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Short communication

# Characterization of nitrogen-fixing moderate halophilic cyanobacteria isolated from saline soils of Songnen Plain in China

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**Abstract**

Twenty out of 200 isolates of cyanobacteria mainly from saline soils of Songnen Plain of China were successfully grown on BG11 N-free medium. The nitrogen-fixing activity was then demonstrated for the 20 isolates in modified BG11 medium using the acetylene reduction assay. All of them possessed appreciable nitrogenase activity (acetylene reduction) under non-saline conditions; however, at 5% NaCl only 60% of the isolates exhibited a high rate of this activity and 25% were completely negative under these conditions. The cyanobacteria isolates grew well in BG11 medium; nevertheless, growth of the majority of isolates was reduced by about 25–85% in the same medium containing 5% NaCl. Cellulolytic activity was detected in 50% of the 20 strains, amylolytic in 45%, and pectinolytic in 10% of the isolates. The cyanobacteria isolates showed also enzymatic activity under saline conditions (6%). The preliminary identification indicated that seven isolates were *Nostoc*, two were *Microcystis,* four were *Oscillatoria,* six were *Anabaena*, and one isolate was *Synechococcus*.


*Keywords:* Isolation; Songnen Plain of China; Nitrogenase activity; Salt tolerance; Filamentous and globular cyanobacteria

## 1. Introduction

Nitrogen fixation is considered as one of the significant biological processes in soil. Together with other events of the N cycle, e.g. ammonification, nitrification and denitrification, it is influenced drastically by environmental factors such as temperature, pH, oxygen, and mineral nutrients [1–3]. Factors, such as salinity of soil may affect soil fertility by inhibiting the activity of microorganisms mediating the nitrogen turnover.

In saline soil and salt marshes, high rates of nitrogen fixation were detected [4–12]. *Anabaena torulosa* could successfully grow and fix nitrogen on moderately saline "Kharland" soils (soil conductivity 5–8.50 dS $m^{-1}$), typical of Indian coastline [13]. The cyanobacteria showed high rates of nitrogen fixation at 10–20% NaCl [14]. Salinity and alkalinity of the soil were found to diminish the population and nitrogen-fixing activity of cyanobacteria and other diazotrophs, although the survival of these organisms was evident at the highest salt levels [15–17]. Species of halotolerant and haloalkaliphilic bacteria of the genus *Bacillus* were isolated from the salt-affected soils of the Nile Valley [18] and salt marsh of Wadi-Al-Natrun, Egypt [19]. However, nitrogen-fixing halotolerant or haloalkaliphilic cyanobacteria have never been reported.

This investigation aimed to isolate halotolerant or haloalkaliphilic heterotrophic nitrogen-fixing cyanobacteria from saline environments of Songnen Plain in China. The growth characteristics and the nitrogen-fixing activity, as well as some enzymatic activities were investigated under salt stress.

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## 2. Materials and methods

### 2.1. Isolation of cyanobacteria

Samples were collected from three saline soils of Songnen Plain in China (light saline soil, median saline soil, heavy saline soil) and from one non-saline cultivating land in March, 2005. The cyanobacteria were isolated using BG11 medium (with nutrient agar), which were supplemented with various concentrations of NaCl in the range of 0–30% to allow isolation of halotolerant or halophilic cyanobacteria. After incubation for appropriate periods, the plates were visually examined and separate colonies were picked out and subcultured for maintenance and further studies.

### 2.2. Screening of isolates for nitrogen fixation

The isolates recovered from above cultures were screened in a BG11 nitrogen-free medium [20]. The nitrogen-fixing activity of those isolates which were able to grow in this medium was investigated using the acetylene reduction assay to detect nitrogenase activity [21]. The 100 ml Stanier's N-poor medium (g/l: $K_2HPO_4$, 0.4 g; $MgSO_4{\cdot}7H_2O$, 0.75 g; $CaCl_2{\cdot}2H_2O$, 0.36 g; Citric acid, 0.06 g; ammonium ferric citrate, 0.06 g; EDTA, 0.01 g; $Na_2CO_3$, 0.2 g; trace elements: $H_3BO_3$, $61.0 \times 10^{-6}$ mg; $MnSO_4{\cdot}H_2O$, $169.0 \times 10^{-6}$ mg; $ZnSO_4{\cdot}7H_2O$, $287.0 \times 10^{-6}$ mg; $CuSO_4{\cdot}5H_2O$, $2.5 \times 10^{-6}$ mg; $(NH_4)Mo_7O_{24}{\cdot}4H_2O$, $12.5 \times 10^{-6}$ mg) amended with 5 g/l glucose and 0.1 g/l yeast extract without and with 5% NaCl were dispensed in 250 ml Erlenmeyer flasks [22]. About 0.2 ml of a 12–24 h old culture was used as inoculum and the cultures were incubated at $25 \pm 2$ °C for 48 h. The acetylene reduction assay was carried out in serum bottles (25 ml) containing 5 ml pregrown culture. Acetylene was injected into the bottles to give a final concentration of 10%, and the bottles were incubated for 2 h at $25 \pm 2$ °C. The reaction was terminated by injecting 1 ml of 6 M HCl. Acetylene reduction was detected in a Pye 104 gas chromatograph (with dual flame ionization detector) fitted with a 5 feet·1/8 in. glass column filled with activated alumina. The column was run at 150 °C with nitrogen as carrier gas at a flow rate of 45 ml/min. Peak heights were measured and the amount of ethylene product was calculated.

