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Isolation and Characterization of Beneficial Bacterium from the Rhizosphere of *Suaeda nudiflora* in Sundarbans, India

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A study was carried out to isolate and characterize salt-tolerant beneficial bacteria from the soil sample collected from the rhizosphere of a halophytic plant (*Suaeda nudiflora*) from Gosaba, 24 Parganas (South), West Bengal, India located in the Sundarbans delta during January 2018. The physicochemical analysis of the soil showed that the soil was alkaline in nature with high salinity. The organic carbon, total nitrogen and available phosphorus status of the soil were low while the available potassium content was high. Bacterium N8 isolated from the rhizospheric soil possessed multiple growth-promoting activities *i.e.,* the highest phosphate solubilizing efficiency (8.52 mg P g^{-1} of sugar), nitrogen-fixing capacity (0.7 mg of N g^{-1} of sugar), phosphatase enzyme activity and tolerance to salinity at 6% NaCl. A morphological study of this bacterium indicated that it was gram-positive, rod-shaped and was present in both single and chain form. The PCR amplification of genomic DNA (using 16S rDNA specific primers) showed a clear visible band at 1.5Kb. Sequencing of the amplicon followed by BLAST analysis depicted that the bacterium N8 exhibited the highest similarity (99%) with the genus *Bacillus*. The NCBI-Gen Bank accession number of the bacterium was SUB416213 N8 MH489431. The presence of members of the genus *Bacillus* in the rhizosphere soil of *Suaeda nudiflora* from Sundarbans is being reported for the first time. This study has led to a significant avenue for the use of the N8 bacterium as a potential salt-tolerant phosphate solubilizing bio-inoculum for improving nutrient availability in the coastal region.

(***Key words:*** *Nitrogen fixation, Phosphate solubilising bacteria, Salinity, Suaeda nudiflora,* 16S *rDNA*)

Sundarbans has the largest biodiversity of mangrove plants in the world. Approximately 0.82 million ha of land is salt affected in the coastal region of West Bengal (87º25´ E and 89ºE latitude and 21º30´ N and 23º15´ N longitude), covering the districts of North and South 24 Parganas, Howrah and East Midnapur (Yadav *et al.,* 1983). *Suaeda nudiflora* is a succulent halophyte, commonly occurring in saline marshes of Sundarbans. Inherently, it exhibits luxuriant growth under high saline conditions. In certain areas of Sundarbans, people use this plant as a vegetable substitute due to its high amino acid content and used as an alternative to oilseed crops (Cherian and Reddy, 2000). Several studies have been conducted on endophytic bacteria of this plant (Arora *et al.,* 2014) and their antimicrobial activities (Makadia and Panchal, 2016). A study on comparative leaf epidermal morphology and foliar Na:K accumulation in *Suaeda nudiflora* revealed that low Na:K ratio and high water storage capacity in leaves of *S. nudiflora* make this species tolerant to high salinity (Farooqui *et al.,* 2009).

A plethora of information on the effects of salinity on soil physical and chemical properties is available (Bandyopadhyay *et al.,* 2003; Sardinha *et al.,* 2003) but

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soil microbial and biochemical aspects of the natural saline environment were scantily studied (Jaiswal *et al.,* 2022; Rietz and Haynes 2003). Studies revealed that salinity has influenced the microbial and biochemical properties of the coastal soil of Sundarbans (Tripathi *et al.,* 2006, 2007). According to Oren (2022), non-halophiles are those microorganisms that grow best below 2% salt concentration. So categorically those halophiles best grow in media containing 2 to 5%, 5 to 20%, and 20 to 30% are called slight, moderate, and extreme halophiles respectively. The rhizospheric soil is a storehouse of a huge number of plant growth-promoting rhizobacteria (PGPR), especially phosphate solubilizing bacteria (PSB) that can promote the dissolution of insoluble phosphorous (P) in soil, enhancing the availability of soluble P (Wang *et al.,* 2017).

Phosphorous is an important limiting factor for agricultural production (Asea *et al.,* 1988; Goldstein 1951; Jha *et al.,* 2013). Large amounts of phosphate fertilizer added to the soil not only causes environmental hazard but also increase the cost of agriculture and are unfavourable to the sustainable development of agriculture (Hanif *et al.,* 2015; Majeed *et al.,* 2015; Shahid *et al.,* 2012). PSB has a great ability to transform insoluble P in the soil into an available form and have great application prospects for eco-agriculture. The secretion of organic acids, enzymes and chelation are major P-solubilization mechanisms of PSB. For the effective promotion of crop growth and sustainable agricultural development, an important aspect is the occurrence of numerous PSB in the soil (Fernandez *et al.,* 2012).

