African Journal of Microbiology Research Vol. 7(3), pp. 211-219, 15 January, 2013
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1802
ISSN 1996-0808 ©2013 Academic Journals

Full Length Research Paper

Aerobic denitrifying characteristics of duckweed rhizosphere bacterium RWX31

Zhou Ying-ru[1,2], Lu Yu-fang[1,2], Zhang Hai-lin[3] and Shi Wei-ming[1*]

[1]State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, The Chinese Academy of Sciences, Nanjing 210008, China.
[2]Graduate School of Chinese Academy of Sciences, Beijing 100081, China.
[3]Department of Plant and Soil Sciences, Oklahoma State University, Stillwater, OK 74078, USA.

Accepted 5 October, 2012

Aerobic denitrifying characteristics of duckweed rhizosphere bacterium RWX31 was studied in this paper. Bacterium RWX31 exhibited efficient growth stability and nitrogen removal ability, with nitrate removal efficiency of 81.3% at an initial 140 mg l^{-1} NO_3-N. Under aerobic conditions, the maximum nitrate-N removal rates were 30.9, 24.3 and 11.5 mg l^{-1} h^{-1}, with nitrate, nitrite and ammonium as sole nitrogen source, respectively. The optimal conditions for aerobic denitrification were C : N ratio of 8, pH 7.2 and 27°C when nitrate served as the substrate, and shaking speed had no significant impact on nitrate removal efficiency within the range tested (dissolved oxygen concentration 3.5 to 7.3 mg l^{-1}). Stationary test suggested that the association of duckweed and strain RWX31 performed better in nitrate-removal than by itself or in association with other bacteria species. According to the morphological observation and 16S rRNA gene analysis, strain RWX31 was preliminarily identified as *Pseudomonas* sp. Strain RWX31 is the first aerobic denitrifier isolated from duckweed *Lemna minor* rhizosphere with the potential to reduce nitrate leaching.

Key words: Aerobic denitrification, nitrogen removal, *Pseudomonas,* rhizobacteria.

INTRODUCTION

Nitrogen (N) losses from agricultural fields have become a major source of eutrophication in water bodies and high nitrate concentration in drinking water can cause methemoglobinemia in infants (Bruning and Kaneene, 1993). Many treatment processes have been developed to remove nitrate from water (Kapoor and Viraraghavan, 1997), and denitrification is considered to be the most preferred method because of its less labor-intensive and more environmental-friendly nature. Denitrification is a microbial process that convert nitrate to nitrous oxide and nitrogen gas. It has been widely accepted that denitrification requires complete anoxic conditions until the famous aerobic denitrifier *Thiosphaera pantotropha* (*Paracoccus denitrificans*) was isolated (Robertson and Kuenen, 1983). Since then, the studies on aerobic denitrification have drawn more and more attentions. Aerobic denitrifying bacteria isolated from various environmental sites, such as canals, ponds, soils and activated sludge, have been reported (Lloyd, 1993; Wan et al., 2011; Song et al., 2011).

Recent work has also indicated that rhizo-bacteria can continuously degrade the pollutants while using nutrients such as carbohydrates, amino acids and organic acids from the plant root exudates and oxygen released from the root system (Kuiper et al., 2004). This “rhizosphere effect” is well known in terrestrial systems. Accelerated degradation of ammonium (Eriksson and Weisner, 1999) as well as organic chemicals including pesticides (Toyama et al., 2011) and chlorinated solvents (Anderson and Walton, 1995) has been reported in the rhizosphere or rhizoplane of terrestrial plants by certain plant-microbial

*Corresponding author. E-mail: wmshi@issas.ac.cn. Tel: +86 025 86881566. Fax: +86 25 86881000

associations.

Lemna minor is a species of *Lemnaceae* (duckweed family) which grows broadly in natural environment of still waters with the presence of N (Cheng et al., 2002). *L. minor* has been widely used as an assay plant for removing N from polluted water due to its rapid propagation. The removal of N by duckweed is probably through its rhizosphere microbes (Zhou et al., 2010). Meanwhile, abundant exudates are available around duckweed niches because all parts of root surfaces and ventral sides of fronds have the same opportunity to have contact with microbes. Therefore, Fan et al. (2012) proposed that *L. minor* was a promising tool for investigating the plant-microbial interactions. Although, the exact composition of duckweed rhizosphere denitrifiers and the relationship with their host plant is not well documented, we infer that there might be aerobic denitrifying bacteria in duckweed rhizosphere from their habitat environment and oxygen secretion characteristics.

The present work describes the identification of duckweed rhizosphere bacterium as well as the investigation of its aerobic denitrification characteristics. In addition, the role of the duckweed-rhizobacteria association in accelerating nitrate removal was elucidated. The final aim of this study was to develop strategies for the efficient and continuous removal of nitrate from polluted water with minimum impact on the environment.