### 2.3. Growth of cyanobacteria without and with NaCl

Growth of the isolates was measured in Staner's medium supplemented with glucose (5 g/l), $NH_4Cl$ (1 g/l) and nicotinic acid (0.1 mg/l). The cyanobacteria were grown in test tubes containing liquid medium without and with 5% NaCl at $25 \pm 2$ °C for 48 h. Growth (optical density) was measured spectrophotometrically (Spectronic 601 Milton and Roy Co. USA) at 660 nm wave length. The salt tolerance of the nitrogen-fixing isolates was determined using nutrient broth containing NaCl at the range of 0–20%. Growth was visually monitored by appearance of turbidity or changing the colour of the medium. The survival of cyanobacteria at the highest salt concentration was checked by subculture on nutrient agar.

### 2.4. Enzymatic activities of cyanobacteria

The activity of three extracellular enzymes was investigated under non-saline and saline (5% NaCl) conditions. Amylase activity was detected in NA medium supplemented with 1% soluble starch using Gram's iodine solution to stain the undigested starch. Cellulase activity was detected in a medium containing microcrystalline cellulose as a substrate [23]: clear zones appeared around the cellulolytically active colonies after the addition of chloroiodide of zinc as indicator. Pectinolytic activity was tested by growing the organisms on a solid mineral salt medium containing 5 g/l citrus pectin [24]: appearance of clear zones around and beneath the colonies after flooding the plates with lead acetate (10%) and 6 M HCl indicate the production of pectinase.

## 3. Results

### 3.1. Isolation of nitrogen-fixing cyanobacteria

About 200 cyanobacterial isolates were isolated from different saline and non-saline soils using various isolation media and subsequently screened for their ability to grow in N-free medium. Only 20 isolates were able to grow in this medium (Table 1). While two strains were isolated from non-saline soil (NSS), 12 came from median saline soil (MSS), two from heavy saline soil (HSS) and four were recovered from light saline soil (LSS). The majority of strains were isolated from nutrient agar (9 isolates) and yeast-extract mannitol (10 isolates), whereas only one from sato medium (SM). It is noteworthy that the majority of the strains (i.e. 17) were originally isolated from non-saline media whereas only three from media containing 5% or 20% NaCl, respectively; the isolates have therefore to be regarded as facultative halophilic. With the exception of one strain, which was isolated from an alkaline medium (pH 10.5) all others were obtained with media of neutral pH. The cyanobacteria were mostly fastgrowers, appearing on the medium after 1–3 days of incubation; only few appeared after 2–12 days.

### 3.2. Nitrogenase activity (acetylene reduction)

As shown in Table 1 the cyanobacteria exhibited a substantial activity of $N_2$-ase, and the $C_2H_4$ produced was about 15–85 nM/2 ml/2 h at non-saline medium and 10–60 nM/ml/h at saline conditions (5% NaCl). Whereas the acetylene reduction was only slightly reduced by 5% NaCl in six of the 20 isolates, it was inhibited by 30–80% in nine isolates and completely inhibited in five.