According to Suraj Singh *et al.* (2013) for plant growth, nitrogen is commonly considered one of the foremost restrictive nutrients. In the biosphere, nitrogen is available in a form of atmospheric nitrogen (molecular nitrogen) that cannot actually be utilised by the plant. After photosynthesis, the second most important biological process on earth is biological nitrogen fixation, which is the reduction of atmospheric nitrogen (N_2) to ammonia or ammonium ion depending upon the pH of the medium (Kizilkaya, 2009). According to Sylvia *et al.* (2005) independent, free-living nitrogen-fixing microbes have some associations of differing degrees of complexity with other microbes and plants.

Generally, long-term use and oversupply of inorganic fertilizer cause eutrophication in surface water, nitrate pollution in groundwater, loss of soil organic matter, biological activities, decrease in soil fertility and deterioration in structure (Zhong and Cai, 2007; Ju *et al.,* 2009). Given the impending cost of fertilizers, the application of PSB and nitrogen fixers as biofertilizers can reduce the consumption of fertilizers and aid in sustainable agriculture development (Wang *et al.,* 2017). Soil salinity, imbalance of nutrients, unfavourable pH, and lack of good quality irrigation water are the major problems for crop production (Sarkar *et al.,* 2022) in Sundarbans. Little effort has been directed to isolate and characterize efficient salt-tolerant phosphate solubilizing bacteria (PSB) and nitrogen-fixing bacteria from the rhizosphere of halophytic plants like (*Suaeda nudiflora*) from the coastal region.

It was hypothesized that agriculturally beneficial phosphate solubilizing bacteria, as well as nitrogen fixers, may be present at the rhizosphere of *Suaeda nudiflora,* some of which might act as efficient salt-tolerant phosphate solubilizers and nitrogen fixers. The objective of the present study was to isolate and characterize an efficient salt-tolerant PSB and nitrogen fixer strain from the rhizosphere soil of *Suaeda nudiflora* of Sundarbans which may be used as a bioinoculant in coastal agriculture.

MATERIALS AND METHODS

Study site

The study site was situated at Gosaba (22°10′ 11″ N and 88°47′69″ E), (Sundarbans), 24 Parganas (South), West Bengal, India. The rhizospheric soil along with the plant (*Suaeda nudiflora*) was collected from the river banks at the study site in January 2018. The samples were brought to the laboratory in the field moist condition for physico-chemical and microbiological study. Each of the analyses was made in triplicates.

Physico-chemical properties of the rhizosphere soil

The rhizospheric soil of (*Suaeda nudiflora*) was separated from the roots aseptically to assess their microbiological properties. A part of rhizospheric soil was air-dried and used for physico-chemical properties

analysis. The pH and EC of the soil samples were measured using a digital pH meter and EC meter (Systronics) in 1:2.5 (w/v) aqueous solutions (Deionized water). The total organic carbon (TOC) was estimated by the method of Walkley-Black (Walkley and Black 1934). Total Kjeldahl nitrogen (TN) was measured using the method described by Jackson (1967) using Kelplus Nitrogen Estimation System (Model: DISTYL-EM(VA), Pelican Equipments, Chennai, India). Available phosphorus was measured according to Olsen (1994). The available potassium was determined using a flame photometer (Jackson, 1967).

Isolation and morphological identification of bacterial stains

Salt tolerant bacteria were isolated from the rhizospheric soil of plant samples by serial dilution plate technique on both Pikovskaya's agar plates and Jensen's agar plate containing 1% NaCl (Cappuccino and Sherman, 2005). The plates were incubated at 30±2ºC for 48 hours. Visible discrete single colonies with clear zone indicating insoluble phosphate solubilisation on Pikovskaya Agar (for PSB) and single smooth gummy colonies on Jensen's agar plate (for N_2 fixing bacteria), were identified and isolated. Purification of the isolated colonies was done using the standard method. Colony morphology of selected bacterial strains has been observed on agar plates and agar slants. Different types of staining have been done following the standard method for morphological characterization of pure strains (Cappuccino and Sherman, 2005).

