

*Full Length Research Paper*

# **Aerobic denitrification of a *Pseudomonas* sp. isolated from a high strength ammonium wastewater treatment facility**

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Accepted 30 December, 2010

**An aerobic denitrifier (termed X31 in this study) isolated from a wastewater treatment facility treating high strength ammonium wastewater was genetically identified, and morphologically and physiologically characterized. Its aerobic denitrification performance was also studied under various levels of DO, pH, temperature, and C/N ratios, as well as three carbon sources. Experimental results showed that: 1) the 16S rDNA of X31 has a 99% similarity to *Pseudomonas stutzeri*, but it is suggested to be a different strain due to its capability to carry aerobic denitrification at high DO levels that are normally inhibitory to *P. stutzeri*; 2) X31 is a mesophile and prefers a neutral to slightly alkaline environment to perform its aerobic denitrification process; 3) No  $\text{NO}_2^-$ -N accumulation was observed during X31 aerobic denitrification process; 4)  $\text{NO}_3^-$ -N removal by X31 appeared to be a zero-order reaction over  $\text{NO}_3^-$ -N concentrations when X31 grows exponentially, which needs further investigation; 5) As a heterotrophic bacteria, X31 growths and utilization of  $\text{NO}_3^-$ -N varied between different organic carbon sources.**

**Key words:** Aerobic denitrification, *Pseudomonas* sp., wastewater

## **INTRODUCTION**

From a macro perspective, biological denitrification is more favorably to be carried without the presence of oxygen, and this concept has been consistently followed by the wastewater industry to operate denitrification process (Rittmann and McCarty, 2000; Robertson et al., 1988; VanNiel, 1991). However, denitrification can be extended into aerobic conditions by bacteria that can perform denitrification under aerobic conditions. Some of those reported aerobic denitrifiers are *Alcaligenes faecalis* (Joo et al., 2005, 2006), *Citrobacter diversus* (Huang and Tseng, 2001), *Microvirgula aerodenitrificans* (Patureau et al., 1998), *Pseudomonas nautica* (Bonin and Gilewicz, 1991), *Pseudomonas stutzeri* (Körner and

Zumft, 1989), *Thaurea mechernichensis* (Scholten et al., 1999), and *Thiosphaera Pantotropha* (Robertson et al., 1988). This unique feature of aerobic denitrifiers is believed to be associated with the presence of periplasmic nitrate reductase whose expression is less influenced from oxygen inhibition, instead of the membrane-bound nitrate reductase which is usually favorably expressed under low DO environment (Bell et al., 1990; Patureau et al., 1988).

The reason for not seeing any practical industrial utilization of aerobic denitrifiers is mainly due to their slow growths that normally put them incompetent with the other organisms in conventional activated sludge systems (Rittmann and McCarty, 2000; Robertson et al., 1988). Also, aerobic denitrification seems contrary to the primary interest of the currently popular wastewater treatment processes that focus on either denitrification through nitrite (Abeling and Seyfried, 1992; Fux et al.,

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2003; Turk and Mavinic, 1986) and/or simultaneous nitrogen and phosphorus removal (Arun et al., 1988; Seviour et al., 2003). However, these cannot be used to conclude a dim future for aerobic denitrifiers, and the application possibilities lies within the progress of genetic engineering and appropriate engineering combination (Srivastava and Majumder, 2007). The study of aerobic denitrifiers should not be discontinued.

In this study, an aerobic denitrifier (termed X31 in this study) isolated from a wastewater treatment facility treating high strength ammonium wastewater was genetically identified, and morphologically and physiologically characterized. Its aerobic denitrification performance was also studied under various levels of DO, pH, temperature, and C/N ratios, as well as three carbon sources. The purpose of this study is to have a general picture of X31 aerobic denitrification performance, rather than quantifying its practical applicability.