Table 1
Location, isolation media, nitrogenase activity (nM $C_2H_4$/ml/h), growth and maximum salt tolerance for cyanobacterial isolates

| Isolate no. and location | Cyanobacteria | Nitrogenase activity | | Growth (OD) | | Maximum salt tolerance (NaCl%) |
|---|---|---|---|---|---|---|
| | | Without NaCl | With 5% NaCl | Without NaCl | With 5% NaCl | |
| 1 LSS | *Nostoc* | 28.06 | – | 0.78 | 0.51 | 15 |
| 2 LSS | *Nostoc* | 30.21 | 17.24 | 0.74 | 0.25 | 15 |
| 3 LSS | *Oscillatoria* | 29.89 | 26.18 | 0.43 | 0.13 | 15 |
| 4 LSS | *Microcystis* | 28.52 | – | 1.72 | 0.51 | 15 |
| 5 MSS | *Anabaena* | 33.21 | 11.20 | 0.84 | 0.48 | 10 |
| 6 HSS | *Nostoc* | 7.56 | 5.59 | 1.24 | 0.43 | 10 |
| 7 NSS | *Oscillatoria* | 24.21 | 20.48 | 1.29 | 0.58 | 10 |
| 8 NSS | *Microcystis* | 35.01 | 30.19 | 0.63 | 0.31 | 10 |
| 9 HSS | *Anabaena* | 32.59 | 27.54 | 1.01 | 0.86 | 15 |
| 10 MSS | *Anabaena* | 27.21 | 5.01 | 1.12 | 0.28 | 10 |
| 11 MSS | *Anabaena* | 20.14 | 21.59 | 1.21 | 0.24 | 15 |
| 12 MSS | *Anabaena* | 30.25 | 26.15 | 1.04 | 0.31 | 15 |
| 13 MSS | *Anabaena* | 30.68 | 22.63 | 0.87 | 0.37 | 10 |
| 14 MSS | *Oscillatoria* | 13.60 | – | 1.23 | 0.51 | 10 |
| 15 MSS | *Oscillatoria* | 26.32 | – | 0.96 | 0.57 | 15 |
| 16 MSS | *Nostoc* | 24.13 | – | 1.14 | 0.41 | 15 |
| 17 MSS | *Nostoc* | 42.18 | 26.85 | 1.18 | 0.36 | 15 |
| 18 MSS | *Nostoc* | 26.57 | 19.87 | 1.16 | 0.31 | 15 |
| 19 MSS | *Nostoc* | 35.18 | 27.63 | 0.99 | 0.46 | 15 |
| 20 MSS | *Synechococcus* | 30.21 | 22.04 | 0.68 | 0.13 | 15 |

Sample from: LSS, light saline soil; MSS, middling saline soil; HSS, heavy saline soil; NSS, non-saline soil.

### 3.3. Growth of cyanobacteria

As shown in Table 1, the cyanobacteria exhibited a fairly high rate of growth in the modified Stanier's medium, the optical density ranging from 0.62 to 1.71 under non-saline conditions and from 0.10 to 0.85 with 5% NaCl, i.e. growth of the majority of cyanobacteria was inhibited by 30–80%. The bacterial isolates possessed a substantial salt tolerance when grown in NB supplemented with up to 20% NaCl. About 25% of the isolates tolerated 10% NaCl and 75% even 15% NaCl. The cyanobacteria were viable under these conditions, therefore, they can be regarded as moderate halophilic.

### 3.4. Enzymatic activities of cyanobacteria

As shown in Table 2, the cyanobacteria were enzymatically active under both normal (non-saline) and saline (5% NaCl) conditions: the amylolytic activity was detected in 10 of the 20 isolates (13 filamentous cyanobacteria and 7 globular cyanobacteria); only isolate No.11 failed to demonstrate amylase activity under salt stress conditions; 13 isolates showed cellulolytic activity under non-saline conditions, however, under salt stress only 6 isolates. The heterocystous cyanobacteria were very rare in saline soils, only two of the 20 isolates being positive.

### 3.5. Preliminary identification of cyanobacterial isolates

Among the 20 isolates 13 proved to be filamentous cyanobacteria, endospore-forming rods belonging to the genus *Nostoc* and *Anabaena*. The other seven isolates (two isolates were *Microcystis,* four isolates were *Oscillatoria,* one isolate was *Synechococcus*) were globular cyanobacteria of varying morphology. Two may be related to *Microcystis* as indicated by colony appearance and cell morphology.

## 4. Discussion

The ecology of nitrogen-fixing microorganisms has been the subject of considerable interest for many years. In nature, these microorganisms are rarely vigorous and they are seldom notably successful [25,26]. In saline ecosystems the conditions created by the density of the soil and the water regime are limiting for nitrogen fixation [27,28]; nevertheless, nitrogen fixation has been demonstrated in salt marsh and saline lands (see Section 1).

In this investigation, among the 200 cyanobacterial isolates from mainly saline soils, only 20 (i.e. 10%) were able to grow in nitrogen-free medium. These isolates were later shown to be nitrogenase positive in the acetylene reduction assay.

