Journal of Environmental Sciences Vol.17, No.4, pp.589—592, 2005

ISSN 1001—0742
CN11—2629/X

Article ID: 1001-0742(2005)04-0589-04 **CLC number:** X703 **Document code:** A

Isolation and characterization of heterotrophic nitrifying bacteria in MBR

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Abstract: The study presented the method for isolating the heterotrophic nitrifiers and the characterization of heterotrophic nitrification. Continuous tests via a membrane bioreactor (MBR) were operated under the controlled conditions to proliferate the nitrifiers. Heterotrophic nitrifying bacteria were isolated from the system in which the efficiency of total nitrogen (TN) removal was up to 80%. Since no autotrophic ammonium and nitrite oxidizers could be detected by fluorescence *in situ* hybridization (FISH), oxidized-N production was unlikely to be catalyzed by autotrophic nitrifiers during the heterotrophic nitrifiers' isolation in this study. The batch test results indicate that the isolated heterotrophic bacteria were able to nitrify. After 3 weeks incubation, the efficiencies of the COD removal by the three isolated bacterial strains B1, B2, and B3 were 52.6%, 71.7%, and 77.7%, respectively. The efficiencies of the TN removal by B1, B2, and B3 were 35.6%, 61.2% and 68.7%, respectively.

Keywords: membrane bioreactor (MBR); simultaneous nitrification and denitrification (SND); heterotrophic nitrification; aerobic denitrification

Introduction

Nitrification in wastewater treatment has been one of the most intensively studied reactions in the nitrogen cycle. It is generally thought to be performed by a suite of autotrophic bacteria over a long time. Since the growth rate of heterotrophs is much higher than that of autotrophs, it would be important to find some heterotrophs that have nitrification activity (Geraats, 1990). The phenomenon of heterotrophic nitrification was described as early as 1894 as a fungus (Stutzer, 1984). 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, 1984; Robertson, 1988; 1995; Papen, 1989; Arts, 1995). Furthermore, heterotrophic nitrification of bacteria can take place during the entire exponential growth phase (Papen, 1989). 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 associated with cell lysis (Brierly, 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 aims of this work were: (1) to develop a method for isolating aerobic heterotrophic fraction; (2) to probe into the heterotrophic microorganisms responsible for nitrification in MBR by isolating pure cultures of nitrifying organisms; and (3) to obtain information about characterization of these isolates in terms of the ability of heterotrophic nitrification.

1 Materials and methods

1.1 Apparatus and operating conditions

A membrane bioreactor (MBR) with 20 L total volume was used in the experiments and its working volume was kept at 15 L throughout the study. The experimental set-up is shown in Fig.1. A high-flux (HF) membrane (Tianjin Motian Membrane Engineering and Technology Co., Ltd. Tianjin, China) with 0.2 μm pore size, made of polyethylene, was employed in this study. The surface area of the laboratory membrane module was 0.4 m^2. The flux for the membrane module was maintained at 0.15 $m^3/(m^2 \cdot d)$. To minimize membrane fouling, filtration was performed semi-continuously by alternating 10 min suction with a 2 min pause. The air diffuser was aligned with the membrane module to optimize the contact between the air bubbles and the membrane surface. The number and the size of the holes were made to create sufficient turbulence.

The MBR was put into a chamber with temperature controlled at 25 ± 2℃. The pH was adjusted to 7.2—8.0 with addition of H_2SO_4 (0.5 mol/L) or NaOH (1.0 mol/L). The dissolved oxygen (DO) concentration was set to 0.8—1.2 mg/L. The extra sludge was intermittently withdrawn from the bioreactor and the solids retention time (SRT) was controlled as 50 d. The hydraulic retention time (HRT) for the entire study was 6 h. The system was operated at chemical oxygen demand (COD), NH_4^+-N and total nitrogen (TN) volumetric loading rates of approximately 2 $kgCOD/(m^3 \cdot d)$, 0.132 $kgNH_4^+-N/(m^3 \cdot d)$ and 0.192 $kgTN/(m^3 \cdot d)$, respectively.