## MATERIALS AND METHODS

### Strain isolation

The isolate (here termed X31) was obtained from the aerobic activated sludge of a cyclic activated sludge system (CASS) treating a high strength ammonium ( $\text{NH}_4^+\text{-N}$  200-400  $\text{mg L}^{-1}$ ) wastewater at a fertilizer manufacturing plant in Harbin, China (Ma et al., 2005). Briefly, the activated sludge was domesticated in a bench scale sequential batch reactor (SBR) under aerobic conditions using a cultural medium composed of the following ingredients ( $\text{g L}^{-1}$ ) (Scholten et al., 1999):  $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$ , 7.9;  $\text{KH}_2\text{PO}_4$ , 1.5;  $\text{NH}_4\text{Cl}$ , 0.3;  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ , 0.1; and 2  $\text{mL L}^{-1}$  trace element solution. The trace element solution had a pH of 7 and was composed of the following ingredients ( $\text{g L}^{-1}$ ): EDTA, 50.0;  $\text{ZnSO}_4$ , 2.2;  $\text{CaCl}_2$ , 5.5;  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ , 5.06;  $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ , 5.0;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ , 1.1;  $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ , 1.57;  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ , 1.61. The pH of the cultural medium was controlled between 7.0 - 7.5. Sodium succinate was used as the carbon source at 840  $\text{mg C L}^{-1}$ . Potassium nitrate and sodium nitrate were used as the nitrogen source at 185  $\text{mg N L}^{-1}$ . After the SBR effluent stabilized, 10 ml sludge was put into a 50 ml vial with glass beads and shaken thoroughly to break down the sludge. Serial dilution and agar streaking were then used to isolate distinct colonies. Those isolated colonies were further screened for their aerobic denitrification capabilities. Finally, one isolate (X31) capable of aerobic denitrification was selected for further characterization in this study.

### Morphologic and physiologic characterization, and genetic identification

The X31 physiology characteristics were determined by the procedures outlined in 'Manual of Methods for General Bacteriology (Smbert and Krieg, 1981). The 16S rDNA of X31 was amplified by PCR using the universal primer BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and BSR1541/20 (5'-AAGGAGGTGATCCAGCCGCA-3'). DNA amplification was performed in a 50- $\mu\text{L}$  reaction mixture containing 40 ng of template DNA, 0.3 U rTaq DNA polymerase, 0.3  $\text{mmol L}^{-1}$  dNTPs, 0.1  $\mu\text{mol L}^{-1}$  primer in a hot lid thermal cycler (MJ Research, USA). The reaction procedure was: pre-denaturation at 94°C for 5 min; 30 temperature cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, elongation at 72°C for 1.5 min; final elongation at 72°C for 10 min. The amplified PCR product was measured by

Agarose/EtBr gel electrophoresis and photographed with a UVG Image Analysis System (USA). Target fragments were then cut from the gel and purified using an Agarose Gel DNA Purification Kit (TaKaRa, China). After this, the purified products were ligated to a pGEM-T vector (Promega, USA). The ligated products were then transformed to *Escherichia coli* competent cell TOP10, and the transformed *E. coli* was selected on a LB solid culture medium with Amp (50  $\mu\text{g mL}^{-1}$ ) and X-gal, and was analyzed using the universal primers T7 and SP6. Then the 16S rDNA were sequenced on an ABI 377 genetic analyzer (PE Applied Biosystems, USA) using a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, USA).

The obtained 16S rDNA sequence was compared with non-redundancy nucleotides database by BLAST to judge the homology of 16S rDNA sequence. Multiple sequences alignment was conducted using BioEdit v5.06 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and a phylogenetic tree was constructed with the neighbor-joining method through MEGA (Version 3.1, USA).

### Aerobic denitrification performance

X31 aerobic denitrification performance was studied in a 2-L bench scale SBR (Figure 1) under various levels of DO (3.2  $\text{mg L}^{-1}$  - saturated), pH (5.0-10), temperature (20 - 40°C), and C/N ratios (3-14), as well as three organic carbon sources (acetate, malate, and succinate). For each test, 1-L cultural medium prepared the same as during the previous colony isolation process (Ma et al., 2005) was filled into the flask. The initial nitrogen, carbon, DO, and pH levels of the cultural medium were already adjusted to the requirement of each test. Then, a 100-mL X31 inoculum solution ( $7.9 \times 10^8$  cfu  $\text{mL}^{-1}$ ) was inoculated to the cultural medium. The inoculums solution was prepared by taking one colony of X31 from an agar plate into a 100-mL cultural medium and then incubated at 30°C for 24 h. After inoculation, the flask was immediately sealed with a septum to prevent nitrogen gas from re-entering the flask. The gas inlet and outlet were each equipped with a filter (0.25  $\mu\text{m}$ ) to intercept possible outside bacterial interference. One gas sampling port and one cultural medium sampling port were also made available. A DO meter (YSI model 200) was inserted into the mixed liquor to monitor DO. A combo pH/ORP meter (Orion 370) was inserted into the mixed liquor to monitor oxidation-reduction potential (ORP) and pH. The water temperature during each test was controlled by putting the flask into a water bath. Gas and liquid samples were periodically taken from the flask to monitor the concentrations of total organic carbon (TOC), nitrogen gas ( $\text{N}_2$ ),  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$ . All solutions and apparatus were autoclaved before putting into use. Each test was replicated for at least three times.