Study of salt tolerance by isolated strains

Salt tolerant ability of the isolated bacterial strains was determined by exposing them to increasing concentrations of salt in media. Isolated bacterial strains were streaked on Pikovskaya's and Jensen's growth medium plates, respectively, supplemented with different concentrations (1%, 2%, 3%, 4%, 5% and 6%) of NaCl. The observations were taken after 48 hours of incubation at 30±2ºC. All isolated strains were tested and compared against the control.

Phosphate solubilizing efficacy of bacterial strains

For determination of phosphate solubilizing efficiency of selected bacterial strains, a loop full of pure bacterial strain was transferred to 50 mL of Pikovskaya's broth containing 1% NaCl in a 100 mL Erlenmeyer flask and incubated at 30±2ºC for 7 days in a shaker (180 rpm). After completion of the incubation period, 10 mL of culture was centrifuged at 13,000 x g for 10 minutes to obtain cell free supernatants. To quantify the soluble phosphate, the supernatant was analyzed by molybdate blue (Modified Olsen's) method with the absorbance measured at 750 nm using a spectrophotometer (Systronics UV-VIS Spectrophotometer 117). The standard curve was prepared using standard protocol. Similarly, a control setup was prepared without bacterial cell culture. All the observations were taken in triplicates. The strain exhibiting maximum phosphate solubilisation was screened for further analysis.

Total nitrogen fixing efficacy of bacterial strains

In the micro-Kjeldahl method, a loop full of pure bacterial strain was transferred to 50 mL of Jensen's broth containing 1% NaCl in a 100 mL Erlenmeyer flask and incubated at 30±2ºC for 7 days in a shaker (180 rpm) for quantification of nitrogen-fixing efficacy of selected bacterial strain as described by Jackson (1967). Simultaneously control was prepared without bacterial inoculation into Jensen's broth. After 7 days, 8 mL broth culture was transferred into a digestion tube along with 10 mL concentrated H_2SO_4 and 10 g digestion mixture ($CuSO_4$:K_2SO_4=1:10). Then the solution was boiled at 420ºC temperature until the solution turned green. A parallel set of blanks was performed with conc. H_2SO_4 and a digestion mixture without bacterial strain. After completion of the digestion process, the solution was cooled at room temperature, 40% NaOH and 4% boric acid were used in the distillation process followed by titration with 0.02 N H_2SO_4. The titration values were recorded and the strain exhibiting maximum nitrogen fixation was screened for further analysis.

Growth curve study

Based on the previous study, the same selected strain was grown in Luria broth. To analyse the growth pattern of the selected bacterial strain, 0.1 mL of fresh culture was transferred to 100 mL of Luria broth containing 1% of NaCl and incubated at 30±2ºC in a shaker (180 rpm). Optical density (O.D) was measured at 660 nm duration of 0, 12, 24, 36, 48, 60 and 72 hours using a spectrophotometer (Systronics UV-VIS Spectrophotometer 117). Simultaneously control

was prepared without the addition of bacterial culture (Cappuccino and Sherman, 2005).

Phosphatase enzyme activity of the selected strain

The phosphatase activity of screened bacterial strain was estimated by Modified Universal Buffer (MUB) method (Tabatabai and Bremner, 1969). A loop-full culture of the selected strain was transferred in 25 mL of Pikovskaya's broth containing 1% NaCl and incubated at 30±2ºC for 7 days in a shaker (180 rpm). After 7 days, Pikovskaya's broth culture was centrifuged at 10,000 rpm for 10 min at 4ºC. One millilitre of bacterial cell free culture was taken in an Erlenmeyer flask (50 mL) and treated with toluene, MUB (pH 6.5 or 11), p-nitrophenyl phosphate solution and incubated for 1 hour at 37°C. After incubation, $CaCl_2$ and NaOH were added, mixed properly in the flask and then filtered through Whatman no. 42 filter paper. The absorbance of the filtrate solution was recorded at 420 nm using a spectrophotometer (Systronics UV-VIS Spectrophotometer 117). Similarly, for control, p-nitrophenyl phosphate solution was added, after the addition of $CaCl_2$ and NaOH immediately before filtration of the suspension. For blank, 5 mL distilled water, 1 mL 0.5 (M) $CaCl_2$ and 4 mL 0.5 (M) NaOH were added into the flask and then filtered through Whatman no. 42 filter paper. The standard curve was prepared using standard protocol.