1.2 Synthetic wastewater and seed sludge

The composition of the synthetic wastewater was as follows: soluble starch (Shanghai Chemical Reagent Co., Ltd. Shanghai, China, same as the follows except otherwise described) 0.133 g/L, glucose 0.33 g/L, peptone 0.06 g/L, KH_2PO_4 0.053 g/L, $MgSO_4 \cdot 7H_2O$ 0.066 g/L, $MnSO_4 \cdot 7H_2O$ 0.006 g/L, $FeSO_4 \cdot 7H_2O$ 0.0003 g/L, $CaCl_2$ 0.006 g/L, NH_4Cl 0.14 g/L, $NaHCO_3$ 0.67 g/L. The influent characteristics are described in Table 1.

The seed activated sludge was taken from the aeration tank of a domestic wastewater treatment plant located in Minhang Shanghai. It was cultivated for two weeks in aerobic and ammonium containing medium before feeding of the synthetic wastewater.

1.3 Isolation of bacterial strains capable of heterotrophic nitrification

Isolation of heterotrophic nitrifying bacteria was performed when the MBR showed steady simultaneous nitrification and denitrification (SND) at COD/TN ratio 10

Foundation item: The National Natural Science Foundation of China (No. 20176027) and the National High-Tech Research and Development Program (863) of China (No. 2002AA601220); * Corresponding author

and DO concentration 1.0 mg/L.

Table 1 Synthetic sewage composition

Component	Average concentration
COD_{Cr}, mg/L	500
NH_4^+-N, mg/L	30—36
NO_X^--N(NO_2^--N/NO_3^--N), mg/L	0—2.5
TN, mg/L	45—50
MLSS, mg/L	0—10
Alkalinity($CaCO_3$), mg/L	340—360
pH	7.2—8.0

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, beef extract 10 g/L and sodium chloride 5 g/L. The resulting isolates of bacteria were tested for their ability to produce nitrite or nitrate by inoculation into the liquid medium containing 10 ml glucose-ammonium chloride medium. Spot tests for total oxidized-N(nitrite and nitrate) were made on approximately 2 ml medium using the Griess-losvay method(Keeney, 1982) every week. When the test proved positive for total oxidized-N, a 2 ml aliquot of the enrichment cultures was transferred to fresh medium. This procedure of transfer to fresh medium was repeated once more when the spot tests again proved positive.

It should be noted that the nitrite/nitrate accumulating in glucose-ammonium chloride medium could only have been produced by heterotrophic bacteria, since autotrophic ammonia oxidizers and nitrite oxidizers are unable to grow in PM medium plate containing high amounts of carbon substrates. To validate this, 0.5 ml aliquots from the inoculated test tubes containing glucose-ammonium chloride 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. The probes used for FISH were as follows: NSO1225 (Mobarry, 1996) and NIT3 (Wagner, 1996). The results were determined by confocal laser scanning microscopy 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 present in the test tubes and, therefore, could not be responsible for the nitrite/nitrate production observed in glucose-ammonium chloride cultures.

1.4 Tracking study

The tracking studies for the aerobic condition were conducted to investigate the ability of heterotrophic nitrification by the pure cultures isolated. COD and NH_4^+-N concentrations in the mixing liquid were 500 mg/L, and 30 mg/L, respectively. Samples of approximately 5 ml mixed liquid was added to duplicate 500 ml conical flasks containing 200 ml of the sterilized medium; the pH was adjusted to 7.0—8.0 by the addition of filter sterilized 1 mol/L H_2SO_4 or NaOH. The flasks were incubated at 30 ± 2℃ on a rotary shaker at around 110 r/min.

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.

1.5 Analytical methods

pH, chemical oxygen demand (COD_{Cr}), TN, MLSS, NH_4^+-N, NO_2^--N and NO_3^--N were measured according to the standard methods as described in APHA(APHA, 1995). 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-losvay method(Keeney, 1982).

2 Results and discussion

2.1 Simultaneous nitrification and denitrification(SND) in MBR

In the experimental system, simultaneous nitrification and denitrification was consistently observed when COD/TN ratio was 10 and DO concentration was 1.0 mg/L. COD and total nitrogen removal efficiency was 95.5% (effluent COD concentration was about 21.6 mg/L) and 80.3% (effluent TN concentration was about 9.41 mg/L) in the MBR, respectively. The MBR was considered to be highly efficient in simultaneous removal of COD and nitrogen at small-scale treatment of domestic wastewater.