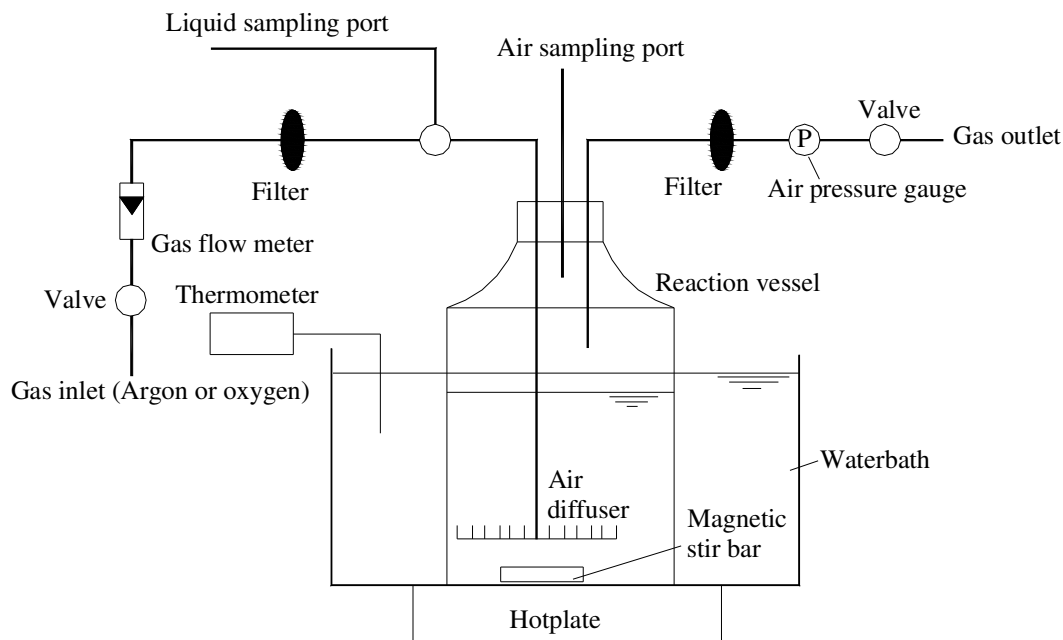
### Analytical methods

Nitrogen gas ( $\text{N}_2$ ) was measured by an Agilent HP4890D gas chromatography (GC) equipped with a thermal conductivity detector (TCD). Nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) were measured by an ion chromatography (DIONEX-100). TOC was measured by a TOC analyzer (Shimadzu, Japan). X31 biomass concentration in the mixed liquor was estimated by filtering a 5-mL water sample through a Whatman® filter and then measured the weight of the dry content left on the filter after oven dried for 24 h at 105°C.

