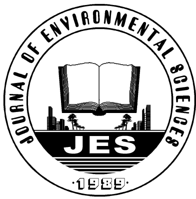

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Denitrification characteristics of a marine origin psychrophilic aerobic denitrifying bacterium

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Abstract

A psychrophilic aerobic denitrifying bacterium, strain S1-1, was isolated from a biological aerated filter conducted for treatment of recirculating water in a marine aquaculture system. Strain S1-1 was preliminarily identified as *Psychrobacter* sp. based on the analysis of its 16S rRNA gene sequence, which showed 100% sequence similarity to that of *Psychrobacter* sp. TSBY-70. Strain S1-1 grew well either in high nitrate or high nitrite conditions with a removal of 100% nitrate or 63.50% nitrite, and the total nitrogen removal rates could reach to 46.48% and 31.89%, respectively. The results indicated that nitrate was mainly reduced in its logarithmic growth phase with a very low leve1 accumulation of nitrite, suggesting that the aerobic denitrification process of strain S1-1 occurred mainly in this phase. The GC-MS results showed that N_2O was formed as the major intermediate during the aerobic denitrifying process of strain S1-1. Finally, factors affecting the growth of strain S1-1 and its aerobic denitrifying ability were also investigated. Results showed that the optimum aerobic denitrification conditions for strain S1-1 were sodium succinate as carbon source, C/N ratio15, salinity 10 g/L NaCl, incubation temperature 20°C and initial pH 6.5.

Key words: nitrogen removal; aerobic denitrification; marine environment; low temperature; *Psychrobacter* sp. S1-1

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Introduction

Nitrogen pollution, which can lead to eutrophication of water bodies and make the aquatic plants and algae grow out of control, will cause water quality deteriorate rapidly and seriously threaten the survival of aquatic organisms. Moreover, excessive nitrate or nitrite in the water will greatly affect human health. Therefore, nitrogen removal has become an important aspect of wastewater treatment.

It is well known that biological nitrogen removal technology is the most effective way (Sun, 2003). Conventional biological nitrogen removal consists of two separated processes, nitrification through oxidation of ammonia to nitrate via the nitrite intermediate production by autotrophic nitrifiers, and denitrification involving the heterotrophic conversion of nitrate to nitrogen gas by denitrifiers under anaerobic condition. Autotrophic nitrifiers grow slowly and cannot compete with heterotrophic denitrifiers in nutrient. Moreover, nitrification is an aerobic process, whereas denitrification is restricted to anoxic conditions, therefore conventional biological nitrogen removal system requires one aerobic tank for autotrophic nitrifiers and one anaerobic tank for heterotrophic denitrifiers.

In recent decades, more and more studies on oxidation ditch (Bruce and Wayne, 1985), biological aerated filter (Chui et a1., 2000), biofilm (Helmer and Kunst, 1998) and sequencing batch reactor (Münch et al., 1996) systems have all discovered the loss of total nitrogen under aerobic conditions, and the phenomenon of aerobic denitrification has been continuously reported later. Meanwhile, some special bacteria that have aerobic denitrification ability were isolated and characterized, such as *Thiosphaera pantotropha* (Robertson and Kuenen, 1990; Gupta and Kshirsagar, 2000), *Alcaligenes faecalis* (Joo et al., 2005), *Pseudomonas nautica* (Bonin et al., 1989), *P. aeruginosa* (Chen et al., 2003), *Microvirgula aerodenitrificans* (Patureau et al., 1998) and *Bacillus cereus* (Kim et al., 2005). Comparing with traditional denitrification that occurred only in anoxic environment, new type of aerobic denitrification has advantages as follows: (1) nitrification and denitrification could occur concurrently in one reactor, which will greatly reduce the reactor volumes and construction cost; (2) the alkali produced by aerobic denitrification could neutralize the acid produced by nitrification, which will reduce the extra addition of

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pH-adjusting chemical substances and decrease the treating cost; (3) aerobic denitrifying bacteria are easier to control (Huang and Tseng, 2001). The discovery of aerobic denitrification and the isolation of aerobic denitrifiers provide a promising approach for biological nitrogen removal and also lay a good theory foundation for the establishment of submerged denitrification reactor. However, most of the aerobic denitrifiers including those above mentioned were from fresh water or soil environments, rare marine origin aerobic denitrifiers were reported. Furthermore, to our knowledge, all of the described aerobic denitrifiers were mesophilic, and none of them could perform efficient denitrification at low temperatures.