2.2 Microbial population levels in MBR and isolation using enrichment cultures

The numbers of autotrophic nitrifiers, denitrifiers, heterotrophs and heterotrophic nitrifiers in the MBR are summarized in Table 2. Results show that the autotrophic and heterotrophic nitrifiers in the system proliferated after two weeks incubation. Meanwhile, the quantity of the specific heterotrophic nitrifiers was only 10^2 times lower than the heterotrophs. It indicates a greater accumulating function of heterotrophic nitrifiers in this MBR system.

Table 2 Numbers of autotrophic nitrifiers, denitrifiers, heterotrophs and heterotrophic nitrifiers in MBR

Organism	Number
Autotrophic nitrifiers*, MPN/ml	3.5×10^7
Denitrifiers, MPN/ml	3.5×10^3
Heterotrophs, CFU/ml	1.8×10^8
Heterotrophic nitrifiers, MPN/ml	6.4×10^6

Note: * Autotrophic ammonia oxidizers and nitrite oxidizers

Based on the phenomenon of SND in the system, three bacterial strains were isolated from the mixed enrichment

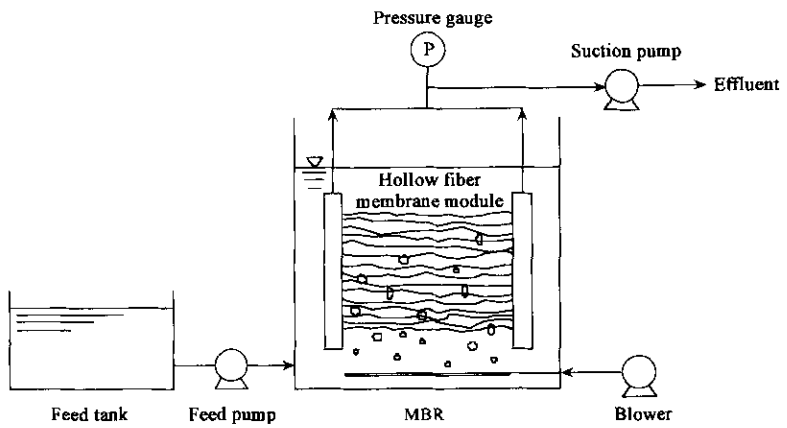

Fig.1 Sketch of the experimental set-up

culture in the MBR, named as B1, B2 and B3. Among them strain B2 was provisionally identified as a nonmotile, Gram-positive coryneform rod (Fig. 2). It exhibited milk-white colonies on the medium.

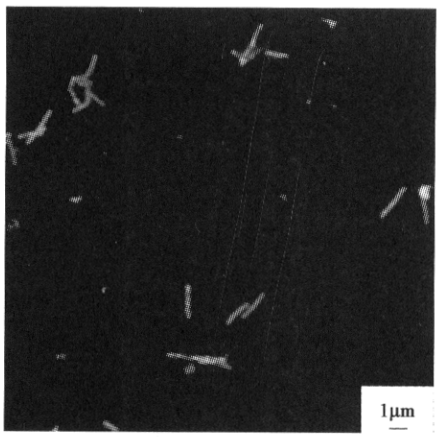

Fig. 2 FISH micrograph of strain B2 using probe Eub338-fitc for the profile of aggregates in samples

2.3 Heterotrophic nitrifying ability of the isolates

During characterization of heterotrophic nitrifying bacteria, glucose and ammonium chloride were used as carbon and nitrogen source. As shown in Fig.3 and Fig.4, the COD concentration declined significantly and the OD_{600} value increased at the beginning of incubation. These phenomena indicate that at the beginning of incubation, isolated microorganisms utilized a growing portion of the carbon content in the mixed liquid to gain energy for growth. It was a course of assimilation for the bacteria. After 8 d incubation, COD concentration remained stable while the OD_{600} did not change much for strain B2 and B3. After three weeks, the COD removal rates by microorganisms of strain B1, B2 and B3 were 52.6%, 71.7% and 77.7%, respectively(Table 3). Above mentioned results indicate that during the course of incubation, strain B1, B2 and B3 have the special heterotrophic ability to utilize organic substrate as the energy source to fulfill the course of assimilation.