Molecular identification of the selected strain

For molecular characterization, genomic DNA isolation from the screened bacterial strain was carried out by lysozyme-SDS-phenol/chloroform method (Moore *et al.,* 2004). The isolated DNA was subjected to PCR amplification for the 16S rDNA gene, using universal bacterial 16S rDNA primers (27 Forward primer: 5′-AGAGTTTGA TYMTGGCTCAG-3′); [M=A/C] and (1492 Reverse primer: 5′-TACCTTGTTAYGACTT-3′); [Y= C/T]. Amplicons were confirmed for their size on 1.2% agarose gel and purified using a QIAquick Gel Extraction Kit. The gel eluted DNA product was subsequently sequenced and the derived sequence was compared to known bacterial 16S rDNA gene sequences in the National Centre for Biotechnology Information NCBI GenBank database using the BLAST algorithm.

RESULTS AND DISCUSSION

Characterization of rhizospheric soil

The physico-chemical analysis of the rhizosphere soil of *Suaeda nudiflora* under study indicated that it was alkaline in nature (pH 8.4). It was highly saline as evident from the EC of the soil (8.8 dS m^{-1}). The organic carbon content, total nitrogen status, available phosphorus and available potassium content of the soil were 6.64 g kg^{-1}, 0.42 g kg^{-1}, 9.9 kg ha^{-1} and 1258 kg ha^{-1}, respectively. This halophytic plant is a common habitat of Sundarbans and usually grows around the char land, which experiences regular inundation by saline water. The rhizospheric soil was alkaline which might be due to the presence of alkaline earth metal ions in this soil. As the soil receives regular tidal inundations, the organic carbon, total nitrogen and available phosphorus content were low. As the salinity level of the soil was high and potassium is one of the major components of soil salinity, the rhizospheric soil had high potassium content.

The rhizospheric soil is a storehouse of various types of organisms such as bacteria, fungi, protozoa, algae etc. but among them, plant growth promoting rhizobacteria (PGPR) were found to be most abundant (Bahadur *et al.,* 2017). The total nitrogen content of this rhizospheric soil was comparatively low since nitrogen fixation by the diazotrophic bacterial population was moderate. As the soil was highly alkaline (pH>8.0), it might be high in sodium chloride, borate and bicarbonate, often associated with high salinity, which in turn reduced nitrogen fixation (Bordeleau and Prévost, 1994). In Sundarbans, salt is the most common factor that hampers crop growth and coastal agricultural development (Yadav *et al.,* 1983).

Isolation and efficacy assay of bacterial strain

During the study, total thirty two bacterial colonies were initially isolated on NaCl containing Pikovskaya's and Jensen's media plates from the rhizospheric soil of this halophytic plant. Out of total thirty two bacterial colonies, twenty two bacterial strains were purified and selected for further studies. The study of tolerance to different concentrations of NaCl revealed that out of twenty two pure isolated bacterial strains, twelve bacteria strains showed the highest salt tolerance at 6% NaCl. These twelve salt tolerant bacterial strains showed that a minimum amount of salinity (1% NaCl) was required for their growth in the media. Without salt

