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Isolation and Characterization of a New Heterotrophic Nitrifying *Bacillus sp.* Strain[1]

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Objective To characterize the heterotrophic nitrifying bacteria. **Methods** The bacteria were isolated from membrane bioreactor for treating synthetic wastewater using the method newly introduced in this study. Fluorescence *in situ* hybridization (FISH) was used to validate the nonexistence of autotrophic ammonia oxidizers and nitrite oxidizers. Batch tests were carried out to investigate the capability of heterotrophic nitrification by the pure culture. Phylogenetic analysis of the pure culture was performed. **Results** A heterotrophic nitrifier, named *Bacillus sp. LY*, was newly isolated from the membrane bioreactor system in which the efficiency of TN removal was up to 80%. After 24-day, incubation, the removal efficiency of COD by *Bacillus sp. LY* was 71.7%. The ammonium nitrogen removal rate after assimilation nearly ceased by *Bacillus sp. LY* was 74.7%. The phylogenetic tree of *Bacillus sp. LY* and the neighbouring nitrifiers were given. **Conclusions** The batch test results indicate that *Bacillus sp. LY* can utilize the organic carbon as the source of assimilation when it grows on glucose and ammonium chloride medium accompanying the formation of oxidized-nitrogen. It also can denitrify nitrate while nitrifying. *Bacillus sp. LY* may become a new bacterial resource for heterotrophic nitrification and play a bioremediation role in 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 first described in 1894 for a fungus[1]. Since then, numerous reports have 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[2-5]. Furthermore, heterotrophic nitrification of bacteria can take place during the entire exponential growth phase[4]. Meanwhile, it was also reported that heterotrophic nitrification of bacteria is not restricted to exponential growing cultures, as previously assumed, but occurs after growth of what has ceased and is associated with cell lysis[6].

However, these assumptions remain speculative since methods are lacking to demonstrate the presence of microorganisms with the potential of heterotrophic nitrification 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 achieved[7].

The aim of this work was 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 the characterization of *Bacillus sp. LY* in terms of the ability of heterotrophic nitrification.

MATERIALS AND METHODS

Enrichment of Nitrifiers

The compositions of the enrichment medium

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were as follows: 0.133 g/L soluble starch, 0.33 g/L glucose, 0.06 g/L peptone, 0.053 g/L KH_2PO_4, 0.066 g/L $MgSO_4 \cdot 7H_2O$, 0.006 g/L $MnSO_4 \cdot 7H_2O$, 0.0003 g/L $FeSO_4 \cdot 7H_2O$, 0.006 g/L $CaCl_2$, 0.14 g/L NH_4Cl, and 0.67 g/L $NaHCO_3$. 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 H_2SO_4 or NaOH. The dissolved oxygen (DO) concentration was at 0.8-1.2 mg/L. The initial cell concentration used was approximately 7.5 g dry wt/L. The MBR was put into a chamber with temperature controlled at 25℃±2℃.

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 compositions were identical to the liquid medium with the addition of 2% agar. The compositions of the PM liquid medium were as follows: 10 g/L peptone, 10 g/L beef extract, and 5 g/L sodium chloride. The resulting isolates of bacteria were tested for their capability of producing nitrite or nitrate by inoculation into 10 mL sterile ammonium sulphate liquid medium. The compositions of the medium were as follows: 0.5 g/L $(NH_4)_2SO_4$, 0.3 g/L NaCl, 1.0 g/L KH_2PO_4, 0.3 g/L $MnSO_4 \cdot 7H_2O$, 0.03 g/L $FeSO_4 \cdot 7H_2O$, and 0.5 g/L $CaCl_2 \cdot 2H_2O$. Spot tests for total oxidized-N (nitrite and nitrate) were carried out on approximately 2 mL medium using the Griess-Ilosvay method[8] every week. When the test proved positive for total oxidized-N, about 2 mL aliquot of the enrichment cultures was transferred to a fresh medium. This procedure was repeated when the spot tests again proved positive.

It should be noted that the nitrite/nitrate accumulation in ammonium sulphate medium could only be produced by heterotrophic bacteria, since autotrophic ammonia 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, was taken and analyzed by fluorescence *in situ* hybridization (FISH) with group specific probes for autotrophic ammonium and nitrite oxidizers. Probes used for FISH were NSO1225, and NIT3. The Nso1225 NSO1225 rRNA-targeted oligonucleotide (CGCCATTGTATTACGTGTGA) was used to target the β-proteobacteria as described by Mobarry *et al*.[9]. 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×oil immersion lens at the National Institute for Environmental Studies, Japan. No autotrophic ammonium and nitrite oxidizers in the mixed liquid were detected, which demonstrated that autotrophic ammonia and nitrite oxidizers could not be presented in the test tubes and therefore were not responsible for the nitrite/nitrate production observed in glucose-ammonium chloride cultures.