In this study, a psychrophilic aerobic denitrifying bacterium, strain S1-1, was isolated from a biological aerated filter conducted for treatment of recirculating water in a marine aquaculture system. Its aerobic denitrification characteristics were investigated and its denitrificational intermediates were detected by GC-MS. Then the effects of culturing conditions on its growth and aerobic denitrification were examined. Such kind of psychrophilic aerobic denitrifying bacteria may play an important role in the nitrogen cycle at the low temperature marine environment, and might be used in the nitrogen removal treatment of wastewater at low temperatures.

1 Materials and methods

1.1 Bacterial strain, medium and growth conditions

Strain S1-1 was isolated from a biological aerated filter conducted for treatment of recirculating water in a marine aquaculture system in Tianjing Haifa Inc., China. The strain showed the ability of aerobic denitrification.

Denitrification medium (DM) (Robertson, 1988) (g/L): KNO_3 1.0; $MgSO_4{\cdot}7H_2O$ 0.2; $CaCl_2$ 0.01; EDTA (0.5 mol/L) 0.5 mL; KH_2PO_4 0.5; Na_2HPO_4 0.5; $FeSO_4$ 0.01; NaCl 20; trace element solution 5 mL; pH 7.0–7.5; the amount of carbon source changes according to the requests of experiment.

Nitrite reduction medium (NRM, g/L): $NaNO_2$ 0.276 (NO_2^--N 56 mg/L); sodium succinate 3.16; $MgSO_4{\cdot}7H_2O$ 0.2; $CaCl_2$ 0.01; EDTA (0.5 mol/L) 0.5 mL; KH_2PO_4 0.5; Na_2HPO_4 0.5; $FeSO_4$ 0.01; NaCl 20; trace element solution 5 mL; pH 7.0–7.5.

Trace element solution (g/L): EDTA 50.0; $ZnSO_4$ 2.2; $CaCl_2$ 5.5; $MnCl_2{\cdot}4H_2O$ 5.06; $FeSO_4{\cdot}7H_2O$ 5.0; $(NH_4)6Mo_7O_2{\cdot}4H_2O$ 1.1; $CuSO_4{\cdot}5H_2O$ 1.57; $CoCl_2{\cdot}6H_2O$ 1.61; pH 7.0.

LB medium (g/L): yeast extract 5; peptone 10; NaCl 20; agar 15–20.

Unless otherwise stated, all the cultivations were conducted on a rotary shaker at 20°C and 160 r/min.

1.2 Aerobic denitrifying assay

For aerobic denitrifying assay, 250 mL flasks containing 100 mL of DM were inoculated with 5 mL of preculture of strain S1-1 grown in DM to the log phase, with the inoculation of 5 mL sterile distilled water as negative controls. Then the flasks were incubated under aerobic condition (with rotation speed 160 r/min) at 20°C. Samples after inoculation were taken at an interval of 4 hr for the determination of bacterial growth (OD_{600}) and the concentrations of NO_2^--N and NO_3^--N. And last, after incubation for 4 days, the final biomass nitrogen, NO_3^--N, and culture total nitrogen (TN) were measured.

1.3 Aerobic nitrite reducing assay

For aerobic nitrite reducing assay, 250 mL flasks containing 100 mL of NRM were inoculated with 5 mL of preculture of strain S1-1 grown at the log phase. Then the flasks were incubated under aerobic condition (with rotation speed 160 r/min) at 20°C for 5 days. The liquid culture was sampled for measuring OD_{600}, and the removal rates of NO_2^--N and TN.

1.4 Effects of culturing conditions on aerobic denitrification

The effects of cultivation conditions on aerobic denitrification performance of strain S1-1 were carried out by single factor tests (Tang et al., 2008), including carbon source, C/N ratio, initial pH and temperature, concentration of NaCl.

