

Isolation of a new heterotrophic nitrifying *Bacillus* sp. strain

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Abstract: A heterotrophic nitrifier, named *Bacillus* sp. LY, was newly isolated from a membrane bioreactor (MBR). *Bacillus* sp. LY could utilize the organic carbon as the source of assimilation when it grew on glucose and ammonium chloride medium accompanying the formation of oxidized-nitrogen. It also could denitrify nitrate while nitrification. After 24 days incubation, the removal efficiencies of the COD and TN by *Bacillus* sp. LY were 71.7 % and 61.2 %, respectively. The phylogenetic analysis of *Bacillus* sp. LY was performed and the phylogenetic tree of *Bacillus* sp. LY and the neighbouring nitrifiers was given. *Bacillus* sp. LY could become a new bacterial resource for heterotrophic nitrification and might play a bioremediation role for nutrient removal.

Key words: 16S rDNA, Aerobic denitrification, *Bacillus*, Heterotrophic nitrification, Phylogenetic analysis.

Introduction

Nitrification in environments which provide unfavourable conditions for autotrophic nitrifying bacteria may result from the activity of heterotrophic microorganisms. The phenomenon of heterotrophic nitrification was described as early as 1894 for a fungus (Stutzer and Hartleb, 1894). Since then, numerous reports demonstrated unequivocally that nitrite/nitrate production is not restricted to autotrophic ammonia oxidizers (e.g. *Nitrosomonas*) or nitrite oxidizers (e.g. *Nitrobacter*) but is a widespread phenomenon among different genera of fungi and heterotrophic bacteria (Johnsrud, 1978; Castignetti and Hollocher, 1984; Papen *et al.*, 1989; Robertson, *et al.*, 1995). Furthermore, heterotrophic nitrification of bacteria can take place during the entire exponential growth phase (Papen *et al.*, 1989). Meanwhile, it was also reported that heterotrophic nitrification of bacteria is not restricted to exponential growing cultures, as was previously assumed, but occurs after growth of what has ceased and associated with cell lysis (Brierly *et al.*, 2001). However, these assumptions remain speculative, since methods were lacking to demonstrate that microorganisms with the potential of heterotrophic nitrification are present in these systems. Furthermore, there is no selective enrichment or isolation method for heterotrophic nitrifying microorganisms. A better understanding of the inherent theory responsible for these (and other) unconventional principles of nitrogen elimination needs to be reached (Gupta, 1997).

The aim of this work were to develop a method for isolating aerobic heterotrophic nitrifiers, to probe into the heterotrophic microorganisms responsible for nitrification in MBR by isolating pure cultures of nitrifying organisms, and to obtain information about characterization of *Bacillus* sp. LY in terms of the ability of heterotrophic nitrification.

Materials and Methods

Enrichment of nitrifiers: The composition of the enrichment medium was as follows: soluble starch 0.133 g l⁻¹, glucose 0.33

g l⁻¹, peptone 0.06 g l⁻¹, KH₂PO₄ 0.053 g l⁻¹, MgSO₄·7H₂O 0.066 g l⁻¹, MnSO₄·7H₂O 0.006 g l⁻¹, FeSO₄·7H₂O 0.0003 g l⁻¹, CaCl₂ 0.006 g l⁻¹, NH₄Cl 0.14 g l⁻¹, NaHCO₃ 0.67 g l⁻¹. The seed activated sludge taken from the aeration tank of a domestic wastewater treatment plant located in Minhang Shanghai was cultivated in a membrane bioreactor (MBR) with 15 l working volume. A high-flux (HF) membrane with 0.2 µm pore size, made of polyethylene, was utilized. The pH was adjusted to 7.2-8.0 with addition of 1N H₂SO₄ or NaOH. The dissolved oxygen (DO) concentration was at 0.8-1.2 mg l⁻¹. The initial cell concentration used was approximately 7.5 g dry wt l⁻¹. The MBR was put into a chamber with temperature controlled at 25±2 °C.