MATERIALS AND METHODS

Medium

Denitrification medium (DM, g/l): sodium citrate 5, KNO_3 1.011, KH_2PO_4 1, K_2HPO_4 0.5, $Mg_2SO_4{\cdot}7H_2O$ 0.2, pH 7.2. Nitrite denitrification medium (NDM, g/l): sodium citrate 5, KNO_2 0.851, KH_2PO_4 1, K_2HPO_4 0.5, $Mg_2SO_4{\cdot}7H_2O$ 0.2, pH 7.2. Ammonium nitrification medium (ANM, g/l): sodium citrate 5, $(NH_4)_2SO_4$ 0.661, KH_2PO_4 1, K_2HPO_4 0.5, $Mg_2SO_4{\cdot}7H_2O$ 0.2, pH 7.2. Luria-Bertani medium (LB, g/l): tryptone 10, yeast extract 5, NaCl 5, pH 7.0. For preparation of LB plates, 1.5% (w/v) agar was added. Modified Steinberg solution (Tkalec et al., 1998) (mg/l): NH_4Cl 12.5, KH_2PO_4 1.756, $Mg_2SO_4{\cdot}7H_2O$ 100, $Ca(NO_3)_2{\cdot}4H_2O$ 98.9 , $Na_2EDTA{\cdot}2H_2O$ 1.5, $ZnSO_4{\cdot}7H_2O$ 0.18, $MnCl_2{\cdot}4H_2O$ 0.18, H_3BO_3 0.12, $NaMoO_4{\cdot}2H_2O$ 0.04, $FeCl_3{\cdot}6H_2O$ 0.76, pH 6.8.

Bacteria

Mature duckweed plants (*L. minor*) were collected from ponds of Tai Lake region and cultivated in modified Steinberg solution, the duckweed rhizo-bacterial strain RWX31 was isolated with 2 g/l KNO_3 as the sole nitrogen source (unpublished data).

Pseudomonas fluorescens ACCC01047 was obtained from the Agricultural Culture Collection of China (ACCC) and used as a control strain.

Identification of the isolated bacteria

The bacterium RWX31 was grown at 28°C on LB plates. Standard physiological and biochemical characteristics were examined according to the methods described in Bergey's Manual of Systematic Bacteriology (Garrity et al., 2004).

Total bacterial DNA was extracted with a genomic DNA extract kit (Tian Gen, China) following the manufacturer's instructions. The 16S r RNA gene was amplified from the extracted genomic DNA by PCR using universal primers 27F and 1492R (Johnson, 1994). PCR reaction mixture (50 µl) was composed of 1 µl of DNA template, 4 µl dNTP (2.5 mmol/l), 5 µl 10×PCR buffer, 0.5 µl Taq DNA polymerase, 1 µl of each primer (10 µmol/l) and sterile water to volume.

PCR recycling was carried out in a thermal cycler (Bio-Rad, USA) under the following conditions: an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40s and elongation at 72°C for 2 min; cycling was completed by a final extension at 72°C for 10 min. The PCR products were purified and sequenced by a commercial company (Genscript, China). BLAST searches were performed through the National Center for Biotechnological Information online service. The 16S rRNA gene sequences of related species were retrieved from the GenBank database to construct a phylogenetic tree with MEGA 4.0 software using the neighbor-joining method.

Cell growth and substrates utilization properties of strain RWX31

The cell growth curve and nitrogen removal profile of the strain RWX31 in 140 mg/l nitrogen from different N sources (nitrate, nitrite and ammonium) were performed. One hundred milliliters of DM, NDM and ANM inoculated with 1% preculture of strain RWX31 grown in LB medium was cultured at 30°C and 160 r/min. Specific growth rate (µ) was calculated from the growth curve, plotted as OD_{600} as a function of time.

N_2O production under aerobic incubation condition

Strain RWX31 was inoculated in 100 ml medium of 140 mg/l nitrogen (DM and NDM) in 250 ml flasks capped with butyl-rubber septa, then a small amount of laboratory glue was placed around the rim of the flasks to reduce any potential leaks to evaluate N_2O production. Before cultivation, the flasks were repeatedly evacuated and filled with mixed gas of 80% He and 20% O_2 (three cycles) for aerobic incubation through a membrane filter (pore size, 0.22 µm). The flasks were incubated at 30°C with shaking speed of 160 r/min, and the gas phase was monitored periodically by a gas chromatography (GC).

Orthogonal test to study the factors influencing the denitrification performance of strain RWX31

Orthogonal tests were designed to illustrate the effect of different factors on the aerobic denitrification performance of strain RWX31. The selected factors and their levels were detailed in Table 1. Differences were considered to be significant when $P < 0.05$. One milliliter cell suspension was inoculated into 100 ml DM. All the experiments were done in triplicates.

Continuous nitrate-N removal by duckweed/rhizosphere denitrifiers

The mature plants of duckweeds *L. minor* were washed three times for 10 min with sterilized water. For duckweed-bacteria association treatments, 1 ml of strains RWX31 or ACCC01047 cells from the late exponential growing phase were recovered by centrifugation (10,000 r/min, 10 min) and suspended in distilled water, then the

Table 1. $L_9(3^4)$ orthogonal test design for aerobic denitrification of strain RWX31.

Experiment numbers	Factor A (Temperature, °C)	Factor B (Initial pH)	Factor C (C/N)	Factor D (Shaking speed, r/min)
1	1(27)	1(6.8)	1(8)	1(100)
2	1(27)	2(7.2)	2(10)	2(150)
3	1(27)	3(7.6)	3(12)	3(200)
4	2(30)	1(6.8)	2(10)	3(200)
5	2(30)	2(7.2)	3(12)	1(100)
6	2(30)	3(7.6)	1(8)	2(150)
7	3(33)	1(6.8)	3(12)	2(150)
8	3(33)	2(7.2)	1(8)	3(200)
9	3(33)	3(7.6)	2(10)	1(100)

bacterial suspension and 30 fronds of duckweed were transferred to 100 ml of Steinberg medium containing 10 mmol NO_3-N. For the degradation test by strains RWX31 or ACCC01047 alone, 1 ml bacterial suspension was introduced to the above medium. For duckweed alone treatment, 30 fronds of duckweed were placed in the same medium. The cultivation was conducted statically at 23°C for 72 h at 16/8 h light/dark photoperiod. Subsamples of culture medium were collected at different times. After 48 h, 8 mmol NO_3-N was added to the bacteria alone and duckweed-bacteria association treatments to enable them to perform continuous NO_3-N removal (Yamaga et al., 2010). All treatments were replicated 3 times and the experiment was repeated 3 times to ensure reproducibility.