## RESULTS AND DISCUSSION

### Characterize the isolate

The observed morphological and physiological



**Figure 1.** The setup of the reaction vessel. (The YSI DO meter and the pH/ORP meter were not shown).

**Table 1.** Morphological and physiological characteristics of the X31.

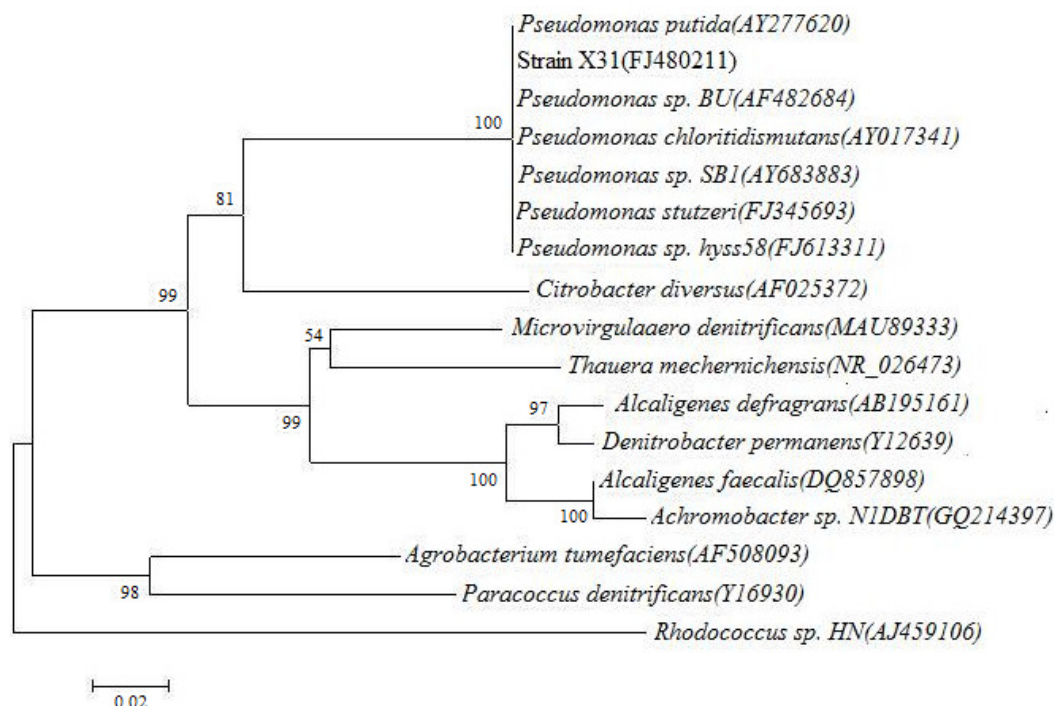
<b>Morphological</b>	
Flagellum	Single polar
Size ( $\mu\text{m}$ )	0.4×1.1
Shape	Rod
Gram	-
<b>Physiological</b>	
Catalase reaction	+
Starch hydrolysis	+
Gelatine hydrolysis	-
Acid from Glucose	+
Citrate utilization	+
Hydrogen sulfide production	-
Production of ammonia	+
Oxidase reaction	+
Methyl red	-
Indole production	+
Nitrate reduction	+
Urea hydrolysis	+
Voges-Proskauer	-

+, positive reaction; -, negative reaction.

characteristics are summarized in Table 1. X31 is rod-shaped with a size of 0.4×1.1 $\mu\text{m}$ . It is gram-negative. The tests for assimilation of glucose, acetate, succinate, citrate, glutamate, malate, formate, and caprate were positive. The tests for assimilation of arabinose, sucrose,

maltose, and mannose were negative. According to the 'Manual of Methods for General Bacteriology (Smibert and Krieg, 1981), the X31 isolate belongs to *Pseudomonas* sp.

The phylogenetic tree of X31 is illustrated in Figure 2.



**Figure 2.** The phylogenetic tree of X31 based on its 16S rDNA sequence.

It is indicated that X31 is in the same branch with *Pseudomonas* sp. and has a 99% similarity to *P. stutzeri*. The obtained 16S rDNA sequence was registered in the genebank at NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov), accession: FJ480211).

#### **Aerobic denitrification under varying DO, temperature, and pH levels**

The 24-h  $\text{NO}_3^-$ -N removal (%) by X31 under varying DO levels are illustrated in Figure 3a. It was observed that the final  $\text{NO}_3^-$ -N removals were consistently maintained above 90% as the DO was increased from as low as  $3.2 \text{ mg L}^{-1}$  till saturation. It is reported that *P. stutzeri* cannot perform aerobic denitrification when DO is above  $5.0 \text{ mg L}^{-1}$  (Körner and Zumft, 1989). However, this study showed that not only X31 was capable of aerobic denitrification under DO levels greater than  $5.0 \text{ mg L}^{-1}$ , but also the final  $\text{NO}_3^-$ -N removals had no obvious influence from DO variations. Therefore, despite the fact that the 16S rDNA of X31 has a 99% similarity to *P. stutzeri* (Figure 1), this study suggests that X31 probably is a new strain of *Pseudomonas* sp. capable of aerobic denitrification.