In carbon source experiments, sodium succinate, trisodium citrate, glucose, seignette salt, sucrose and sodium acetate were used as sole carbon source, respectively. The other conditions were set as: initial concentration of NO_3^--N 140 mg/L, C/N ratio 15, NaCl 20g/L, initial pH 7.0, temperature 20°C, rotation speed 160 r/min. In C/N ratio experiments, the initial concentration of NO_3^--N was set at 140 mg/L, and sodium succinate was used as the sole carbon source at different C/N ratios (2.5, 5, 10, 15, 20), whereas the other conditions were the same as the carbon source effect experiments. In salinity, initial pH, temperature, and rotation speed effect experiments, sodium succinate was used as the sole carbon source with C/N ratio 15 and the initial concentration of NO_3^--N 140 mg/L, and the concentration of NaCl in the medium ranged from 0 to 30 g/L (at an interval of 10 g/L), the initial pH from 6 to 9 (at an interval of 0.5 pH unit) and the temperature from 10 to 30°C (at an interval of 5°C). All these experiments were performed with the same inoculating quantity (5%, *V*/*V*). After incubation for 4 days, samples were taken for determination of bacterial growth (OD_{600}), biomass-N, NO_2^--N, NO_3^--N and TN.

1.5 Determination of bacterial growth, protein content and biomass nitrogen

Bacterial growth was monitored by measuring the turbidity (OD_{600}) using a spectrophotometer (UV-2802H, Shanghai Optical Company, China).

The protein content in the culture supernatant was determined according to Bradford (1976) with bovine serum albumin as standard.

Bacterial cells were harvested by centrifugation at 12,000 r/min for 5 min and then washed twice with sterile saline solution. The biomass nitrogen was determined by analyzing its TN.

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1.6 Chemical analytical methods

Nitrate (NO_3^--N), nitrite (NO_2^--N), ammonium (NH_4^+-N), and biomass nitrogen and culture TN were determined according to standard methods (APHA, AWWA, WPCF, 1998). pH value was detected by precise pH-meter (PB-10, Shanghai Leici Instrument Inc., China).

1.7 Monitoring of gaseous nitrogen products

For this assay, strictly sealed infusion bottles were used. A 500 mL bottle containing 100 mL of DM was inoculated by injection of 5 mL of preculture of strain S1-1 grown in DM to log phase. Then the bottles were directly incubated in a shaker at 20°C and 160 r/min for 10 days. Then the headspace gas was sampled for measuring the gaseous nitrogen products such as N_2O by gas chromatography-mass spectrometry (GC-MS, 7890A/5975C, Agilent, USA). Conditions were as follows: GS-carbon plot column (0.1 μm × 0.25 mm × 30 m) was used with He as carrier gas at a flow rate of 1.2 mL/min; sample injection port and detector temperature was set at 120°C and the programmed column temperature from 35 to 120°C was used at a rate of 25°C/min (kept at 35 and 120°C for 5 and 3 min, respectively); the added quantity was 600 μL.

2 Results and discussions

2.1 Identification of strain S1-1

Strain S1-1 was strictly heterotrophic and aerobic. Colonies on LB medium were semitransparent, slabby, smooth and white circular with 2 mm in diameter after incubation at 20°C for 2 days. Cells were Gram-negative, non-motile, non-spore forming short rods with 0.4–0.5 μm in width and 0.7–0.8 μm in length. Almost complete 16S rRNA gene (1405 nt) was PCR-amplified by using bacterial universal primers (27F and 1492R; Lane, 1991) and sequenced, it was deposited in GenBank under accession number of HQ693280. It was compared with available 16S rRNA gene sequences in GenBank databases using the BLASTn program (Altschul et al., 1990) on NCBI (http://www.ncbi.nlm.nih.gov). The result indicated that strain S1-1 was closely related to members of the genus *Psychrobacter*, showing highest 16S rRNA gene sequence similarity to *Psychrobacter* sp. TSBY-70 (100%). Phylogenetic analysis also showed that strain S1-1 was grouped together with *Psychrobacter immobilis* IC040 in the tree (data not shown). By combination of the above results, the strain was preliminarily identified as *Psychrobacter* sp. S1-1. Up to date, none of the members of genus *Psychrobacter* was reported to possess the aerobic denitrifying ability.