Analytical methods

The cell density was measured using a spectrophotometer (Bio-Rad 3000; USA) at an absorbance of 600 nm. Samples for chemical analysis were centrifuged (10,000 r/min, 10 min) and analyzed for NO_3-N, NO_2-N and NH_4-N following standard methods (The State Environmental Protection Administration of China, 2003). DO concentration was measured by a DO meter (Inesa, China). N_2O gas was measured using a gas chromatograph (column: Porapak Q, 3m×3mm) equipped with an electron capture detector (ECD). All results from batch tests were compared at the 95% confidence level ($p<0.05$) using Duncan's test (SPSS 13.0 for MS Windows).

RESULTS AND DISCUSSION

Isolation and identification of the strain RWX31

Rhizo-bacterium RWX31 was selected from 53 different denitrifying bacteria because it had the highest and the most denitrification efficiency of 81.3% from an initial 140 mg/l DM medium in 24 h incubation. The strain was rod-shaped, facultative aerobic, Gram-negative with good growth on sucrose, glucose, sorbitol and arginine. It had the following physiological properties: catalase-positive, oxidase-positive, urease-positive and gelatin protease-positive; methyl red (MR) test - negative, voges proskauer (VP) test - negative. The 16S rRNA gene was amplified and sequenced, and deposited in GenBank under accession numbers JN020938. BLAST searches revealed that strain RWX31 showed 99.7% similarity with *P. fluorescens*, and a phylogenetic tree was constructed with MEGA 4.0 software using the neighbor-joining method (Figure 1).

Growth and substrate utilization properties of strain RWX31 in batch culture

Aerobic denitrification capabilities of strain RWX31 were assessed in 140 mg/l N medium (DM, NDM and ANM). Growth curves of the isolates were established by measurement of the OD value at 600 (Figure 2a). The growth of the strain in all N sources resulted in sigmoidal curves, where three distinguished phases were found.

The bacterial growth was found to be directly affected by the availability of certain N sources. Ammonium-N in the medium resulted in higher OD_{600}, indicating higher levels of growth than on other N sources. Cell proliferation occurred after 6 h of incubation, and it reached its maximum growth in 20 h. However, 12 h lag phase was observed in DM and NDM. When grown in DM, strain RWX31 took 22 h to reach its maximum growth, whereas in NDM, the isolate grew the slowest and took 24 h or more to reach its maximum growth. The calculated maximum specific growth rates (μ_{max}) of the strain were 0.16, 0.11 and 0.17 h^{-1} in DM, NDM and ANM, respectively. Robertson et al. (1989) reported that μ_{max} of several bacteria was 0.21 to 0.41 h^{-1} when NO_3^- and O_2 were supplied. Wan et al. (2011) reported that μ_{max} of *Pseudomonas* sp.yy7 was 0.34 h^{-1} with NO_2^- as the sole nitrogen source. The μ_{max} determined by our study was lower than the bacteria reported in the literature. The difference may be due to the different components in the medium used.

Furthermore, as depicted in Figure 2b to d, an excellent nitrogen removal was achieved by all isolates in 24 h. In DM, a slow decrease in NO_3^- concentrations was observed in the first 15 h (Figure 2b), which can be attributed to aerobic assimilatory of nitrate. When biomass was forming in the system, NO_3^- concentration and total nitrogen (TN) concentration began to decline swiftly which was the denitrification process of converting nitrate into gas. At 24th h incubation, the remaining NO_3^- concentration was 10.4 mg/l. Most of the nitrate was

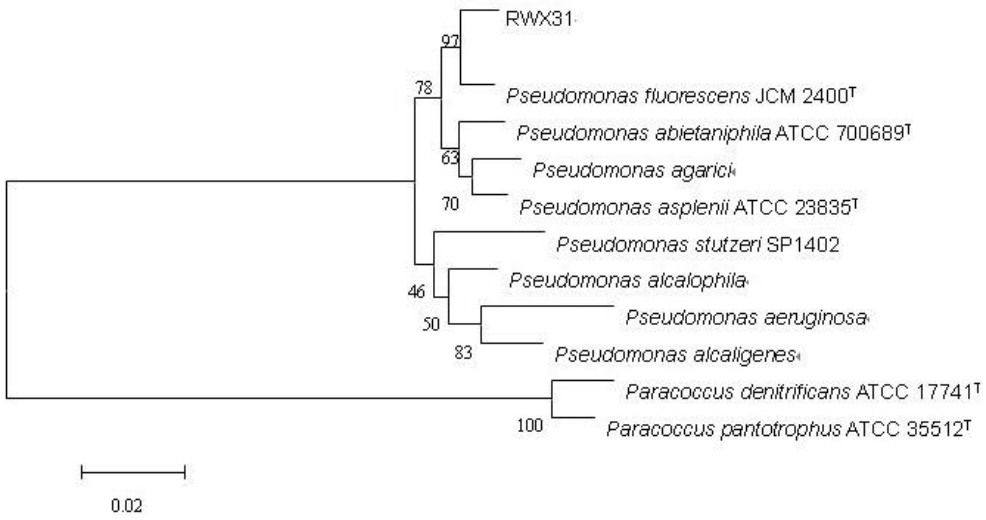

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequence of the four denitrifying bacteria and their related species. The scale bar shows 0.02 nucleotide substitutions per site.