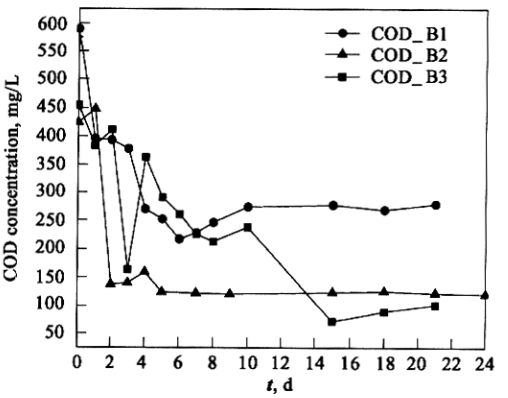

Fig. 3 Changes in COD concentrations in the culture medium during the batch test of strain B1, B2, B3

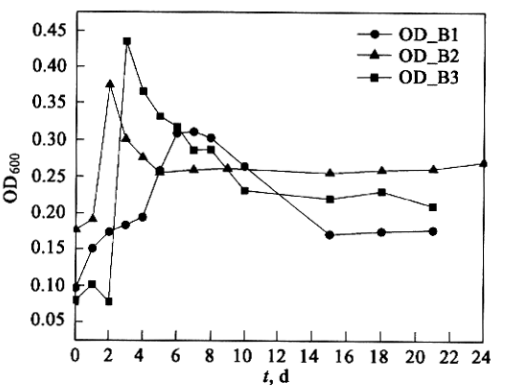

Fig. 4 Changes in OD_{600} values in the culture medium during the batch test of strain B1, B2, B3

Table 3 The average removal rates of COD, TN, and ammonium nitrogen after three weeks incubation

Isolate	COD		TN		NH_4^+-N		NOx^--N	
	Initial/final concentration, mg/L	Efficiency, %	Initial/final concentration, mg/L	Efficiency, %	Initial/final concentration, mg/L	Efficiency, %	Initial concentration, mg/L	Oxidized-N formation, mg/L
B1	589.63/279.58	52.6	60.71/39.12	35.6	40.71/19.88	51.2	0	3.30
B2	424.28/120.00	71.7	65.20/25.32	61.2	45.30/6.89	84.8	0	0.63
B3	454.18/101.12	77.7	57.88/18.12	68.7	37.87/14.95	60.5	0	1.16

Fig.5 represents the changes in TN concentrations in the culture medium during the batch test of strain B1, B2, B3. In the initial three days, the TN concentration in the medium of the stain B2 and B3 decreased significantly, with the TN removal rates of 38.3% and 33.9%, respectively. At the same time, the concentration of ammonium nitrogen decreased rapidly, with the removal rates of 39.9% and 51.2%. At the end of the day 5, the removal rates of TN and ammonium nitrogen by strain B1 were 24.4% and 26.0%, respectively. From the day 8 and the day 10 afterwards, the TN and ammonium nitrogen concentrations decreased slowly while the COD concentration and OD_{600} value kept stable(Fig.3 and Fig.4).

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 days reflect the assimilation into biomass to some extent(Fig. 5 and Fig. 6); however, after 3 or 5 d incubation, with a constant or even decreasing biomass quantity(reflected by the OD_{600} value, Fig.4), 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 strain B1, B2 and B3 were 19.1%, 74.7% and 46.2%, respectively. The pure microorganisms which could nitrify via a heterotrophic pathway were likely responsible for nitrification in that case. The data shown in Fig.7 also indicate the heterotrophic

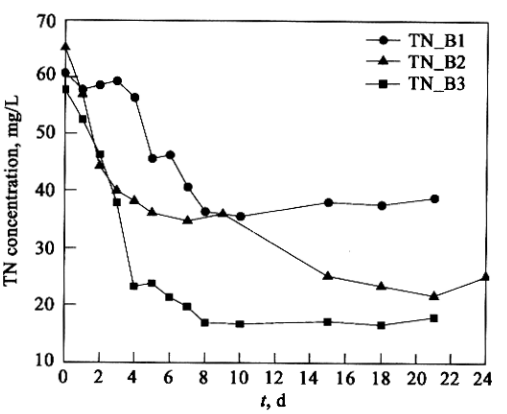

Fig. 5 Changes in TN concentrations in the culture medium during the batch test of strain B1, B2, B3

nitrifying ability of the isolates.