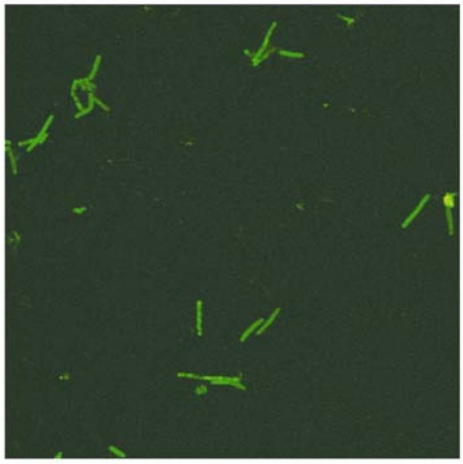

FIG. 1. FISH micrograph of *Bacillus sp. LY* using probe Eub338-fitc for demonstration of presence of sufficient target rRNA in samples.

Characterization of Bacterial Strains Capable of Heterotrophic Nitrification

The tracking studies for the aerobic condition were conducted to investigate the capability of *Bacillus sp. LY* for heterotrophic nitrification. Chemical oxygen demand (COD) and NH_4^+-N concentrations in the mixed liquid were 500 mg/L and 45 mg/L, respectively. After 15-hour, incubation at 35℃ in PM liquid medium, cultures were harvested and washed three times with 10 mmol/L phosphate buffer (pH 7.4). The cell suspensions were added to duplicate 500 mL conical flasks containing 200 mL of the sterilized medium with the pH adjusted to 7.0-8.0 by the addition of filter-sterilized 1N H_2SO_4 or NaOH. The flasks were incubated at 35℃±2℃ on a rotary shaker at around 110 rpm. The medium was filtered and analyzed colorimetrically for ammonium, nitrite, nitrate and total nitrogen each day. 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 cultured on PM medium for 24 hours were transferred to an Eppendorf tube containing 100 μL aseptic double-distilled water. The mixture was centrifuged at 12000 r/min for 5 min after it was kept in boiling water for 7 min, the supernatant of which was directly used for amplification as template DNA.

PCR amplification was done with primers BSF8/20 (5c-AGAGTTTGATCCTGGCTCAG-3c) and BSRl541/20 (5c-AAGGAGGTGATCCAGCCGCA-3c)[10]. The reactions were performed in a final reaction mixture of 50 μL containing 5 μL 10×PCR buffer (containing 20 mmol Mg^{2+} /L), 3 μL $MgCl_2$ (25 mmol $MgCl_2$/L), 2 μL dNTP, 2 μL each primer (20 umol/L), 3 μL template DNA, 0.5 μL Taq DNA polymerase (10000 U/mL), and 32.5 μL ddH_2O. The amplification reactions were performed at 95℃ for 5 min, followed by 35 cycles at 95℃ for 40 s, at 55℃ for 40 s, and at 72℃ for 90 s, with a final extension at 72℃ for 10 min. The amplified products were checked by 2% agarose gel electrophoresis and staining with ethidium bromide. PCR products were purified and sequenced with 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 by BLAST and all sequences were retrieved from GenBank individually and aligned using ClustalX 1.8 with default settings[11]. Phylogenetic analysis was performed by MEGA version 2.1[12] 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 are shown 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 as described in APHA[13]. Growth of bacteria was monitored by measuring the optical density (OD_{600}, 600 nm). The spot tests for total oxidized-N (nitrite and nitrate) were made using the Griess-Ilosvay method[8].

RESULTS

Isolation Using Enrichment Cultures

In the enrichment culture, simultaneous nitrification and denitrification were consistently observed when COD/TN ratio was 10 and DO concentration was 1.0 mg/L. The removal efficiencies of COD and total nitrogen were 95.5% (effluent COD concentration was about 21.6 mg/L) and 80.3% (effluent TN concentration was about 9.41 mg/L), respectively. From the mixed enrichment culture in the MBR, a bacterial strain, named *Bacillus sp. LY*, was isolated. The cells of *Bacillus sp. LY* were 0.5-0.6 μm×1.6-2.6 μm, 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℃ and showed the maximum growth rate at 35℃. It could also grow at pH 6.5-8.8 with an optimal pH of 8.0.

