbitol. Hence, both of these bacterial isolates were considered salt tolerant.

In the present study, the preliminary characterization of the two salt-tolerant bacterial isolates (B-6 and B-7) was done by morphological and various biochemical tests. Both isolates were chosen for Gram's staining to evaluate their morphological or microscopic characteristics. In the results of this study, both the salt-tolerant bacteria (B-6 and B-7) were found Gram's positive, round (cocci) in shape, and arranged in grapes-like clusters. Zinniel *et al*. (2002) also found the presence of Gram-positive endophytic bacteria in agronomic crop plants [41]. In the present study, these Gram-positive cocci were further characterized by various biochemical tests. The results of the biochemical test revealed that both the isolated salt-tolerant bacteria (B-6 and B-7) were shown negative tests for motility and indole tests and positive tests for MR, VP, citrate utilization, nitrate reduction, sugar fermentation tests (for glucose, lactose, and sucrose), catalase, and amylase [42]. In this study, both the salt-tolerant bacterial isolates were shown morphological and biochemical properties of *Streptococcus* Sps based on Bergey's manual studies [43].

In the present work, both the isolated bacteria (B-6 and B-7) were finally identified on a molecular basis using 16 S rRNA sequencing. Based on the 16S rRNA sequencing results, phylogenetic trees were formed for both the isolates B-6 and B-76. The studies revealed that both the salt-tolerant bacterial isolates (B-6 and B-7) belong to the Staphylococcus group, containing *S. aureus* strain GCC_20MS and *S. xylosus* strain AT13, respectively [44].

The results of the present study were supported by various earlier studies, which showed the presence of salt-tolerant staphylococcus sps (namely, *S. aureus* and *S. xylosus*) in the Phyllosphere region of plants [45]. Liu *et al*., 2015 isolated the endophytic bacteria from the various parts of a medicinal plant, Noni (*Morinda citrifolia* L.), and confirmed the presence of *S. aureus* in the above-ground parts like leaves in the plant [46]. In another previous study, Swamy *et al*. 2020 reported the inhabitance of *S. aureus* sps in some plants such as the Ashok tree, Lablab, and Noni [47]. Adeniyi *et al*. 2020, also studied the phyllosphere microbial profile of the Ficus vogelii and confirmed the predominance of *S. aureus* in the plant leaf extract [48]. Similarly, Oluyemi *et al*., 2013 also isolated various multiple drug resistance *staphylococcal species* such as *S. aureus* and *S. xylosus* from the Phyllosphere of the *Ficus sycomorus* Linn [49].

In the present study, salt-tolerant *Staphylococcal sps* were explored in the leaf extract of the *C. asiatica* plants. This study will provide a reference to the phyllosphere bacterial diversity of the plant. In this study, salt-tolerant bacterial sps which were isolated from the plant leaves may have the potential to contribute to plant health by contributing to important plant metabolic activity like nitrogen fixation [50] or by alleviating various abiotic stressors like salinity [51]. The research works are widely documented on the role of plant-associated microorganisms in salinity tolerance mechanism [52,53]. Bian *et al*., 2011 demonstrated that salt-

tolerant bacterial sps like *Staphylococcal sps* cultivated from plant sources were able to produce plant growth hormones like indole acetic acid and contribute to the salinity tolerance mechanism of the host plant under high salt conditions. More studies were also performed which supported the findings of the present studies [54-56]. Komaresofla *et al*. 2019 also reported the presence of *S. aureus* as a rhizospheric and endophytic bacteria of the *Salicornia* plant and also found their positive role in the host plant growth and salinity tolerance ability under salinity stress conditions. Shultana *et al*., 2022 also reviewed the role of *S. xylosus* as a plant growth-promoting rhizobacteria and its role in the promotion of the host plant's growth by influencing salt tolerance adaptation mechanism [57]. Earlier, Afrasayab *et al*., 2010 performed a study that confirmed that the *S. xylosus* influenced the growth characteristics of the *Triticum aestivum* under salinity stress by increasing the seedling length of the plant by 25% at 100mM NaCl concentration [58]. Nagaraju *et al*., 2021 also revealed in their study that the *S. xylosus* inoculants increased the enzyme activity (like superoxide dismutase, and catalase) and proline content in the leaves of chickpea plants and increased the productivity under the salt stress conditions [59].

The main objective of the present study was to reveal the presence of osmotolerant bacterial species as natural inhabitants of the photospheric region of the plants. These osmotolerant bacterial species can be used as a biocontrol agent under salinity stress conditions and helped to induce tolerance mechanisms against high salinity for the host plants.

5. CONCLUSION

The present study explained the presence of a culturable diverse population of bacteria in the leaves of the *C. asiatica* plant. The results of this study reveal that the twelve bacterial isolates were cultured in pure forms, among which only two isolates, namely, *S. aureus* strain GCC_20MS and *S. xylosus* strain AT13 were found salt tolerant. The phyllosphere is an important habitat scientifically and economically as well. It is crowded with a great diversity of microorganisms that can contribute to various vital processes of the host plant and affect the growth of the host plant in a positive manner. Hence, the study of the phyllosphere microflora of plants always provides a better understanding of plant-microbial interactions and their practical applications in the sustainable growth of plants. The remarkable presence of such salt-tolerant bacteria in the phyllosphere region of the plant (*C. asiatica*) suggests that such bacteria can be used as biocontrol agents in the future application and helped in more effective stress management in the host plants under saline field conditions. The present study provides a strong base for further research to find out the positive role of such salt-tolerant bacteria in salinity stress management for plants and their commercial utility in sustainable agricultural production as a biocontrol agent.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

12. PUBLISHER'S NOTE

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