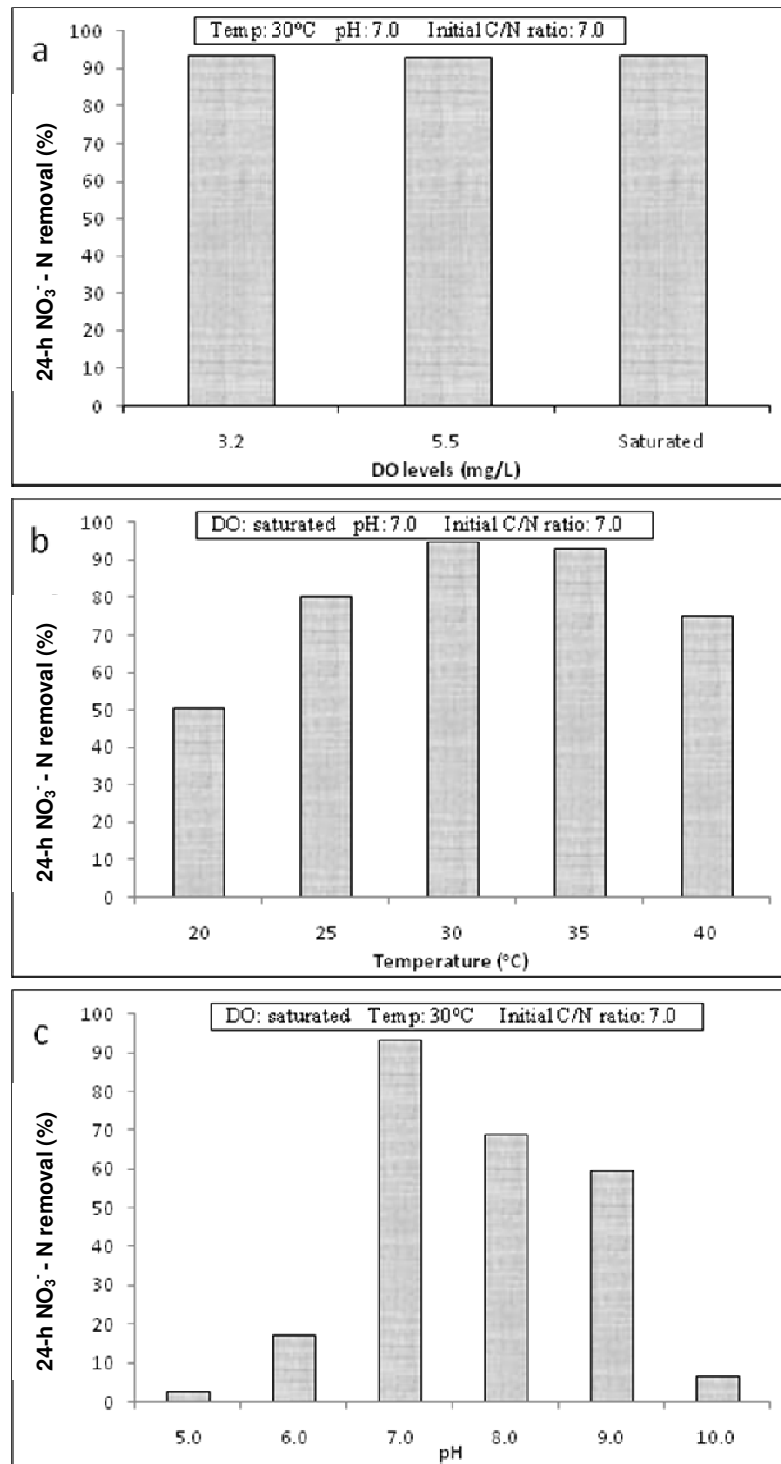
The 24-h  $\text{NO}_3^-$ -N removal (%) by X31 under varying temperatures are illustrated in Figure 3b. Over the testing temperature range, the maximum  $\text{NO}_3^-$ -N removal occurred between  $30 - 35^\circ\text{C}$ . Compared to the DO tests (Figure 3a), the temperature variation showed a much

stronger influence on X31 aerobic denitrification performance. These observations suggest that X31 is a mesophile sensitive to temperature changes.

The 24-h  $\text{NO}_3^-$ -N removal (%) by X31 under varying pH levels are illustrated in Figure 3c. Compared to the temperature tests (Figure 3b), the pH variation showed an even higher control over  $\text{NO}_3^-$ -N removal. The observed maximum  $\text{NO}_3^-$ -N removal occurred at a pH of 7.0, but at pH levels below 7.0 the  $\text{NO}_3^-$ -N removals were lower than at pH levels greater than 7.0. These observations suggest that in terms of aerobic denitrification, X31 is more efficient under a neutral to slightly alkaline environment.