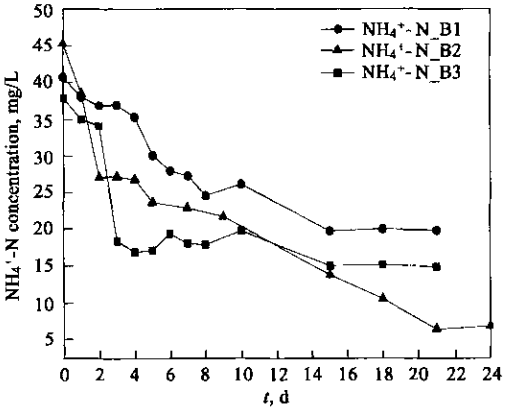

Fig.6 Changes in NH_4^+-N concentrations in the culture medium during the batch test of strain B1, B2, B3

Fig.7 shows the changes in NOx^--N concentrations in the culture medium during the batch test of strain B1, B2, B3. On the day 3 or 5, nitrification occurred and assimilation of the bacteria ceased, which caused the NH_4^+-N concentration decrease and oxidizing-N concentration increase. The concentration of oxidizing-N peaked at the day 8, 4 and 3 for strain B1, B2, B3 respectively, and then declined rapidly. In addition, as the NOx^--N concentration increased, the COD concentration increased at the same time. This indicates 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 was associated with cell lysis (Brierly, 2001). It is unlikely that heterotrophic nitrification provides energy and, therefore, it might be unessential for growth.

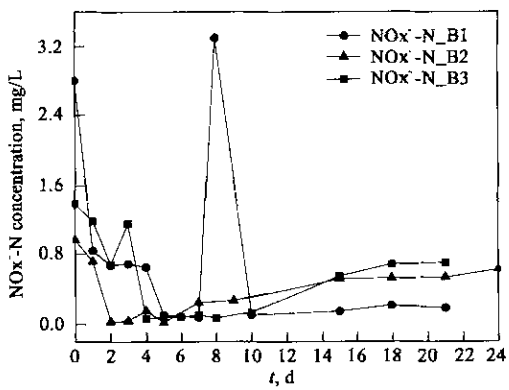

Fig.7 Changes in NOx^--N concentrations in the culture medium during the batch test of strain B1, B2, B3

In addition, at the beginning of incubation, there was some oxidized-N existed in the system, which was brought from the inoculated mixture. Within the initial 2 to 5 d, this part of oxidized-N nearly decreased to the value under detection limit, which showed the effect of denitrification under aerobic condition by the isolates. Moreover, for strain B2 and B3, in spite of the ammonium nitrogen removal rates due to nitrification observed, there were still no apparent products of nitrification accumulated. That also indicates a function of denitrifying under aerobic condition by the two pure isolates. The oxidized-N concentration for B1 peaked on the day 8, and then declined rapidly to nearly non-detected also illustrates the denitrifying ability under aerobic condition by this pure isolate.

3 Conclusions

Several important conclusions from this research could be achieved as follows.

The MBR system in this research had a significant accumulating function of the heterotrophic nitrifiers and autotrophic nitrifiers. The quantity of the specific heterotrophic nitrifiers was only 10^2 times lower than the heterotrophs.

Three bacteria, named B1, B2 and B3 in this paper, were isolated from the mixed enrichment culture in MBR. The isolates exhibited a high efficiency in utilizing the organic substrates during the batch tests. After 3 weeks incubation, the efficiencies of the COD removal by the strain B1, B2 and B3 were 52.6%, 71.7%, and 77.7%, respectively. Moreover, the three isolates obtained displayed a high TN removal with the efficiencies of 35.6%, 61.2% and 68.7%, respectively.

Three microorganisms showed a good ability of heterotrophic nitrifying in aerobic condition. The oxidized-N formation by the strain B1, B2 and B3 were 3.30 mg/L, 0.63 mg/L and 1.16 mg/L, respectively. NH_4^+-N removal was due to heterotrophic nitrification after growth ceased.

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(Received for review September 16, 2004. Accepted November 22, 2004)