The cyanobacterial isolates showed nitrogenase activity under non-saline and saline conditions. Salt stress (5% NaCl) slightly reduced nitrogenase activity of 7 isolates and caused 25–85% inhibition of nitrogenase activity of other 8 isolates. The activity of 5 isolates was completely inhibited by salt stress. Nitrogen fixation is particularly sensitive to salt stress, the nitrogenase activity of *Klebsiella pneumoniae* was sharply decreased as soon as the osmolarity was increased, with no activity detected at NaCl concentrations higher than about 3% [29]. However, some of the cyanobacterial isolates were able to exhibit high nitrogenase activity at 5% NaCl. This is in agreement with results previously reported for *Nostoc* and *Anabaena* isolated from

Table 2
Enzymatic activities without and with 5% NaCl and preliminary identification

| Isolate no. | Amylase | | Cellulase | | Pectinase | | Identity |
|---|---|---|---|---|---|---|---|
| | Without NaCl | With 5% NaCl | Without NaCl | With 5% NaCl | Without NaCl | With 5% NaCl | |
| 1 | + | + | + | + | – | – | *Nostoc* |
| 2 | + | + | + | + | – | – | *Nostoc* |
| 3 | + | + | + | + | – | – | *Oscillatoria* |
| 4 | + | + | + | + | + | – | *Microcystis* |
| 5 | + | + | + | + | – | – | *Anabaena* |
| 6 | + | + | + | + | – | – | *Nostoc* |
| 7 | – | – | + | – | + | – | *Oscillatoria* |
| 8 | – | – | + | – | – | – | *Microcystis* |
| 9 | + | + | + | – | – | – | *Anabaena* |
| 10 | – | – | + | – | – | – | *Anabaena* |
| 11 | + | – | + | – | – | – | *Anabaena* |
| 12 | + | + | – | – | – | – | *Anabaena* |
| 13 | + | + | + | – | – | – | *Anabaena* |
| 14 | – | – | – | – | – | – | *Oscillatoria* |
| 15 | – | – | – | – | – | – | *Oscillatoria* |
| 16 | – | – | – | – | – | – | *Nostoc* |
| 17 | – | – | – | – | – | – | *Nostoc* |
| 18 | – | – | – | – | – | – | *Nostoc* |
| 19 | – | – | – | – | – | – | *Nostoc* |
| 20 | – | – | + | – | – | – | *Synechococcus* |

saline soil or salt marsh. The nitrogen-fixing activity of this salt-tolerant *Azotobacter* was maximal at 5–25% NaCl [30–32].

Growth of all of the cyanobacterial isolates was reduced by 5% NaCl to the extent of 25–85%. The maximum salinity level which inhibits growth of a certain organism can be higher or lower than that of inhibiting cellular activities such as respiration, nitrogen fixation, etc. Some of the cyabacterial isolates were able to grow at 5% NaCl but failed to express nitrogenase activity at the same level, whereas other isolates showed reduced growth while nitrogenase activity was not or only slightly affected. This is consistent with the observation that the respiration of *Nostoc* and *Anabaena* attained its maximum at a salinity level which was lower than that for nitrogen fixation [33,34].

The persistence of cyanobacteria in the extreme saline conditions may exert upon them some ecological challenge, e.g., the production of some extracellular enzymes. Therefore, the cyanobacteria under investigation were screened for their enzymatic activity: about 70% of the isolates possessed cellulase activity, 50% amylase activity and few were able to produce pectinase. These enzymes were also active under salt stress conditions. Enzymatically active filamentous cyanobacteria were previously isolated from salt-affected soil [18] or from salt marsh [19], the cyanobacteria showing substantial enzymatic activity under salt stress (10% NaCl).

Nitrogen fixation in salt marsh sediments and saline soils has been attributed to sulphate reducing cyanobacteria, clostridia, cyanobacteria and *Nostoc* and *Anabaena* (see Section 1). In this investigation, the preliminary identification of the cyanobacterial isolates indicated that the filamentous cyanobacteria belong to genus *Nostoc* and *Anabaena,* whereas the other isolates were globular cyanobacteria of varying cell morphology, some of them showed resemblance to *Nostoc* and *Anabaena* and the remaining were not identified.

In conclusion, nitrogen-fixing cyanobacteria of the genus *Nostoc* and *Anabaena* may play a role in maintaining fertility and productivity of saline soil. The results demonstrate the persistence of these cyanobacteria in saline soil and emphasize that the search for new heterotrophic nitrogen-fixing (filamentous cyanobacteria) halotolerant cyanobacteria colonizing saline soils is worth further investigation.

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