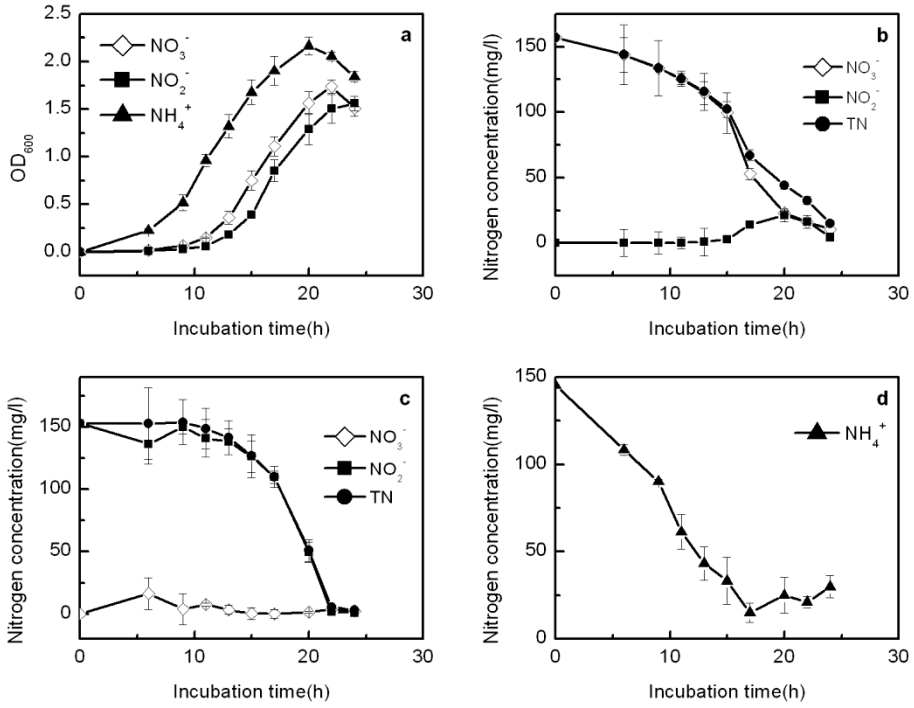

Figure 2. Bacterial growth curve and nitrogen concentration profile in denitrification medium. Bacterial growth (OD_{600}) (a) was conducted in DM (b), NDM (c) and ANM (d) at 30°C with shaking at 160 r/min for 24 h. Data points represent mean and standard deviation of triplicate samples.

successively reduced to nitrite, nitric oxide, nitrous oxide and nitrogen gas in the denitrification process. When nitrite is formed, the nitrite reductase will be activated and transform into reduced form, so the accumulation of nitrite in the medium is negligible during NO_3^- removal. When cultivated in NDM, strain RWX31 did not produce NO_3^- in the process of NO_2^- removal (Figure 2c), but its removal rate was much lower than that in DM. The slow transformation in NDM can be explained by the inhibitory effect of high NO_2^- concentration on bacterial growth and denitrification activity (Hunter, 2003). As a result of that, a distinct lag phase was found in the first 15 h, but NO_2^-

Table 2. Comparison of denitrification rates of strain RWX31 (this study) with other reported strains.

Bacteria	Initial nitrogen concentration (mg/l)	Denitrification rate (mg l^{-1} h^{-1})	Reference
Pseudomonas stutzeri SU2	332 (NO_3^-)	6.89	Su et al., 2001
P. stutzeri ASM-2-3	225.8 (NO_3^-)	45.01	Kariminiaae-Hamedaani et al., 2004
P. fluorescens Strain RWX31	140 (NO_3^-)	30.91	This study
Providencia rettgeri YL	300 (NH_4^+)	8.33	Taylor et al., 2009
Comamonas sp.GAD4	65 (NH_4^+)	5.33	Chen and Ni, 2010
Alcaligenes faecalis sp.NO.4	1200 (NH_4^+)	29	Joo et al., 2005
P. fluorescens Strain RWX31	140 (NH_4^+)	11.53	This study
Ps. sp.yy7	18 (NO_2^-)	0.76	Wan et al., 2011
Bacillus coagulans YX-6	10 (NO_2^-)	0.71	Song et al., 2011
P. fluorescens Strain RWX31	140 (NO_2^-)	24.28	This study

concentration decreased from 126.2 to 49.6 mg/l between 15 and 20 h incubation. After the bacterial rapid growth, a greater denitrification activity using nitrite as substrate was also revealed. Strain RWX31 removed the NO_2^- completely within 22 h, but no decrease in the concentrations was observed afterwards. In ANM, NH_4^+ was assimilated or converted continuously without a lag period. The final NH_4^+ concentration was as low as 30 mg/l, in addition there was no NO_3-N accumulation in the whole process which might be due to the continuous assimilation and transformation activity or the simultaneous nitrification and denitrification (SND).