Isolation of bacterial strains capable of heterotrophic nitrification: Pure isolates were obtained from the system by plating onto a peptone-meat extract (PM) agar; the composition was identical to the liquid medium with the addition of 2% agar. The composition of the PM liquid medium was as follows: peptone 10 g l⁻¹, beef extract 10 g l⁻¹ and sodium chloride 5 g l⁻¹. The resulting isolates of bacteria were tested for their ability to produce nitrite or nitrate by inoculation into 10 ml sterile ammonium sulphate liquid medium. The composition of the medium was as follows: (NH₄)₂SO₄ 0.5 g l⁻¹, NaCl 0.3 g l⁻¹, KH₂PO₄ 1.0 g l⁻¹, MnSO₄·7H₂O 0.3 g l⁻¹, FeSO₄·7H₂O 0.03 g l⁻¹, CaCl₂·2H₂O 0.5 g l⁻¹. Spot tests for total oxidized-N (nitrite and nitrate) were made on approximately 2ml medium using the Griess-Ilosvay method (Keeney and Nelson, 1982) every week. When the test proved positive for total oxidized-N, about 2 ml aliquot of the enrichment cultures was transferred to fresh medium. This procedure of transfer to fresh medium was repeated when the spot tests again proved positive.

It should be noted that the nitrite/nitrate accumulating in ammonium sulphate medium could only have been produced by heterotrophic bacteria, since autotrophic ammonia oxidizers and nitrite oxidizers are unable to grow on PM medium plate containing high amounts of carbon substrates. To validate this, 0.5 ml aliquots from the inoculated test tubes containing ammonium sulphate medium, which tested positive for

nitrite/nitrate accumulation, were taken and analyzed by fluorescence *in situ* hybridization (FISH) with group specific probes for autotrophic ammonium and nitrite oxidizers. Probes used for FISH were as follows: NSO1225, and NIT3. The Nso1225 rRNA-targeted oligonucleotide (CGCCATTGTATTACGTGTGA) was used to target the β -Proteobacteria as described by Mobarry *et al.*, (1996). The NIT3 (CCTGTGCTCCATGCTCCG) was specific for *Nitrobacter* species. The general bacterial probe EUB338 (GCTGCCTCCCGTAGGAGT) was used to demonstrate the presence of sufficient target rRNA and good permeation of cells of interests (Fig. 1). The results were determined by confocal laser scanning microscopy (TCS4D; Leica Lasertechnik, Germany) equipped with an Ar-Kr ion laser (488, 568 and 647 nm) and magnified using 100 $\times$ oil immersion lens at the National Institute for Environmental Studies, Japan. No autotrophic ammonium and nitrite oxidizers in the mixed liquid were detected. This demonstrates that autotrophic ammonia and nitrite oxidizers were not possible to present in the test tubes and, therefore, could not be responsible for the nitrite/nitrate production observed in glucose-ammonium chloride cultures.

Characterization of bacterial strains capable of heterotrophic nitrification: The tracking studies for the aerobic condition were conducted to investigate the ability of heterotrophic nitrification by *Bacillus* sp. LY. COD and NH_4^+ -N concentrations in the mixing liquid were 500 mg l⁻¹, and 45 mg l⁻¹, respectively. After 15 hrs incubation at 35 °C in PM liquid medium, cultures were harvested and washed three times with 10 mM phosphate buffer (pH 7.4). The cell suspensions were added to duplicate 500 ml conical flasks containing 200 ml of the sterilized medium; the pH was adjusted to 7.0 to 8.0 by the addition of filter sterilized 1N H₂SO₄ or NaOH. The flasks were incubated at 35 $\pm$ 2°C on a rotary shaker at around 110 rpm. The medium was filtrated and analyzed colorimetrically for ammonium, nitrite, nitrate and total nitrogen each day, and after one week of incubation, these cultures were plated onto sterilized PM agar in order to confirm purity. All results were expressed relative to uninoculated, incubated medium.