2.2 Aerobic denitrification performance of strain S1-1

The growth of strain S1-1 and the changes of NO_3^--N and NO_2^--N in DM at C/N ratio 15 and 20°C are shown in Fig. 1. The results indicated that strain S1-1 experienced four typical growth phases in DM, postponing phase, log phase, stable phase and deadline phase. During the first 40 hr, strain S1-1 grew slowly and the removal rate of NO_3^--N was low. From 40–80 hr, strain S1-1 was in logarithmic

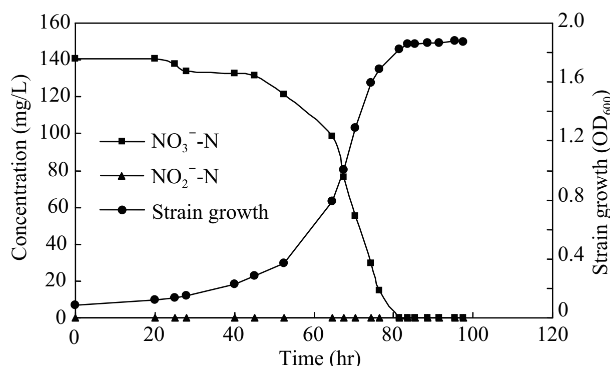

Fig. 1 Growth of strain S1-1 and the changes of NO_3^--N and NO_2^--N in denitrification medium (DM) at C/N ratio 15 and 20°C.

growing phase, and the consumption of NO_3^--N mainly occurred in this period, whereas NO_2^--N accumulation was always kept at a very low leve1. Besides, it was also proved that there was no NH_4^+-N or protein nitrogen produced in the supernatant during the whole growing phase and NO_3^--N was mainly converted to biomass nitrogen and gaseous nitrogen. Among the consumed NO_3^--N (140.86 mg/L, 100%) when strain S1-1 grew into its stable phase, about 76.20 mg/L (54.09%) was used as nitrogen source for its growth, and the removal rate of TN could reach up to 46.48% (Table 1).

All the data indicated that strain S1-1 grew quite well (OD_{600}=1.866) with high concentration of NO_3^--N (140.86 mg/L) as sole nitrogen source, and its denitrifying performance was efficient. Moreover, analysis on the monitoring results of the growth of strain S1-1 and the changes of NO_3^--N and NO_2^--N showed that aerobic denitrification process occurred mainly in its log phase, maybe due to the fast growth, and energy and reduction force were largely and mainly consumed in this period (Shao and Yu, 2008).

2.3 Aerobic nitrite reducing activity of strain S1-1

After incubation of strain S1-1 in NRM under aerobic condition for 5 days, strain growth (OD_{600}), biomass nitrogen and NO_2^--N were determined and the results are summarized in Table 2. Under aerobic condition, S1-1 grew well with high concentration of NO_2^--N (60 mg/L) as sole nitrogen source, and there were no NO_3^--N, NH_4^+-N or protein nitrogen produced. The removal rates of NO_2^--N

Table 1 Aerobic denitrifying performance of strain S1-1 when it grew into stable phase

	Initial	Final	Removal (%)
Strain growth (biomass-N, mg/L)	1.43	76.20	
NO_3^--N (mg/L)	140.86	0	100.00
TN (mg/L)	142.29	76.20	46.48

Table 2 Aerobic nitrite reducing activity of strain S1-1

	Initial	Final	Removal (%)
Strain growth (biomass-N, mg/L)	1.40	19.92	
NO_2^--N (mg/L)	60.00	21.90	63.50
TN (mg/L)	61.40	41.82	31.89

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and TN were up to 63.50% and 31.89%, respectively, and there was about 33.20% (19.92 mg/L) of NO_2^--N converted to biomass nitrogen.