The relationship between the strain RWX31 biomass and OD_{600} data followed the equation: Biomass (mg/ml) = $0.07205 \times OD_{600} + 0.0581$ (R^2=0.8291), where the biomass nitrogen content was calculated. According to the nitrogen balance of nitrogen removal by strain RWX31 incubated in DM, NDM and ANM under aerobic condition, a comparison of the initial and final TN mass in this system revealed that 19.1% of input nitrate-N, 19.3% of input nitrite-N and 21.8% of input ammonium-N was changed to biomass. Therefore, the nitrogen assimilation was very low because about 60% of TN disappeared from the cultural system, most probably through denitrification.

Table 2 outlines the aerobic denitrification rates reported by previous studies. The aerobic denitrification rate of RWX31 was much higher than those strains when nitrite was used as substrate. *Pseudomonas* sp. yy7 showed very poor growth when an initial NO_2-N concentration of 50 mg/l was applied (Wan et al., 2011), but strain RWX31 tolerated as much as 140 mg/l NO_2-N and performed an efficient denitrification, which made this strain a promising candidate in the treatment of wastewater with high nitrite concentration.

N_2O production under aerobic incubation condition

In order to verify the aerobic denitrification activity of strain RWX31, we measured the N_2O produced under aerobic condition. Time-dependent N_2O production of 140 mg/l nitrate or nitrite nitrogen by strain RWX31 was examined at 20% initial O_2 concentration. DO level was decreased as the cell concentration increased, so at the end of the experiment, DO concentration in the incubation system was measured (3.8 to 5.9 mg/l). Based on the DO level, the entire incubation process was considered aerobic. GC results showed that N_2O was produced on both NO_3^- and NO_2^- served as substrate (Table 3). It could aerobically denitrify nitrate or nitrite to form N_2O, and the latter was reported more in fungi than in bacteria (Hayatsu et al., 2008). During the first 14 h incubation, nitrite served as a better electron acceptor, and N_2O concentration was 27.20 ppm for NDM but only 0.658 ppm for DM. This may be due to the fact that nitrite is the reducing form in the denitrification process. But the accumulative N_2O concentration at the end of the incubation was 196.9 ppm in DM and 81.15 ppm in NDM, which suggests strain RWX31 contains most of the reducing steps, from nitrate to nitrous oxide, and preferred the first step (from nitrate to nitrite) by nitrate reductase (Nar).

Table 3. Concentrations of N_2O at different incubation time under aerobic conditions with NO_3^- or NO_2^- as sole nitrogen source.

Incubation time (h)	Accumulative N_2O concentration (ppm)	
	NO_3^- substrate	NO_2^- substrate
0	0.250±0.012	0.250±0.012
4	0.281±0.019	0.522±0.067
8	0.470±0.184	0.913±0.177
11	0.646±0.502	1.659±0.745
14	0.658±0.218	27.20±9.295
20	98.73±12.74	52.44±12.38
24	196.9±23.16	81.15±15.71

Incubation was conducted at 20% initial O_2 concentration and 30°C with shaking at 160 rpm. Data points represent mean of triplicate samples.

Denitrification is traditionally thought to be inhibited in the presence of O_2 as O_2 is preferred over NO_3^- as a terminal electron acceptor during respiration (Lloyd,1993), but Robertson and Kuenen (1984) found that *Thiosphaera pantotropha* could use both NO_3^- and O_2 as terminal electron acceptors for respiration. Later, Bell et al. (1990) found that this strain had a constitutive periplasmic nitrate reductase (Nap) which could operate under both anaerobic and aerobic conditions. The advantage of aerobic denitrification lies in a higher specific growth rate or a greater adaptability to fluctuating degrees of anaerobiosis when applied in the natural environments. Using aerobic denitrifying species to perform simultaneous nitrification/denitrification (SND) has been reported (Chen and Ni, 2010). Strain RWX31 can perform denitrification in aerobic conditions, which suggests that it is a potential candidate for SND application.

Orthogonal test for the aerobic denitrification of strain RWX31

The results of orthogonal test are tabulated in Table 4. Variance analysis (Table 4a) indicated that the significant values of factor A (temperature), B (pH), C (C/N), D (shaking speed) were 0.000, 0.001, 0.000, 0.321, respectively. Four factors had impact on the NO_3-N removal efficiency in the order of A>C>B>D, while factor A, C and B, namely temperature, pH and C/N ratio, were highly significant ($P < 0.001$). The optimal conditions for strain RWX31 to remove nitrate-N were temperature 27°C, C/N ratio of 8, pH 7.2 and shaking speed at 150 r/min, according to the result of estimated marginal means (Table 4b). In this optimal incubation condition, the NO_3-N removal efficiency was 94.3%.

Temperature played an important role in the denitrification reductase activity. The optimum temperature for denitrification reductase was 25 to 35°C, and the activities of denitrification reductase would be inhibited by too high or too low temperature (Song et al., 2011). In this experiment, the optimum denitrification temperature was also within the suggested range.

The optimum pH for denitrification was neutral or slight alkaline. Otherwise, the activity of denitrifying bacteria would be reduced when pH deviated from this optimum pH (Thomas et al., 1994). Denitrification efficiency of RWX31 reached the peak at pH 7.2. Acidic (pH < 6) or alkaline (pH > 7.5) condition was harmful to the growth of strain RWX31.

