Screening identification of aerobic denitrification bacteria with high soil desalinization capacity

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Abstract: In order to study the mechanism of bacteria used in the saline soil remediation process, the aerobic denitrification bacteria were isolated from an agricultural greenhouse soil in a farm in East China's Zhejiang Province. The identification, nitrogen reducing characteristics and the denitrification effect of bacteria from different soils at various locations were investigated. The results showed that the NO₃⁻ removal rate was 91% with bacteria from the greenhouse soil under aerobic conditions in 52 h, and the bacteria were identified as Gram-positive Castellaniella denitrification bacteria.

1. Introduction
Nowadays, the greenhouse vegetable production is an important part of modern agriculture worldwide. Due to the over application of mineral fertilizers and lack of natural leaching, the problem of greenhouse soil salinization has become serious [1]. High NO₃⁻ content in the soil surface layer was the major reason for soil salinization [2,3].

The bacteria are a part of the soil and have a serious impact on soil functions [4]. It has been reported that denitrification bacteria in soil were the main factors affecting nitrogen loss [5]. Therefore, the study on the distribution and activity of denitrification bacteria in vegetable fields is crucially important on understanding the nitrogen cycle. It is of great practical significance to evaluate and control the salinization degree of greenhouse vegetable lands. A large body of literature is available on identifying the denitrification bacteria isolated from soil and sludge which could effectively treat organic pollutants in various water bodies [6,7]. However, relatively little is known about the community structure changes of denitrification bacteria and their ability of reducing soil salinization.

In this study, a top soil layer of 20 cm was selected for taking samples from Greenhouse soil (GS), Outdoor soil (OS) and Cultivated soil (CS) in a farm in Ningbo, China. Denitrification bacteria, which could use NO₃⁻ as the N source and convert NO₃⁻-N effectively, were screened via the processes of enrichment and acclimation. Moreover, the nitrogen removal characteristics of these bacteria were also investigated.

2. Materials and methods
2.1. Soil sampling and treatment
The sampling points were selected from a greenhouse vegetable production farm in Ningbo, and the soil samples were taken in July and August in 2015 in a size of 100*150 m field with the S-Sharped
sampling method. Vertically soil samples (0-20 cm) were collected from 8 different depths. The collected fresh samples were taken to the laboratory in sealed bags and stored at 4 °C for further use.

2.2. Screening of nitrate reducing bacteria and determination of denitrification capacity

10 g of soil sample and 100 mL sterile water were added into a 500 mL triangle flask. After 10 min magnetic stirring, 1 mL soil suspension was injected into 9 mL sterile water tube, and then the 10⁻² soil suspension was obtained. 10mL suspension was inoculated into inorganic salt liquid medium at 30 °C, 120 r/min shaking cultivation bed for 7 d. Moist and sleek single colonies were selected after separation and purification for 3-4 times on a LB culture medium plate. The single colonies were chosen from LB culture medium and cryopreserved for further use after cultivation.

The NO₃⁻ degradation activity of bacteria was determined as follows. In the LB solid medium, single colonies were inoculated in the inorganic salt medium at 30 °C, 130 r/min for 24 h. The 5 % inoculum was then inoculated in a 100 mL NO₃⁻-N liquid culture medium, and set the strain absent as the control group. After 2 days cultivation, the solution was filtered by a 0.45 μm microfilter. The concentration of NO₃⁻-N and NO₂⁻-N in the filtrate was determined for a period of 6 days. The ability of bacteria denitrification of each group was then compared.

The content of NO₃⁻ and NO₂⁻-N in soil samples was determined by the UV-VIS and N- (1-naphthyl) ethylenediamine dihydrochloride spectrophotometric methods, respectively. The total salinity was measured by evaporation weighting method [8].

2.3 Identification of denitrification bacteria community

Single staining and Gram staining methods were used to make slides. Observation and micro photography were conducted by the Motic BA300 optical microscope system. The strain was identified by the 16S rDNA Sequencing and Analysis.

3. Results and discussion

3.1. Growth curve and denitrification ability of denitrification bacteria

Three strains of denitrification bacteria which were isolated from three different soils were inoculated in a LB medium at 30 °C for 48 h, respectively. Each wet lubrication strain was inoculated to the enrichment of inorganic salt medium at 30 °C. The OD600 of bacteria was tested every 2-4 h, and the growth curves were obtained (Figure 1). It could be seen that three kinds of denitrification bacteria all needed a certain buffer time to enter the logarithmic growth phase and the stable growth period afterwards. Moreover, the growth of strains was significantly different after the growth period. The activity and quality of denitrification bacteria from different soils were diverse under the same conditions. The bacteria screened from the GS had the largest biomass.