2.4 Factors affecting aerobic denitrification performance of strain S1-1

2.4.1 Carbon source

The effect of carbon source on the growth and aerobic denitrifying performance of strain S1-1 is shown in Table 3. This strain could hardly grow on trisodium citrate, seignette salt or sucrose with poor nitrogen removal efficiency. Strain S1-1 could grow but not very well on glucose and sodium acetate, and meanwhile the nitrogen removal rate was also not very high. The optimum carbon source for S1-1 was sodium succinate and the TN removal rate could be high up to 34.32%.

Carbon source is one of the most important factors affecting the aerobic denitrification. During the denitrification process, the denitrifiers utilize the carbon source as electron donor, and reduce nitrate progressively to N_2 which could remove organic matter and nitrate simultaneously. Studies conducted by Her and Huang (1995) concluded that the structure and molecular of the carbon source could obviously influence the efficiency of the denitrification, and generally speaking, the carbon source with simple structure and small molecular would be more favorable to the denitrification. The result of this experiment was similar. Comparing to trisodium citrate, seignette salt and sucrose, the structures of sodium succinate, glucose and sodium acetate are simple and the molecular weights are small. When they were used as carbon sources, the denitrification efficiency would be high. As for sodium succinate, it was easy to be converted to succinic acid, which could then enter the tricarboxylic acid cycle (TCA) and provide energy and reduction force rapidly.

2.4.2 Temperature

The results in Fig. 2a showed that both the growth of strain S1-1 and the nitrogen removal rate increased with the increase of temperature to the highest levels (OD_{600} ca. 1.1 and TN removal rate of 39.41%) at 20°C and then followed by a decrease trend. It could be concluded that the optimum temperature was 20°C for the growth of strain S1-1 and its aerobic denitrifying activity. This is in agreement with the description of the genus *Psychrobacter* (Juni and Heym, 1986). This optimum temperature was much lower than that for other aerobic denitrifiers ever described. Up to date, the optimum temperature for all of the reported aerobic denitrifiers ranged from 30 to 40°C, such as *Bacillus licheniformis* (32.5°C, Zeng et al., 2008), *Pseudomonas aeruginosa* (30°C, Li et al., 2008) and *Thiosphaera pantotroph* (37°C, Ma et al., 2005). Furthermore, there still have been no reports about psychrophilic bacteria that possess aerobic denitrifying ability. The results also indicated that the nitrite accumulation was always kept at very low leve1 during all the processes. These properties of strain S1-1 suggested that it will have great application prospect in the nitrogen removal treatment of low temperature wastewater and that psychrophilic denitrifiers like strain S1-1 might play important roles in the nitrogen cycle at low temperature environments, especially low temperature aquatic environments.

2.4.3 Initial pH

The results in Fig. 2b indicated that the strain growth and TN removal rate increased first and then followed by a decrease with the increase of initial pH value. Strain S1-1 exhibited quite well denitrifying ability between pH