The C/N ratio is a measure of electron donor to acceptor ratio in biological denitrification. At an insufficient carbon concentration, the electron flow is too low to provide enough energy for cell growth, but denitrification efficiency shows a very limited increase under high C/N ratio due to the fact that their growth is also inhibited in an extremely high carbon concentration (Huang and Tseng, 2001). The results demonstrated that the denitrification of RWX31 increased with C/N ratio increase, and reached the maximum at C/N of 8.

Dissolved oxygen (DO) concentration is an important factor for aerobic denitrification. Studies have shown that DO concentration is strongly related to shaking speed (Zhang et al., 2012; Taylor et al., 2009). In a previous study, DO concentration was about 3.5 to 5.8 and 6.5 to 7.3 mg/l at shaking speed of 0 to 50 and 100 to 250 r/min, respectively. Aerobic condition for denitrification has been the subject of dissention since the O_2 concentration varies enormously among different organisms. From an environmental point of view, aerobic conditions correspond to those at detectable levels of air saturation (Robertson and Kuenen, 1984), but from the point of view of microbial respiration, aerobic conditions are those in which the organisms use O_2 as the terminal electron acceptor (Chen et al., 2003). Patureau et al. (2000) pointed out that denitrifying reductase and aerobic respiratory systems co-existed in aerobic denitrifiers, and the lack of NO_x^- or O_2 would reduce the bacterial growth rate and denitrification efficiency. From the results, shaking speed (DO concentration) had no significant impact on nitrate removal efficiency within the range tested (3.5 to 7.3 mg/l), but the maximum efficiency was reached when shaking speed was 150 r/min (DO

Table 4. Orthogonal tests of different incubation conditions with SPSS 13.0.

Source	Type III sum of squares	df	Mean square	F	Sig.
a. Tests of between-subjects effects					
Dependent variable: NO_3-N removal efficiency					
Corrected model	13090.35[a]	8	1636.29	50.95	0.000
Intercept	152382.42	1	152382.42	4.75×10^3	0.000
A (Temperature)	11465.15	2	5732.57	178.50	0.000
B (pH)	690.07	2	345.03	10.74	0.001
C (C/N)	857.31	2	428.66	13.35	0.000
D(Shaking speed)	77.83	2	38.91	1.21	0.321
Error	578.07	18	32.12		
Total	166050.85	27			
Corrected total	13668.42	26			

b. Estimated marginal means				
Level	A	B	C	D
1	95.79	73.79	79.98	72.72
2	82.59	81.88	67.22	76.36
3	47.00	69.71	78.17	76.29
Optimal level	27°C	7.2	8	150 r/min

Dependent variable: NO_3-N removal efficiency. Confidence interval: 95%. [a] $R^2 = 0.958$ (adjusted $R^2 = 0.939$).

concentration 6.6 to 7.2 mg/l).

Continuous stationary nitrate-N removal by duckweed/rhizosphere denitrifiers

Removal of NO_3-N by floating plant duckweed *L. minor* and different microbes was evaluated in a stationary cultivation condition. The control and duckweed alone treatments did not change the NO_3^-N concentration much in all the removal experiments (Figure 3) which suggested that the duckweed assimilation was not an important mechanism for NO_3^- removal. The duckweed/RWX31 combination performed continuous NO_3-N removal while ACCC01047 and duckweed/ACCC01047 decreased their removal rate after 8 mmol NO_3-N was added to the four treatments at 48 h. At the end of the experiment (72 h), the remaining NO_3-N in the medium was 78 and 25 mg/l for strain RWX31 and duckweed/RWX31 treatments, and 166 and 134 mg/l for strain ACCC01047 and duckweed/ACCC01047 treatments, respectively.

The main reason for the improved degradation of pollutants in the rhizosphere is presumably the exudate nutrients and root colonization (Kuiper et al., 2004). Plant roots have the ability to transport oxygen and secrete exudates such as sugars and amino acids (Anderson and Walton, 1995), which play important roles in a number of plant-microbial associations. Some exudates can influence the behavior and structure of bacterial communities in the rhizosphere (Kiely et al., 2006). In addition, plant roots provide niches for bacteria to colonize. *P. fluorescens* has the ability to produce extracellular polymeric substance (EPS), which is thought to be crucial for efficient attachment or colonization of plant roots (Allison et al., 1998). Strain RWX31 can secrete more EPS than strain ACCC01047 (data not shown), which may be helpful for its colonization on duckweed roots. Both possibilities may play different roles in continuous nitrate-N removal process by duckweed-rhizobacteria system. Yamaga et al. (2010) previously reported that the beneficial interaction between rhizobacteria and the duckweed *Spirodela polyrrhiza* might be the main contributor for continuous degradation of phenol. Toyama et al. (2011) also found that *Mycobacterium*-root exudate interactions accelerated biodegradation of pyrene and benzo[a]pyrene in *Phragmites australis* rhizosphere sediments. The accelerated removal of NO_3^- by duckweed/RWX31 association might be explained by the stimulatory effect of plant exudates, or strain RWX31's effective attachment to duckweed roots as compared to strain ACCC01047, which warrants further study.

These results verified the assumption that the stimulatory effect of root exudates on rhizobacteria increased the conversion of nitrate and also indicated that the use of strain RWX31 and duckweed association in a natural system is a promising strategy for nitrate reduction to minimize its release into the environment continuously. However, to confirm this finding, more studies, including isolation and purification of exudate material and mass spectroscopy, will be needed.