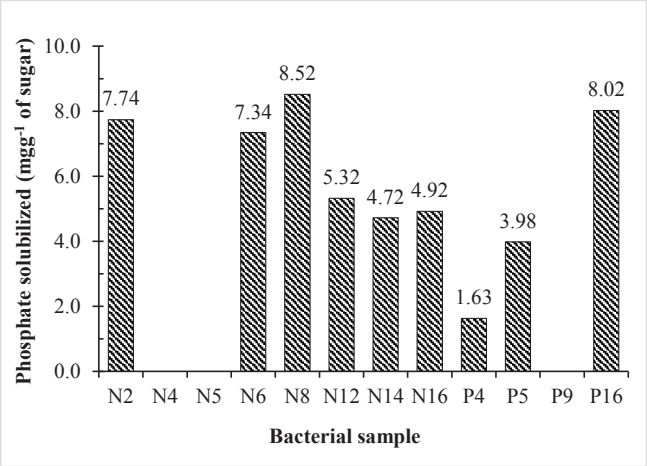

Fig. 1. *Phosphate solubilising capacity of isolated bacterial strains*

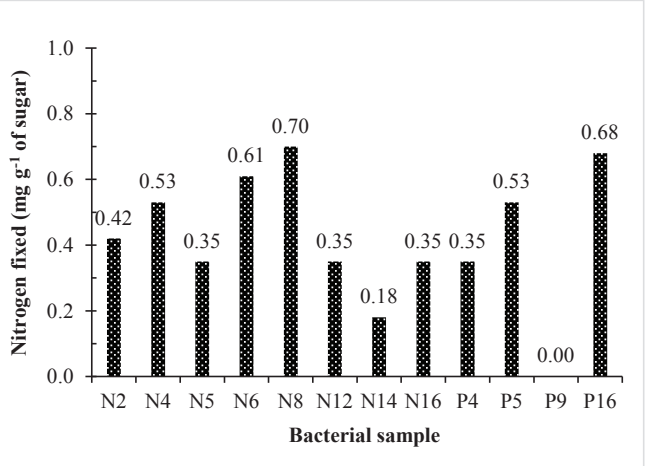

Fig. 2. *Nitrogen fixing capacity of isolated bacterial strains*

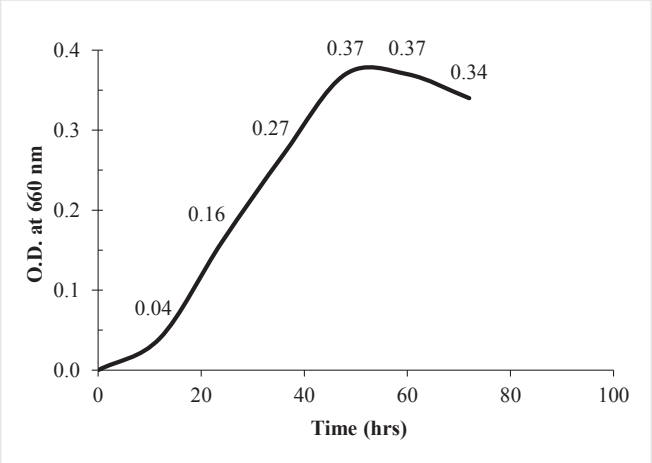

Fig. 3. *Growth curve of N8 bacterial strain*

(WOS) no growth was observed (Plate 1).

These twelve strains were selected for the efficacy study. Phosphate solubilizing efficiency of the twelve strains showed that isolate N8 exhibited the highest phosphate solubilizing efficacy (8.52 mg of P g^{-1} of sugar) (Fig. 1). In Fig. 1, it was observed that isolate N8 (8.52 mg of P g^{-1} of sugar) exhibited highest phosphate solubilizing capacity as compared to others on the Pikovskaya's broth containing 1% NaCl after 7 days of incubation. N4, N5 and P9 did not show any phosphate solubilizing ability, whereas the rest of the bacteria (N2, N6 N12, N14, N16, P4, P5 and P16) showed moderate phosphate solubilizing capacity in the broth medium. In culture medium, the insoluble P is solubilized to release P in an increasing manner depending upon culture time and nutrient consumption, thereby making it available so that plants can absorb it easily (Chatli *et al.,* 2008). According to Zhao *et al.* (2014) in Pikovskaya's broth media inoculated with PSB, soluble P content was found to be consistent over time. PSB might secrete organic acids which degrade TCP in Pikovskaya's culture medium to soluble P, so the content of soluble P does not decrease over time (Li *et al.,* 2015; Osorio and Habte, 2015). Simultaneously, some of these twelve strains also showed nitrogen fixing ability, among them, the same N8 strain exhibited the highest nitrogen fixing (0.7 mg of N g^{-1} of sugar) capacity (Fig. 2). P9 did not fix a sufficient amount of nitrogen in the broth medium, whereas the rest of the bacteria (N2, N4, N5, N12, N14, N16, P4, P5 and P16) showed moderate nitrogen fixing capacity in the broth medium. According to Xie *et al.*