#### **Aerobic denitrification under different initial C/N ratios**

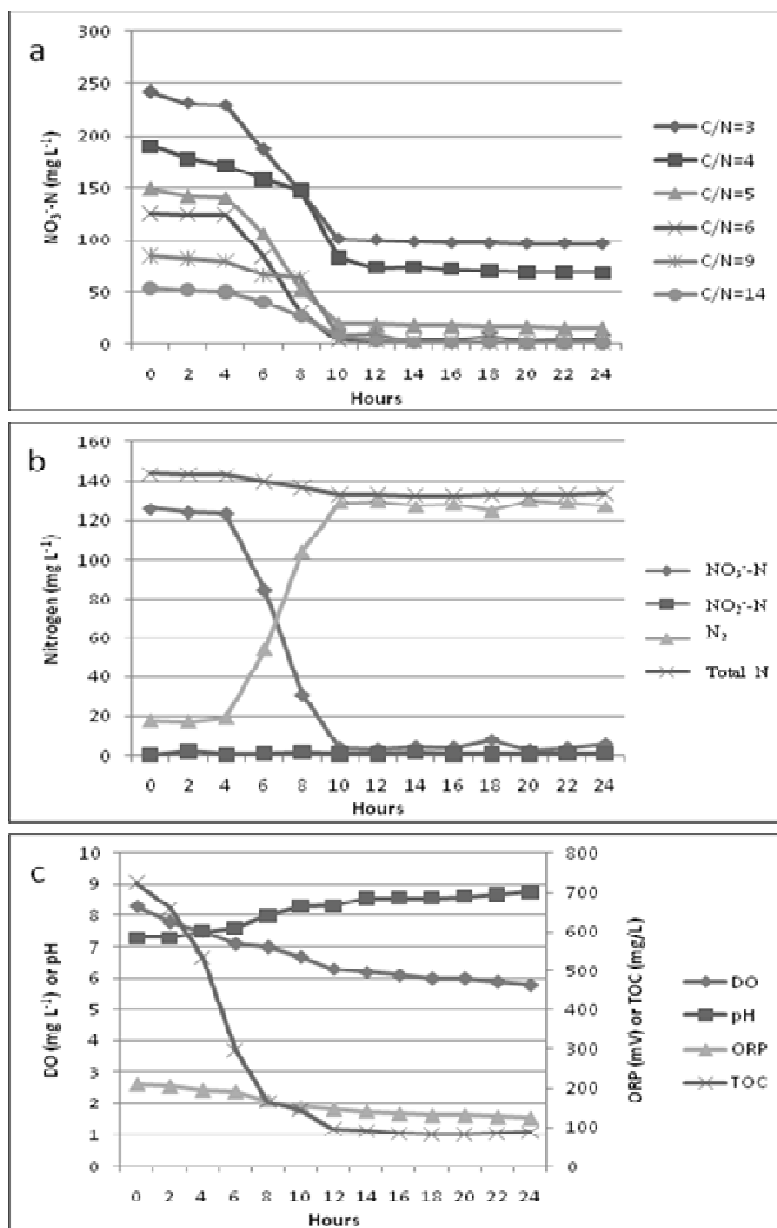
The time courses of mixed liquor  $\text{NO}_3^-$ -N concentrations under different initial C/N ratios are shown in Figure 4a. It was observed that  $\text{NO}_3^-$ -N removal was relatively stagnant in the first four hours and then generally decreased linearly from the 4th hour to the 10th hour, but stalled after the 10th hour under all the tested C/N ratios, showing no obvious dependence on the  $\text{NO}_3^-$ -N concentration. Since this experiment fixed the carbon source concentration and varied the  $\text{NO}_3^-$ -N concentration, these observations suggest that  $\text{NO}_3^-$ -N removal by X31 probably is a zero-order reaction over  $\text{NO}_3^-$ -N concentrations when X31 grows exponentially. However,



**Figure 3.** The 24-h NO<sub>3</sub><sup>-</sup>-N removal (%) by X31 aerobic denitrification under varying levels of DO (a), temperature (b), and pH (c). (Each bar represents the average of three tests).

this hypothesis was not verified in this study, which can be explored by additional experiments on NO<sub>3</sub><sup>-</sup>-N removal by X31 with fixed NO<sub>3</sub><sup>-</sup>-N concentration and varying carbon source concentrations.

It was also observed that the remaining NO<sub>3</sub><sup>-</sup>-N (after the 10th hour) decreased as the initial C/N ratio was increased, but no obvious further decrease was observed once the initial C/N ratio was increased to above 6.0.