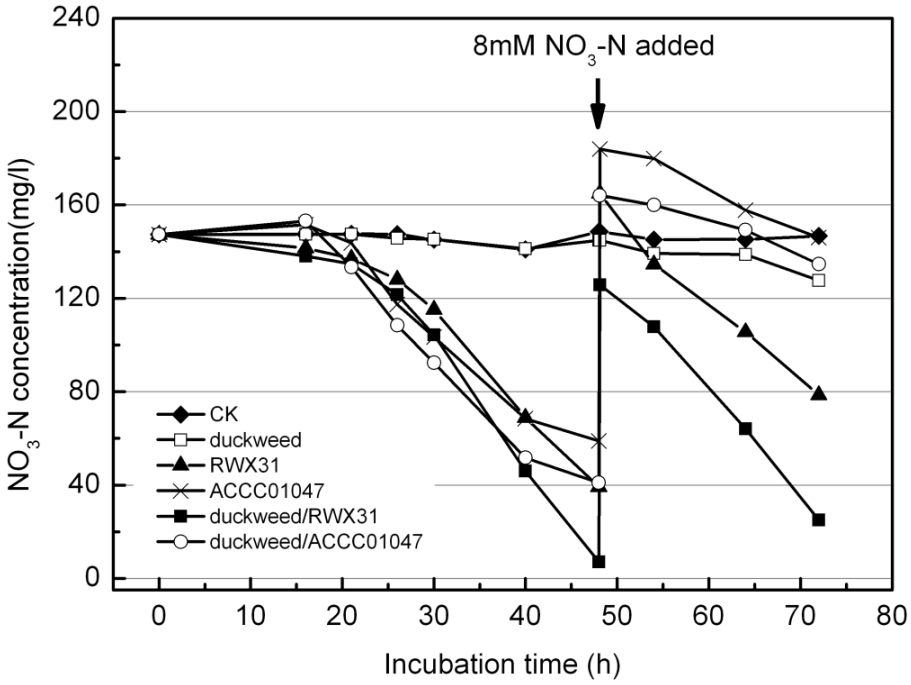

Figure 3. Stationary performance test of NO_3-N removal by duckweed and bacteria associations or bacteria alone treatments. Incubation at 23°C for 72 h at day/night of 16/8 h period. Control was set for the experiment without duckweed and bacteria. Each data point is the average of triplicate.

Meanwhile, it is important to underline that other denitrifying strains in duckweed rhizosphere might also play important roles in nitrogen removal from water body in addition to strain RWX31, and further investigation should be taken to search for higher efficient denitrifying bacteria in duckweed rhizosphere.

Conclusions

In this study, a duckweed *L. minor* rhizosphere bacterium RWX31 was evaluated for its aerobic denitrification potential. Strain RWX31 exhibited efficient aerobic denitrifying ability with maximum NO_3-N removal rate of 30.9 mg l^{-1} h^{-1}, NO_2-N removal rate of 24.3 mg l^{-1} h^{-1} and NH_4-N removal rate of 11.5 mg l^{-1} h^{-1}. Strain RWX31 showed excellent association with duckweed in NO_3-N removal process, probably through exudate nutrients stimulation and root colonization. The optimal conditions for aerobic denitrification of strain RWX31 were C/N 8, pH 7.2 and 27°C when nitrate was the substrate. However, shaking speed had no significant impact on nitrate removal efficiency within the range tested (dissolved oxygen concentration 3.5 to 7.3 mg/l). To our knowledge, strain RWX31 is the first rhizosphere aerobic denitrying bacterium identified from the duckweed *L. minor*, and it has a great potential in practical application of SND system.

ACKNOWLEDGEMENTS

We are grateful to the National Natural Science Foundation of China-Japan Science and Technology Agency (NSFC-JST) Major International Joint Research Project (No. 30821140542) and the Special Fund for Agro-scientific Research in the Public Interest (201003014-1) for financial support.

REFERENCES

Allison DG, Ruiz B, SanJose C, Jaspe A, Gilbert P (1998). Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. FEMS Microbiol. Lett. 167:179-184.

Anderson TA, Walton BT (1995). Comparative fate of [^{14}C] trichloroethylene in the root zone of plants from a former solvent disposal site. Environ. Toxicol. Chem. 14:2041-2047.

Bell LC, Richardson DJ, Ferguson SJ (1990). Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*: The periplasmic enzyme catalyzes the first step in aerobic denitrification. FEBS Lett. 265:85-87.

Bruning F, Kaneene JB (1993). The effects of nitrate, nitrite and N-nitroso compounds on human health: a review. Vet. Hum. Toxicol. 35:521-538.

Chen F, Xia Q, Ju LK (2003). Aerobic denitrification of *Pseudomonas aeruginosa* monitored by online NAD(P)H fluorescence. Appl. Environ. Microbiol. 69:6715-6722.

Chen Q, Ni J (2010). Heterotrophic nitrification–aerobic denitrification by novel isolated bacteria. J. Ind. Microbiol. Biotechnol. pp. 1-6.

Cheng J, Bergmann BA, Classen JJ, Stomp AM, Howard JW (2002).