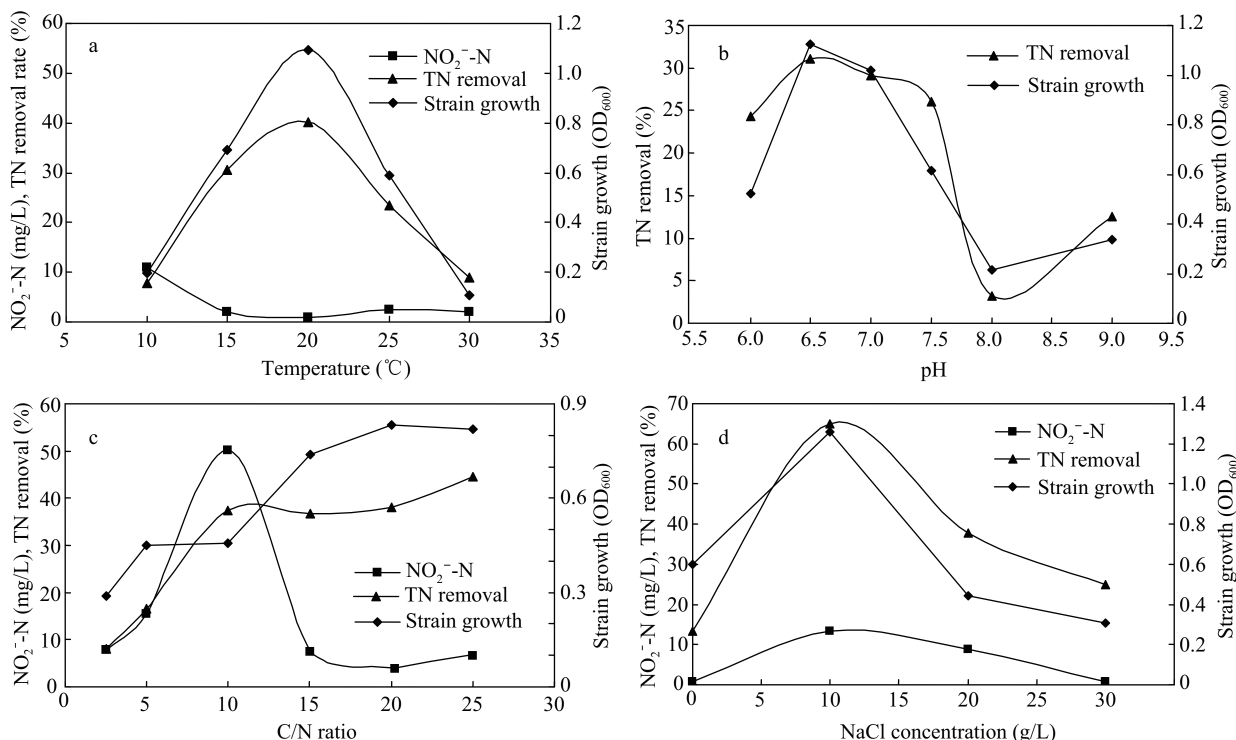

Fig. 2 Effect of temperature (a), Pb (b), C/N ratio (c), and NaCl concentration (d) on the growth of strain S1-1 and its denitrifying performance.

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Table 3 Effect of carbon source on the aerobic denitrifying performance of strain S1-1

Carbon source	Strain growth (OD_{600})	NO_2^--N formed (mg/L)	TN (mg/L)		
			Initial	Final	Removal (%)
Seignette salt	0.010	0.10	142.15	141.44	0.50
Sodium acetate	0.240	4.20	141.97	122.90	13.43
Sodium succinate	0.916	1.75	142.23	93.42	34.32
Trisodium citrate	0.041	0.68	142.06	140.53	1.08
Glucose	0.138	1.10	142.14	13472.	6.80
Sucrose	0.028	0.45	142.18	140.93	0.88

6.0 and 7.5. When pH was 6.5, total nitrogen removal rate reached the highest (ca. 38%). According to Xu et al. (2010), it was found that an aerobic denitrifying bacterium of *Salmonella* had its highest denitrification efficiency at pH 7.0, and under acidic (pH < 5.5) or alkaline (pH > 9) condition, it would lose its denitrification ability.

2.4.4 C/N ratio

Figure 2c shows that when the carbon source was insufficient (C/N < 5), the total nitrogen removal rate would be relatively low (8%–16%). Until C/N ratio was high up to 10, the total nitrogen removal rate increased obviously (37%). This result was consistent with the findings of Robortson and Kuenen (1983) that within a certain C/N ratio range, the higher the organic carbon concentration, the greater the denitrificational efficiency. However, once C/N ratio was higher than 10, its effect on denitrification would be little. As for NO_2^--N, its accumulation was serious and reached the highest at C/N ratio 10. The mechanism for this accumulation needed to be further investigated. By taking total nitrogen removal rate, NO_2^--N accumulation and the utilizing efficiency of carbon source as wastewater treating cost into consideration, 15 was chosen as the optimum C/N ratio for strain S1-1.

2.4.5 Salinity

The growth of strain S1-1 and the total nitrogen removal rate increased first and then followed by a decrease with the increase of NaCl concentration from 0 to 30 g/L (Fig. 2d). The optimum NaCl concentration was 10 g/L for the growth of strain S1-1 and its aerobic denitrifying performance, the total nitrogen removal could reach 64.92%, meanwhile, the accumulation of NO_2^--N was not very high.