The denitrification capacity of bacteria was small in the initial stage (1-2 days) after inoculating in an inorganic salt medium. However, the degradation rate of NO₃⁻ reached more than 60 % in the next three days for all bacteria, as shown in Figure 2. Furthermore, the GS denitrification bacteria had the best growth activity and nitrate degradation capacity among these strains, and would be cultivated for further use. Figure 2 also indicated that the reduction rate of NO₃⁻ reached a high level in fourth days. Compared with the growth rate (Figure 1), the time of the rapid degradation of NO₃⁻ was delayed. This may be ascribed to the strains culture conditions [9]. In order to simulate the actual situation, the temperature in NO₃⁻ degradation experiments was set at 23-27 °C, which was different from the strain culture temperature (30 °C). Such a phenomenon was consistent with the results reported by Verstraete [10] that heterotrophic nitrification aerobic denitrification was commonly presented in aged cells.
Under the condition of oscillation, \( \text{NO}_3^- \) in the solution was degraded rapidly, which indicated that GS denitrification bacteria could effectively degrade \( \text{NO}_3^- \) under aerobic conditions. Furthermore, the concentration of \( \text{NO}_2^- \) was not significantly increased, suggesting that the degradation of \( \text{NO}_3^- \) was quite thorough.

3.2. Identification of strain

The results of physiological/biochemical tests and the morphology of GS denitrification bacteria were shown in Table 1 and Figure 3, respectively. The strain colony had translucent round edge and transparent fluorescent illumination. Furthermore, the bacteria identified as Gram-positive bacteria were short rod, without flagella and spore.

GS denitrification bacteria was identified as *Bacillus sp.* according to its morphology, culture conditions and physiological/biochemical characteristics [11]. Using GS denitrification bacteria as a template, 7F and 1540R as primers, PCR amplification was performed to obtain 1500bp fragments, 16S rDNA sequencing based on the N-J method constructed by rootless phylogenetic tree (Figure 4). It was found that GS denitrification bacteria matched *Castellaniella* strains (16S rDNA sequence similarity 97%) most closely, implying that the GS denitrification bacteria were *Castellaniella* denitrification bacteria.

### Table 1. Physiological and biochemical characteristics of strain DFT

| Identification index | Results | Identification index | Results |
|----------------------|---------|----------------------|---------|
| growth temperature 4°C | −       | starch hydrolysis     | −       |
| growth temperature 15°C | +       | citric acid utilization | −       |
| growth temperature 30°C | ++      | Gram stain          | +       |
| growth temperature 45°C | +       | oxidase              | +       |
| growth pH 4           | − −     | contact enzyme      | +       |
| growth pH 6           | −       | aerobic             | +       |
| growth pH 7.6         | ++      | denitrification     | W       |
| growth pH 10          | +       | \( \text{NO}_2^- \) reduction | +       |
| fructose              | +       | \( \text{NO}_3^- \) reduction | +       |
| glucose               | +       | sucrose            | +       |

Note: + positive, − negative, W weak positive
Figure 3. The morphology of strain DFT by Electron Microscopy (1000×)

Figure 4. Unrooted phylogenetic tree based on the 16s rDNA sequences of strain DFT and related species

4. Conclusions
The aerobic denitrification bacteria which were isolated from the greenhouse soil were *Castellaniella* denitrification bacteria by morphological identification and 16S rDNA sequencing and analysis. Under aerobic conditions, the bacteria could reduce the NO$_3^-$ and NO$_2^-$ effectively, and the reduction rate of NO$_3^-$ in 5 days could be as high as 91%.

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References
[1] Zhang J J and Duan Z Q 2011 Preliminary study on classification, grading standards, causes and hazards of secondary salinization of facility vegetable soils. *Soils* 43 361-66.
[2] Yu H Y, Li T X and Zhou J M 2005 Secondary salinization of Greenhouse soil and its effects on soil properties. *Soils* 37 581-86.
[3] Liu Y B, Pan X B and Li J S 2015 A 1961-2010 record of fertilizer use, pesticide application and cereal yields. *Agron. Sustain. Develop.* 35 83-93.
[4] Lauber C L, Hamady M, Knight R and Fierer N 2009 Pyrosequencing-based assessment of soil
pH as a predictor of soil bacterial community structure at the continental scale. Appl. Environ. Microbiol. 75 5111-20.

[5] Zhao X, Shi W M, Xing G X and Zhu Z L 2011 Nitrogen balance and loss in a greenhouse vegetable system in southeastern China. Pedosphere 21 464-72.

[6] Coban O, Kuschk P, Kappelmeyer U and Spott O 2015 Nitrogen transforming community in a horizontal subsurface-flow constructed wetland. Water Res. 74 203-12.

[7] Kuppusamy S, Thavamanic P and Megharaj M 2016 Kinetics of PAH degradation by a new acid-metal-tolerant Trabulsiella isolated from the MGP site soil and identification of its potential to fix nitrogen and solubilize phosphorous. J. Hazard. Mater. 307 99-107.

[8] Lu R K 2000 Analysis Method of Soil Agricultural Chemistry. Beijing: China agricultural science and Technology Press.

[9] Shen W S, Ni Y Y, Gao N, Bian B Y, Zheng S N, Lin X G and Chu H Y 2016 Bacterial community composition is shaped by soil secondary salinization and acidification brought on by high nitrogen fertilization rates. Appl. Soil Ecol. 108 76-83.

[10] Verstraete W 1975 Heterotrophic nitrification in soils and aqueous media. Biol. Bull. Acad. Sci. USSR 4 541-58.

[11] Shen P and Chen X D 2002 Microbiology Experiment. Beijing: Higher Education Press.