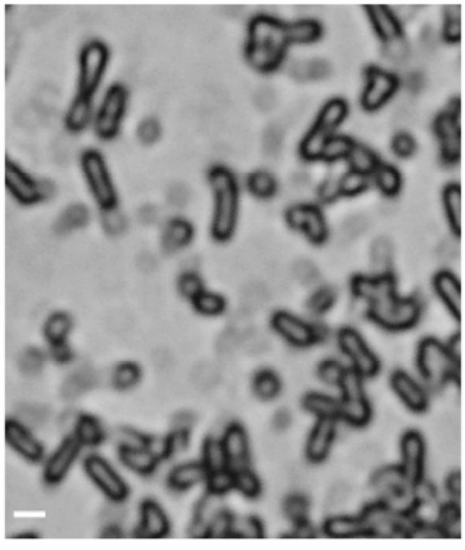

FIG. 2. Microphotograph of *Bacillus sp. LY*. (Bar 1 μm).

Heterotrophic Nitrifying Capability of Isolates

During characterization of heterotrophic nitrifying bacteria, glucose and ammonium chloride were used as carbon and nitrogen source. Figure 3 shows the changes in the COD concentrations and OD_{600} values in the batch test of *Bacillus sp. LY*. In the initial days, the COD concentration decreased significantly with increasing OD_{600} value. After 8-day incubation, COD concentration remained stable while the OD_{600} did not change much. After 24 days, the COD removal rate by *Bacillus sp. LY* was 71.7%.

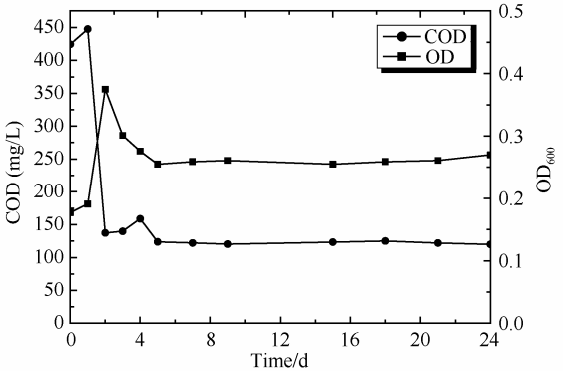

FIG. 3. Changes in COD concentrations and OD_{600} values in culture medium during batch test of *Bacillus sp. LY.*

Figure 4 represents the changes in TN concentrations in the culture medium during the batch test of *Bacillus sp. LY*. In the initial three days, the TN concentration in the medium decreased significantly, and the TN removal rate was 38.3%. At the same time, the concentration of ammonium nitrogen decreased rapidly and the removal rate was 39.9%. From the fifth day, the TN and ammonium nitrogen concentrations decreased slowly while the COD concentration and OD_{600} value kept stable (Figs. 3 and 4).

The changes in oxidizing-N concentrations shown in Fig. 4 indicated the heterotrophic nitrifying capability of the isolate. On day 3, nitrification occurred and assimilation of the bacterium ceased, which caused the decrease in NH_4^+-N concentration and increase in oxidizing-N concentration. The concentration of oxidizing-N peaked on day 4, and then declined rapidly. From day 5, the NO_X^--N concentration kept increasing till the end of 24-day incubation.

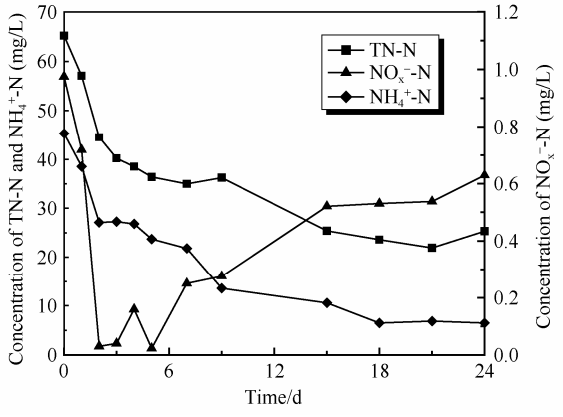

FIG. 4. Changes in concentrations of NO_X^--N, NH_4^+-N, and TN in culture medium during batch test of *Bacillus sp. LY.*

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.