PCR amplification and sequencing of 16S rDNA: *Bacillus* sp. LY cells that had been cultured on PM medium for 24 hr were transferred to an Eppendorf tube containing 100 μ l aseptic double-distilled water. The mixture was centrifuged at 12000r min⁻¹ for 5 min after it was kept in boiling water for 7 min, the supernatant of which was directly used amplification as template DNA.

Amplification was done by PCR with primers (Devereux and Willis, 1995) named BSF8/20 (5c-AGAGTTTGATCCTGGCTCAG-3c) and BSRI541/20 (5c-AAGGAGGTGATCCAGCCGCA-3c). The reactions were performed in a final reaction mixture of 50 μ l containing 5 μ l 10 $\times$ PCR buffer (containing 20 mmol Mg²⁺ l⁻¹), 3 μ l MgCl₂ (25 mmol MgCl₂ l⁻¹), 2 μ l dNTP, 2 μ l each primer (20 μ mol l⁻¹), 3 μ l template DNA, 0.5 μ l Taq DNA polymerase (10000U ml⁻¹), 32.5 μ l ddH₂O. The amplification reactions were performed with the

following cycles of parameters: 95 °C for 5 min, followed by thirty five cycles of 40 s at 95 °C, 40 s at 55 °C and 90 s at 72 °C, with a final extension at 72 °C for 10 min. The amplification products were checked by 2% agarose gel electrophoresis and staining with ethidium bromide. PCR products were purified and sequenced by ABI Prism 3730 Sequencer at Shanghai Bioasia Biologic Technology Co., Ltd., China.

Phylogenetic analysis: The 16S rDNA gene sequence (1465 bp) of *Bacillus* sp. LY aligned with all the sequences available from the GenBank database by BLAST and all sequences were retrieved from Genbank database individually and aligned using ClustalX 1.8 with default settings (Thompson *et al.*, 1997). Phylogenetic analysis was performed by means of MEGA version 2.1 (Kumar *et al.* 2001) software using UPGMA method and selecting Kimura 2-parameter distance model, which was tested by Bootstrap method (1000 repetitions). The 16S rDNA sequences included in the phylogenetic analysis can be seen in Fig. 5.

Analytical methods: Chemical oxygen demand (COD_{Cr}), pH, total-N (TN), mixed liquid suspended solid (MLSS), NH_4^+ -N, NO_2^- -N and NO_3^- -N were measured according to the standard methods (APHA, 1995). Growth of bacteria was monitored by measuring the optical density (OD₆₀₀, 600 nm). The spot tests for total oxidized-N (nitrite and nitrate) were made using the Griess-Ilosvay method (Keeney and Nelson, 1982).

Results and Discussion

Isolation using enrichment cultures: In the enrichment culture, simultaneous nitrification and denitrification was consistently observed. The removal efficiencies of COD and total nitrogen were 95.5 % (effluent COD concentration was about 21.6 mg l⁻¹) and 80.3 % (effluent TN concentration was about 9.41 mg l⁻¹), respectively. From the mixed enrichment culture in the MBR, a bacterial strain, named as *Bacillus* sp. LY, was isolated. The cells of *Bacillus* sp. LY were 0.5-0.6 μ m $\times$ 1.6-2.6 μ m, nonmotile, gram-positive coryneform rod (Fig. 2). It exhibited milk-white colonies on PM medium after 1-2 days. *Bacillus* sp. LY could grow at 10-50 °C, and showed maximum growth rate at 35 °C. It could also grow on pH value of 6.5-8.8 with an optimal pH of 8.0.