2.5 Detecting results of N_2O

The above results about TN removal rate in the denitrification process suggested the possible occurrence of NO_3^--N or NO_2^--N conversion to biomass nitrogen and gaseous nitrogen by the aerobic denitrification which has been known to occur with some species of bacteria such as *Alcaligenes faecalis* (Joo et al., 2005). To verify the possible occurrence of aerobic denitrification, gas chromatography-mass spectrometry tests were conducted to identify the production of N_2O by strain S1-1.

Exploratory experiment by GC proved that the GS-carbon plot column could separate N_2O from other gases such as N_2, O_2 and CO_2 and its peak time was after 2.8 min while N_2, O_2 and CO_2 were all separated before 2.8 min. Therefore, the solvent delay time of GC-MS was set 2.8 min. The total ion currency (TIC) profiles of blank control and the sample of strain S1-1 were obtained as shown in Fig. 3. An obvious peak emerged between 2.946 and 2.998 min and there was a huge increase in the peak area after the inoculation of strain S1-1 in comparing to the blank control. In the MS chromatogram of this peak as shown in Fig. 4, there were 6 characteristic ion peaks at *m/z* 3.9, 13.9, 18, 28, 29.9 and 43.9, which should be He^+, N^+, H_2O^+, N_2^+, Ar^+, and N_2O^+, respectively. From the results of TIC and MS chromatogram, it could be concluded that the peak emerged between 2.946 and 2.998 min was actually the characteristic peak of N_2O, suggesting that there was N_2O formed in the denitrification process of strain S1-1.

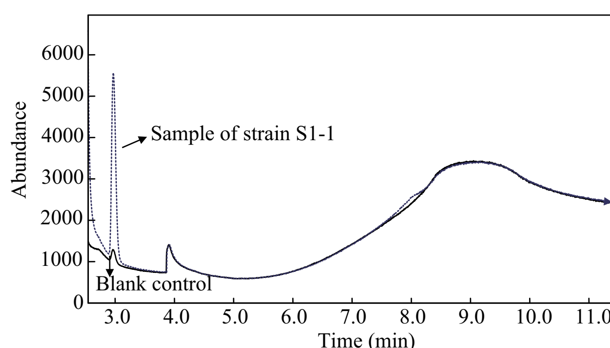

Fig. 3 TIC profile of blank control and sample of strain S1-1.

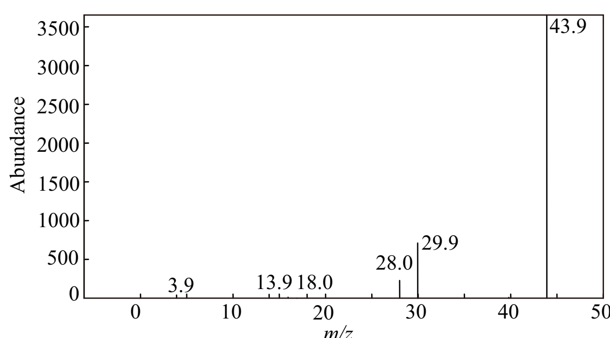

Fig. 4 MS chromatogram of the peak at retention time 2.946–2.998 min.

3 Conclusions

A psychrophilic aerobic denitrifying bacterium, strain S1-1, was isolated from a biological aerated filter conducted for the treatment of recirculating water in a marine aquaculture system. It was preliminarily identified as *Psychrobacter* sp. S1-1 based on the analysis of its 16S rRNA

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gene sequence. Strain S1-1 grew well either in high nitrate or high nitrite conditions with a removal of 100% nitrate or 63.50% nitrite, and the TN removal rates could reach to 45.91% and 30.30%, respectively. This consumption of nitrate or nitrite occurred mainly in its log growing phase. The optimum denitrification conditions for S1-1 were: sodium succinate as carbon source, C/N ratio15, salinity 10 g/L NaCl, incubation temperature 20°C and initial pH 6.5. N_2O was formed as a major intermediate in the aerobic denitrification process of strain S1-1.

Acknowledgments

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