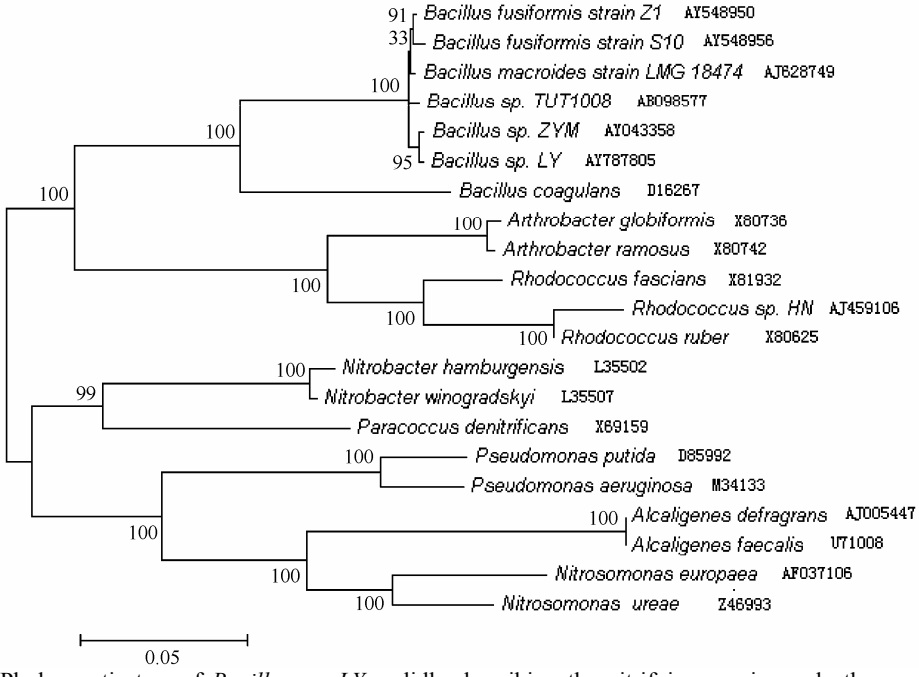

FIG. 5. Phylogenetic tree of *Bacillus sp. LY*, validly describing the nitrifying species and other correlative species based on 16S rDNA sequence comparisons. Bootstrap values obtained by 1000 repetitions are indicated as percentages at all branches.

DISCUSSION

As shown in Fig. 3, the COD concentration declined significantly and the OD_{600} value increased in the initial two days of incubation, indicating that at the beginning of incubation, *Bacillus sp. LY* is capable of utilizing a growing portion of the carbon content in the mixed liquid to gain energy for growth. It is a course of assimilation for the heterotrophic bacterium. Results indicate that during the course of incubation, *Bacillus sp. LY* has the special heterotrophic ability to utilize organic substrate as the energy source to fulfill the course of assimilation.

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 reflected the assimilation into biomass to some extent. However, after 3 days of incubation with a constant or even decreasing biomass quantity (indicated by the OD_{600} value, Fig. 3), the effect of assimilation on ammonium removal could be neglected, suggesting that the removal of ammonia nitrogen during this course is 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 was likely responsible for nitrification in that case. In addition, as the NO_X^--N concentration increased, the COD concentration increased at the same time, indicating that COD might be released *via* decay of biomass which might serve heterotrophic microorganisms to nitrify. It has been proposed that heterotrophic nitrification which occurs after growth ceased is associated with cell lysis[6]. It is unlikely that heterotrophic nitrification provides energy and therefore, is unessential for growth.

In addition, at the beginning of incubation, some oxidized-N existed in the system, which was brought from the inoculated mixture. Within the initial 2 days, this part of oxidized-N was undetectable, nearly showing the effect of denitrifying 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 accumulated nitrification, indicating a function of denitrifying under aerobic condition by *Bacillus sp. LY*.

The sequence similarity between *Bacillus sp. LY* and *Bacillus sp. ZYM* was 99.7%. However, there is still no report on the characterization 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 are *Alcaligences spp.*[14] and *Pseudomonas putida*[15], which are closely related to the autotrophic nitrifying bacteria of *Nitrosomonas spp*. Another reported heterotrophic nitrifier, *Paracoccus denitrificans*[16], is clustered with the autotrophic nitrifier, *Nitrobacter spp.* In this study, *Bacillus sp. LY* isolated by us is a new bacterial resource for nitrification and may have a possible bioremediation role in water contaminated by deoxidized nitrogen.

CONCLUSIONS

A heterotrophic nitrifying bacterium isolated *via* a new isolating method is identified as *Bacillus sp. LY*. The batch test indicates that *Bacillus sp. LY* can utilize the organic carbon as the source of assimilation when it grows on glucose and ammonium chloride medium accompanying the formation of oxidized-nitrogen. After 24 days of incubation, the removal efficiency of COD by *Bacillus sp. LY* is 71.7% and the ammonium nitrogen removal rate after assimilation nearly ceased by *Bacillus sp. LY* is 74.7%. A phylogenetic tree including most published representatives of validly described nitrifying species and other correlative species is given in this study. *Bacillus sp. LY* is a new bacterial resource for nitrification.

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