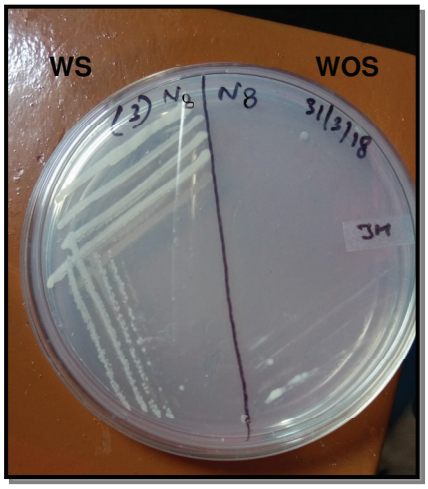

Plate 1. *Growth of N8 strain with 1% NaCl salt (WS) and without 1% NaCl salt (WOS)*

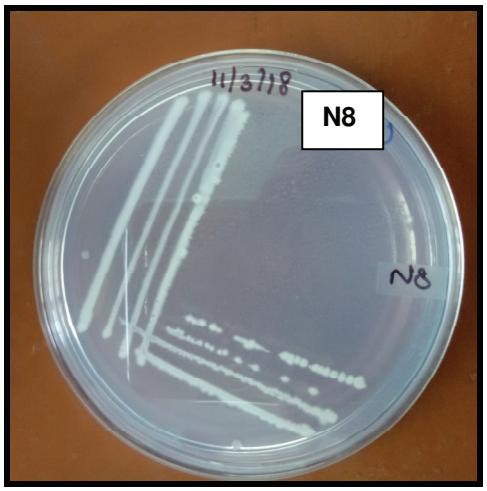

Plate 2. *Pure culture of N8 strain amended with 1% NaCl*

(2003), nitrogen fixing capacity of *Bacillus cereus* was reported to be around 0.014 to 0.239 mg culture^{-1} day^{-1}, which was relevant to this present study. Based on the highest phosphate solubilizing efficacy (8.52 mg of P g^{-1} of sugar) as well as nitrogen fixing capacity (0.7 mg of N g^{-1} of sugar) and high salt tolerance ability (6% NaCl), N8 bacterial isolates were selected for further studies.

Phosphates enzyme activity and growth pattern study of selected bacterial strain

An enzyme which plays an important role in inorganic phosphate solubilization in Pikovskaya's broth was phosphatase. The acid phosphatase activity (11.17 µg PNP produced mL^{-1} of culture medium hr^{-1}) of isolate N8 was higher than alkaline phosphatase activity (6.87 µg PNP produced mL^{-1} of culture medium hr^{-1}). The acidic environment was much more favourable for phosphatase enzyme activity than the alkaline environment for N8 bacterium. As per Tabatabai and Bremner (1969), acid phosphatase was predominant in an acidic environment where as alkaline phosphatase was predominant in an alkaline environment. N8 bacterium might be producing organic acids in the medium. Bacteria that particularly belong to the genus *Bacillus* spp., were reported to be secreting organic acids such as tartaric acid, formic acid, acetic acid, propionic acid, citric acid, glyoxylic acid, ketobutyric acid, malonic acid, succinic acid, gluconic acid, and fumaric acid to convert insoluble phosphate into soluble one (Paul and Rao, 1971). Due to these acids, pH of the culture broth was lower, which in turn resulted in the dissolution of bound forms of phosphate. For effective P solubilization and utilization of phosphates, some hydroxyl acids may chelate with calcium and iron (Paul and Rao, 1971). Therefore, this bacterium efficiently solubilized insoluble phosphate in the medium.

On the basis of these studies, N8 bacterium was selected for growth pattern study and further morphological and molecular analysis. The bacterial growth curve of isolate N8 clearly showed a distinctive change in its growth pattern (Fig. 3). At the initial growth phase, it showed relatively slow growth. After 12 hrs of incubation at 30ºC, isolate N8 showed a steep rise in its growth and the highest growth was observed at 48 hrs. N8 bacterium was a moderately fast growing strain as its highest growth was observed in Luria broth after 48 hrs of incubation at 30°C from the growth study (Fig. 3).