Heterotrophic nitrifying ability of the isolates: During characterization of heterotrophic nitrifying bacterium, glucose and ammonium chloride were used as carbon and nitrogen source. The COD concentration declined significantly and the OD₆₀₀ value increased in the initial two days of incubation. These phenomena indicate that at the beginning of incubation, *Bacillus* sp. LY has the ability to utilize a growing portion of the carbon content in the mixed liquid to gain energy for growth. It was a course of assimilation for the heterotrophic bacterium. After 24 days, the COD removal rates by microorganisms of *Bacillus* sp. LY were 71.7 %.

In general, ammonia nitrogen can be removed by assimilation into biomass or by nitrification. In this study, the changes in concentrations of TN and ammonia nitrogen during the initial three days reflect the assimilation into biomass to

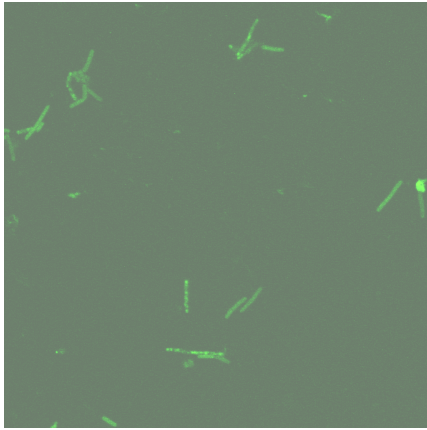


Fig. 1: Fish micrograph of *Bacillus* sp. LY using probe Eub338-ftc for demonstration of the presence of sufficient target rRNA in samples.

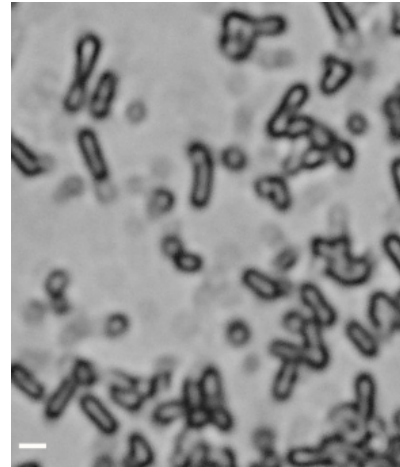


Fig. 2: Microphotograph of *Bacillus* sp. LY. Bar 1 µm.

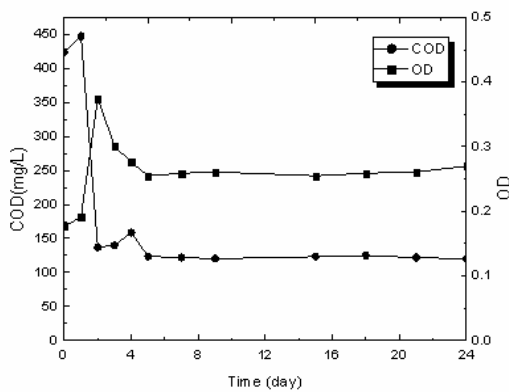


Fig. 3: Changes in COD concentrations and OD₆₀₀ values in the culture medium during the batch test of *Bacillus* sp. LY.

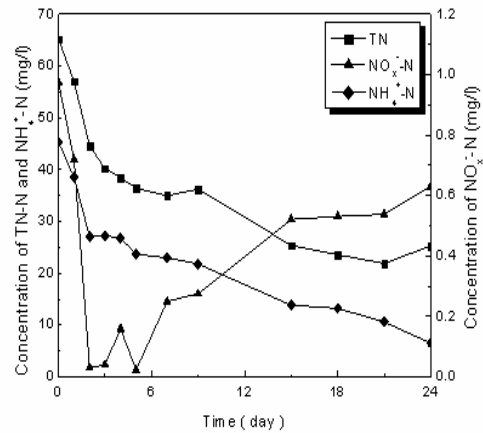


Fig. 4: Changes in concentrations of NO_x-N, NH₄⁺-N and TN in the culture medium during the batch test of *Bacillus* sp. LY.