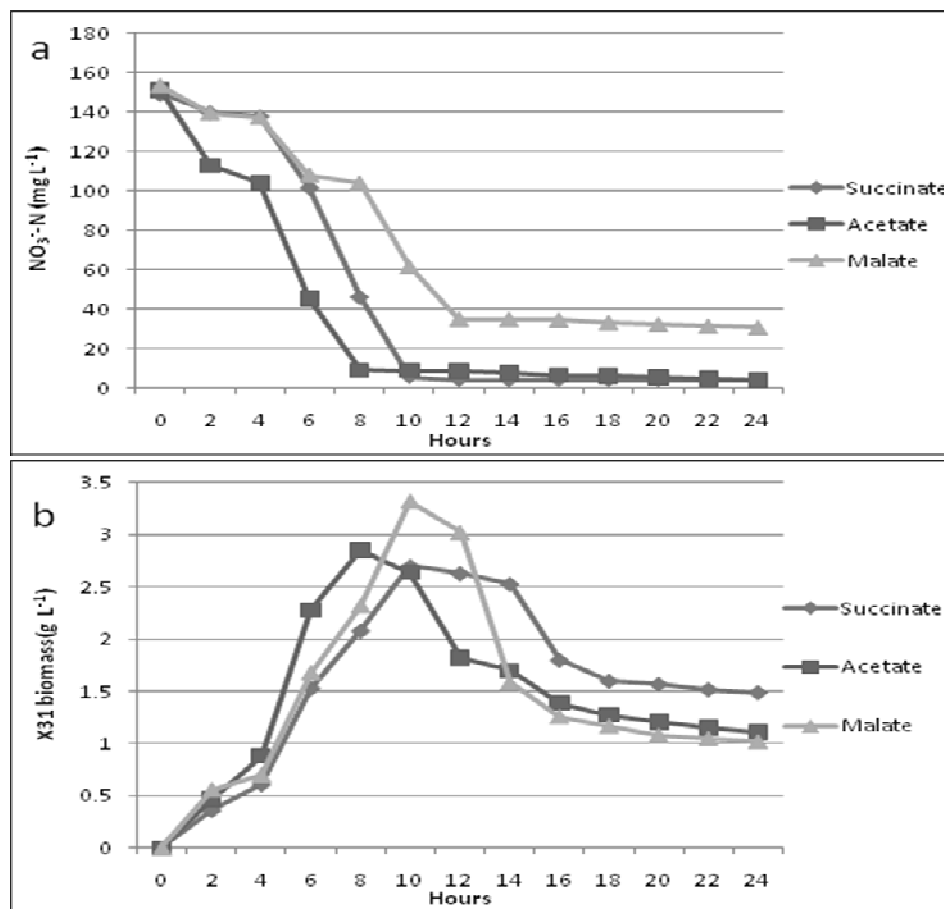
**Figure 4.** (a) The time courses of  $\text{NO}_3^-$ -N under different initial C/N ratios. (b) Nitrogen balance during the test of C/N ratio of 6. (c) The time courses of DO, pH, ORP, and TOC during the test of C/N ratio of 6. (1. Testing conditions are: saturated DO, 30°C temperature; 2. each data point represents the average of three tests; 3.  $\text{NO}_2^-$ -N was consistently observed below 1.0 mg L<sup>-1</sup> and was not plotted here).

Also,  $\text{NO}_2^-$ -N was consistently observed below 1.5 mg L<sup>-1</sup> during each test with no sign of accumulation (data not shown), suggesting  $\text{NO}_2^-$ -N was not a limiting step during X31 aerobic denitrification. These observations indicate there was an upper limit of  $\text{NO}_3^-$ -N removal by X31 under the testing conditions.

To get a more detailed picture of X31 aerobic denitrification process, the nitrogen balance within the 2-L reaction vessel under the test of a C/N ratio of 6 was

monitored and is shown here in Figure 4b. It was noted that the total N was well maintained over time as the  $\text{NO}_3^-$ -N dropping trend corresponding to the increasing trend of  $\text{N}_2$ . Meanwhile, the  $\text{NO}_2^-$ -N concentration did not exceed more than 1.0 mg L<sup>-1</sup>. These are indications that X31 thoroughly denitrified  $\text{NO}_3^-$ -N into  $\text{N}_2$ .