Morpho-molecular characterization of bacterial strain

Morphological characterization of bacterial isolate N8 was done by colony morphology and different staining methods. Colonies of N8 strain on Pikovskaya's agar plates appeared small in size, milky white with entire margins shown (Plate 2). This N8 bacterium was gram-positive with a rod-shaped cell morphology existing in both singly and chain form (Plate 3a and 3b)

Molecular characterization of the screened isolate N8 was carried out. Genomic DNA was isolated using

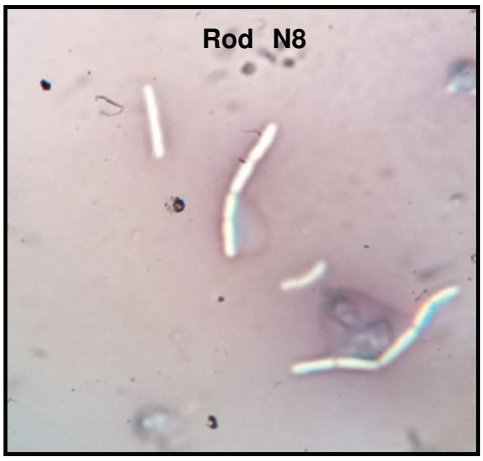

Plate 3a. *Negative staining (1000X)*

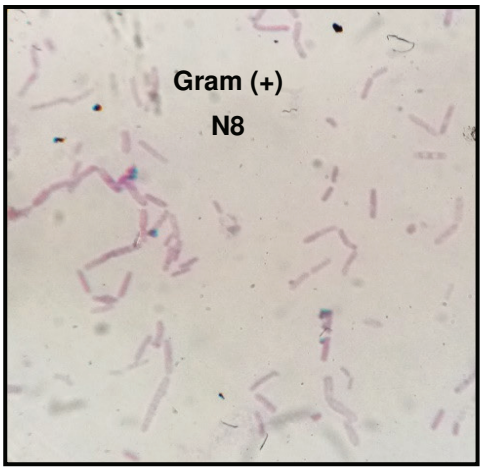

Plate 3b. *Gram staining (1000X)*

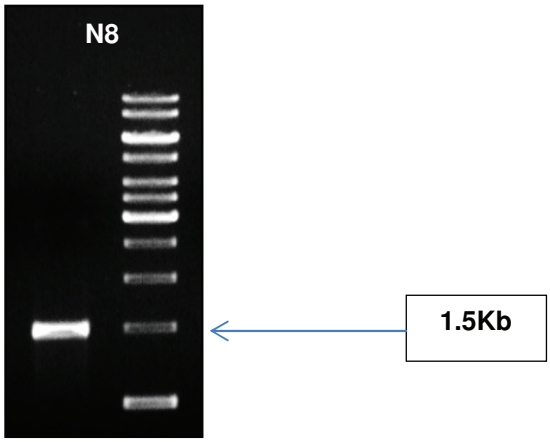

Plate 4. *16s rDNA PCR amplified product of N8 strain*

the standard method, followed by PCR amplification of the 16S rDNA portion was done with 2 universal primers (27 F: 5′- AGA GTT TGA TYM TGG CTC AG-3′); [M=A/C] and (1492R:5′-TAC CTT GTT AYG ACT T-3′); [Y= C/T]. The PCR product was subjected to 1.2% agarose gel electrophoresis along with a 1kb DNA ladder. The gel was observed under Gel doc, which showed a very bright, thick band of PCR product that was about 1.5 kb in size (Plate 4.). The purification of the PCR amplified product was carried out by QIA quick gel extraction kit. Gel eluted DNA products were sent for sequencing. The 16S rDNA gene sequence of strain N8 was submitted to the NCBI GenBank and the accession number was SUB416213 N8 MH489431. BLASTn analysis revealed that 16S rDNA gene sequence of N8 bacterium was 99.9% identical to *Bacillus cereus* strain (Acc. no. SUB416213 N8 MH489431).

N8 strain *Bacillus cereus* was a potent salt tolerant bacterial strain which was identified through morphological and 16S rDNA sequence analysis. The strain exhibited the highest both phosphate solubilizing capacity and nitrogen fixing efficiency. During the solubilization of phosphate, phosphatase enzymes and various organic acids are produced in the medium which leads to a drop in the pH of the medium. N8 strain was isolated and reported for the first time from the rhizosphere of *Suaeda nudiflora* in Sundarbans. This study will help to understand the efficiency of the N8 strain in terms of salt tolerance, N- fixation and phosphate solubilisation and provide useful information for the application of this strain as an effective bioinoculant for coastal agriculture in future. Further research is needed to measure the plant growth promoting properties of the N8 strain under field conditions.

CONFLICTS OF INTEREST

The authors declare no coflicts of interest.

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