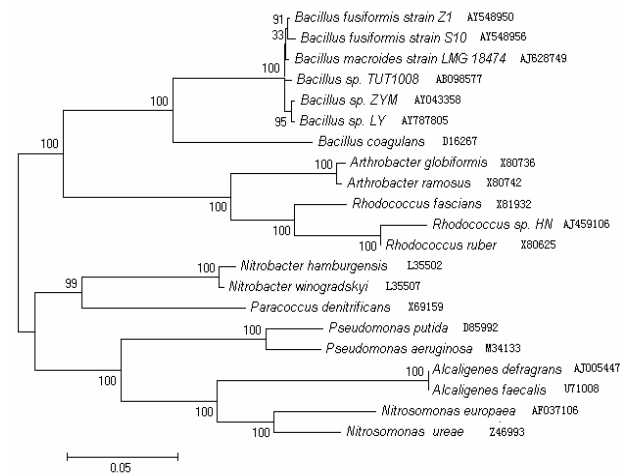


Fig. 5: Phylogenetic tree of *Bacillus* sp. LY, validly described nitrifying species and other correlative species based on 16S rDNA sequences comparisons. Bootstrap values obtained with 1000 repetitions are indicated as percentages at all branches.

some extent; however, after 3 days incubation, with a constant or even decreasing biomass quantity (reflected by the OD₆₀₀ value, Fig. 3), the effect of assimilation on ammonium removal can be neglected, so the removal of ammonia nitrogen during this course was mostly attributed to nitrification. The ammonium nitrogen removal rate after assimilation nearly ceased by *Bacillus* sp. LY was 74.7 %. The pure microorganism which could nitrify via a heterotrophic pathway were likely responsible for nitrification in that case. The changes in oxidizing-N concentrations shown in Fig. 4 also indicate the heterotrophic nitrifying ability of the isolate. On day 3, nitrification occurred and assimilation of the bacteria ceased. The concentration of oxidizing-N peaked at day 4, and then declined rapidly. From day 5 onwards, the NO_x-N concentration kept increasing till the end of 24 days incubation.

In addition, at the beginning of incubation, there is some oxidized-N existed in the system, which was brought from the inoculated mixture. Within the initial 2 days, this part of oxidized-N nearly decreased to under detection limit, which

shows the effect of denitrification under aerobic condition by the isolate. Moreover, in spite of the ammonium nitrogen removal rates due to nitrification observed, there were no apparent products of nitrification accumulated. That indicates a function of denitrifying under aerobic condition by *Bacillus* sp. LY.

Phylogenetic analysis of 16S rDNA sequence: A phylogenetic tree including most published representatives of validly described nitrifying species and other correlative species is given in Fig. 5. Genus *Bacillus* constituted one big cluster on the phylogenetic tree. The 16S rDNA gene of *Bacillus* sp. LY showed high sequence similarity (more than 99%) to the 16S rDNA genes of five strains in *Bacillus* species. The sequence similarity between *Bacillus* sp. LY and *Bacillus* sp. ZYM was 99.7 %. However, there is still no formal report on the characterisation of *Bacillus* sp. ZYM. The 16S rDNA gene sequences of *Arthrobacter globiformis*, *Arthrobacter ramosus* and *Rhodococcus* spp. apparently formed a big cluster. In addition, the pure cultures of bacteria capable of heterotrophic nitrification that have been documented were *Alcaligenes* sp. (Anderson et al., 1993) and *Pseudomonas putida* (Daum et al., 1998), which were closely related to the autotrophic nitrifying bacteria of *Nitrosomonas* spp. Another reported heterotrophic nitrifier, *Paracoccus denitrificans* (Ludwig et al., 1993), is clustered with the autotrophic nitrifiers, *Nitrobacter* sp. In this study, *Bacillus* sp. LY that we have isolated is a new bacterial resource for nitrification and might have a possible bioremediation role for water contaminated by deoxidized nitrogen.

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