In addition, the time courses of pH, ORP, DO, and TOC during the test of C/N ratio of 6 were observed and are shown in Figure 4c. The TOC, DO, ORP were all



**Figure 5.** (a) The time courses of nitrate ( $\text{NO}_3^-$ -N) concentration under acetate, malate, and succinate; and (b) the X31 growth curves under the three carbon sources. (1) Testing conditions are: saturated DO, 30°C temperature, and an initial C/N ratios of 5.0; (2) Each data point represents the average of three tests; (3)  $\text{NO}_2^-$ -N was consistently observed below 1.0 mg L<sup>-1</sup> and was not plotted here).

observed decreasing over time while the pH was found increased, corresponding to a typical denitrification process that consumes organic carbon and electron acceptor, and produces alkalinity. The TOC dropping trend stalled after the 10th hour, corresponding to when  $\text{NO}_3^-$ -N dropping trend stopped (Figure 4b). The average C/N ratio was around 26 after the 10th hour, indicating the organic carbon supply was in surplus and  $\text{NO}_3^-$ -N shortage might be the limiting factor to achieve further  $\text{NO}_3^-$ -N removals. Comparing the dropping trends of  $\text{NO}_3^-$ -N (Figure 4b) and DO (Figure 4c), the  $\text{NO}_3^-$  decreased much quicker than DO, suggesting that  $\text{NO}_3^-$  was more favorably than oxygen to be utilized by X31.

#### Aerobic denitrification under different carbon sources

The time courses of mixed liquor  $\text{NO}_3^-$ -N concentration under the three carbon sources (acetate, succinate, and

malate) are compared in Figure 5a.  $\text{NO}_3^-$ -N removal stalled approximately after the 8th hour under acetate, 10th hour under succinate, and 12th hour under malate. However, the final  $\text{NO}_3^-$ -N removals (%) were almost the same between acetate (97.57%) and succinate (97.31%), with malate being the lowest (80.11%). This relatively lower  $\text{NO}_3^-$ -N removal by malate might be ascribed to the mixed usage of L- and D- malate in this study and X31 may be selective on malate types for metabolism. However, this study did not carry further test to confirm this hypothesis.

The growth curves of X31 under the three carbon sources are shown in Figure 5b. The X31 growth curves are Monod type (Table 2) and reached their peak values (maximum biomass) when the  $\text{NO}_3^-$ -N removal stalled (Figure 5a). Biomass yield was observed highest under malate, followed by acetate and succinate. Since this experiment fixed the concentrations of carbon source and  $\text{NO}_3^-$ -N, these obtained results suggest that  $\text{NO}_3^-$ -N removal by X31 was highly dependent upon the type of

**Table 2.** The fitted Monod equation for the X31 growth under three carbon sources.

	$\mu_{\max}$ (d <sup>-1</sup> )	$K_m$ (mg L <sup>-1</sup> )
Succinate	0.386	28.93
Acetate	0.307	27.25
Malate	0.224	37.13

\* The Monod equation takes the form of  $\mu = (\mu_{\max} \times S) / (K_m + S)$ , where  $\mu$  is the specific growth rate of X31,  $\mu_{\max}$  is the maximum specific growth rate of X31,  $K_m$  is the half saturation constant,  $S$  is the concentration of substrate (carbon source in this study).

carbon sources. Further studies are suggested to explore and quantify the influence from different organic carbon sources on X31 aerobic denitrification performance.

## Conclusion

An aerobic denitrifier (X31) isolated from a wastewater treatment facility treating high strength ammonium wastewater was studied through a series of genetic and biological test. The following conclusions were obtained:

1. The 16S rDNA of X31 has a 99% similarity to *P. stutzeri*, but is suggested to be a different strain due to its capability to carry aerobic denitrification at DO levels that are normally inhibitory to *P. stutzeri*.
2. X31 is a mesophile and prefers a neutral to slightly alkaline environment to perform its aerobic denitrification process.
3. X31 can carry its aerobic denitrification thoroughly without NO<sub>2</sub><sup>-</sup>-N accumulation.
4. NO<sub>3</sub><sup>-</sup>-N removal by X31 appeared to be a zero-order reaction over NO<sub>3</sub><sup>-</sup>-N concentrations when X31 grows exponentially based on the experimental results. However, further test are needed to validate such a conclusion.
5. X31 favorably utilized NO<sub>3</sub><sup>-</sup> instead of oxygen for its metabolism process.
6. As a heterotrophic bacteria, X31 growth and aerobic denitrification efficiency varies between different organic carbon sources.

Successive studies are suggested to explore into the mechanism that makes X31 unique to the others already known aerobic denitrifiers. Some of the unverified hypothesis and inadequate experimental results also deserve further notice.

## ACKNOWLEDGEMENT

This work was financially supported by the National Natural Science Foundation of China (NSFC) (51008239), the Fundamental Research Funds for the Central Universities (5082010) and the Open Project of

State Key Laboratory of Urban Water Resource and Environment (HIT)No.QA200810; QAK201014).

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