Nutrient recovery from swine lagoon water by *Spirodela punctata*. Bioresour. Technol. 81:81-85.
Eriksson PG, Weisner SE (1999). An experimental study on effects of submersed macrophytes on nitrification and denitrification in ammonium-rich aquatic systems. Limnol. Oceanogr. 44:1993-1999.
Fan B, Borriss R, Bleiss W, Wu X (2012). Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 colonizes three types of plants in different patterns. J. Microbiol. 50:38-44.
Garrity GM, Bell JA, Lilburn TG (2004). Bergey's Manual of Systematic Bacteriology. 2th ed. The Williams & Wilkins Co., New York, Berlin, Heidelberg, p. 97.
Hayatsu M, Tago K, Saito M (2008). Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Sci. Plant Nutr. 54:33-45.
Huang HK, Tseng SK (2001). Nitrate reduction by *Citrobacter diversus* under aerobic environment. Appl. Microbiol. Biotechnol. 55:90-94.
Hunter WJ (2003). Accumulation of nitrite in denitrifying barriers when phosphate is limiting. J. Contam. Hydrol. 66:79-91.
Johnson JL (1994). Similarity analysis of rRNAs. In: Gerhardt P, Murray RGE, Wood WA, and Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C. pp. 683-700.
Joo HS, Hirai M, Shoda M (2005). Nitrification and denitrification in high-strength ammonium by *Alcaligenes faecalis*. Biotechnol. Lett. 27:773-778.
Kapoor A, Viraraghavan T (1997). Nitrate removal from drinking water – Review. J. Environ. Eng. 123:371-380.
Kariminiaae-Hamedaani HR, Kanda K, Kato F (2004). Denitrification activity of the bacterium *Pseudomonas* sp. ASM-2-3 isolated from the Ariake Sea tideland. J. Biosci. Bioeng. 97:39-44.
Kiely P, Haynes J, Higgins C, Franks A, Mark G, Morrissey J, O'Gara F (2006). Exploiting new systems-based strategies to elucidate plant-bacterial interactions in the rhizosphere. Microb. Ecol. 51:257-266.
Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ (2004). Rhizoremediation: A beneficial plant-microbe interaction. Mol. Plant Microbe Interact. 17:6-15.
Lloyd D (1993). Aerobic denitrification in soils and sediments: From fallacies to facts. Trends Ecol. Evol. 8:352-356.
Patureau D, Bernet N, Delgenès JP, Moletta R (2000). Effect of dissolved oxygen and carbon–nitrogen loads on denitrification by an aerobic consortium. Appl. Microbiol. Biotechnol. 54:535-542.
Robertson LA, Cornelisse R, Vos P, Hadioetomo R, Kuenen JG (1989). Aerobic denitrification in various heterotrophic nitrifiers. Antonie van Leeuwenhoek 56:289-299.
Robertson LA, Kuenen JG (1984). Aerobic denitrification - old wine in new bottle? Antonie van Leeuwenhoek 50:525-544.
Robertson LA, Kuenen JG (1983). *Thiosphaera pantotropha* gen. nov. sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacteria. J. Gen. Microbiol. 129:2847-2855.
Song ZF, An J, Fu GH, Yang XL (2011). Isolation and characterization of an aerobic denitrifying *Bacillus* sp. YX-6 from shrimp culture ponds. Aquaculture 319:188-193.
Su JJ, Liu BY, Liu CY (2001). Comparison of aerobic denitrification under high oxygen atmosphere by *Thiosphaera pantotropha* ATCC 35512 and *Pseudomonas stutzeri* SU2 newly isolated from the activated sludge of a piggery wastewater treatment system. J. Appl. Microbiol. 90:457-462.
Taylor SM, He Y, Zhao B, Huang J (2009). Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Providencia rettgeri* YL. J. Environ. Sci. 21:1336-1341.
The State Environmental Protection Administration of China (2003). Water quality-determination of nitrogen (nitrite)-spectrophotometric method. 1st ed, Standards press of China, Beijing.
Thomas KL, Lloyd D, Boddy L (1994). Effects of oxygen, pH and nitrate concentration on denitrification by *Pseudomonas* species. FEMS Microbiol. Lett. 118:181-186.
Tkalec M, Vidakovic-Cifrek Z, Regula I (1998). The effect of oil industry “high density brines” on duckweed *Lemna minor L*. Chemosphere 37:2703-2715.
Toyama T, Furukawa T, Maeda N, Inoue D, Sei K, Mori K, Kikuchi S, Ike M (2011). Accelerated biodegradation of pyrene and benzo[a]pyrene in the *Phragmites australis* rhizosphere by bacteria–root exudate interactions. Water Res. 45:1629-1638.
Wan CL, Yang X, Lee DJ, Du M, Wan F, Chen C (2011). Aerobic denitrification by novel isolated strain using NO_2-N as nitrogen source. Bioresour. Technol. 102:7244-7248.
Yamaga F, Washio K, Morikawa M (2010). Sustainable biodegradation of phenol by *Acinetobacter calcoaceticus* P23 isolated from the rhizosphere of duckweed *Lemna aoukikusa*. Environ. Sci. Technol. 44:6470-6474.
Zhang QL, Liu Y, Ai GM, Miao LL, Zheng HY, Liu ZP (2012). The characteristics of a novel heterotrophic nitrification–aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. Bioresour. Technol. 108:35-44.
Zhou YR, Yuan XY, Wang JR, Bao TF, Shi WM (2010). Screening of duckweed (*Lemnaceae*) species for efficient removal of water-body's nitrogen in the Tai Lake region and preliminary study on nitrogen removal mechanism. Soils 42:390-397 (in Chinese).