RSC Advances

Heterotrophic nitrification and aerobic denitrification by a novel groundwater origin cold-adapted bacterium at low temperatures

Journal:	*RSC Advances*
Manuscript ID:	RA-ART-10-2014-013141.R2
Article Type:	Paper
Date Submitted by the Author:	09-Dec-2014
Complete List of Authors:	Qu, Dan; Jilin University, Wang, Cong; Jilin University, Wang, Yangfang; Jilin Agriculture University, Zhou, Rui; Jilin University, Ren, Hejun; Jilin University,

Journal Name

RSCPublishing

ARTICLE

Heterotrophic nitrification and aerobic denitrification by a novel groundwater origin cold-adapted bacterium at low temperatures

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

Dan Qu[a], Cong Wang[a], Yanfang Wang[b], Rui Zhou[a], Hejun Ren[a]*

The cold-adapted bacterium *Pseudomonas migulae* (AN-1), which has *nirS* and *nosZ* genes, was isolated from an aniline-contaminated groundwater system. AN-1 could remove ammonium, nitrate, and nitrite at 1.56, 1.57, and 0.69 mg L^{-1} h^{-1}, respectively, at 10 °C under aerobic conditions. Nitrate removal followed the Monod kinetics under a non-competitive substrate inhibition (k = 270.27 mg NO_3^--N g X^{-1} h^{-1}, K_S = 43.65 mg L^{-1}, and K_{SI} = 185 mg L^{-1}). The putative gaseous denitrification pathway in AN-1 was via nitrite to N_2 using nitrogen balance assays. Moreover, the pH, dissolved oxygen, and NO_3^--N concentration were used as independent variables for the optimization of nitrate removal by AN-1 at 10 °C, and a statistically significant (R^2 = 0.9840, p < 0.0001) quadratic polynomial mathematical model was found. This study highlights the potential of AN-1 for nitrogen removal from wastewaters at low temperatures.

Introduction

In the past decades, nitrogen removal from wastewaters to prevent hazardous environmental effects has become a major concern of wastewater treatment industries. Biological treatment is widely adopted because it is easier to apply as well as has higher efficiency and lower maintenance cost compared with other approaches.[1, 2]

Conventional biological nitrogen removal consists of two processes, namely, nitrification through the oxidation of ammonia to nitrate via nitrite by autotrophic nitrifiers under aerobic conditions and denitrification through the heterotrophic conversion of nitrate to nitrogen gas by anoxic denitrifiers under anaerobic conditions.[3, 4] In this system, autotrophic nitrifiers grow slowly and are sensitive to organic matter. The growth of nitrifiers depends on oxygen, which is toxic to denitrifiers.[5] Thus, nitrification and denitrification should be separated in treatment systems.[6] However, the separation of these processes is time consuming and requires a large area, which increase wastewater-treatment costs.

Compared with traditional nitrogen removal, aerobic denitrification has interesting advantages. First, aerobic denitrifiers have high growth rates and high oxygen tolerance. Thus, nitrification and denitrification can simultaneously occur in one reactor, thereby reducing the costs associated with maintaining an anoxic tank and specific reactor volumes.[7] Moreover, aerobic denitrifiers can be easily controlled during operation and balance the pH changes in the reactor, thus avoiding acidification caused by nitrification.[8] The discovery and isolation of aerobic denitrifying bacteria provide a promising approach for the establishment of simultaneous nitrification–denitrification (SND) reactor and facilitate the denitrification process. Most of the reported aerobic denitrifiers are mesophilic. These microorganisms cannot efficiently perform denitrification at 10 °C (typical wastewater temperature during wintertime of northern China) or lower. Thus, for bioaugmentation using heterotrophic nitrifying-aerobic denitrifying microorganisms from temperate climates, microorganisms must be adapted to cold and capable of efficient denitrification.

In recent years, microorganisms capable of aerobic denitrification at low temperatures have attracted increasing attention. The genes of nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*) are used as functional markers to identify denitrifying bacteria and analyze their corresponding denitrifying pathways.[9, 10] Only a few bacteria that are both heterotrophic nitrifiers and aerobic denitrifiers with excellent tolerance to low temperatures have been isolated and characterized. Among these bacteria are *Acinetobacter* sp. HA2,[11] *Microbacterium* sp. strain SFA13,[12] and *Acinetobacter* sp. Y16.[13] Hitherto, the mechanism of biological nitrogen removal at low temperatures is still difficult to generalize because of the limited number of tested species. Further investigation on a wider range of psychrophilic species is necessary. In addition to the aforementioned denitrifiers, which are obtained from wastewater treatment systems or surface water, rare groundwater-origin aerobic denitrifiers are also reported.

In this study, the strain AN-1 of cold-adapted aerobic denitrifying bacterium was first isolated from an aniline-contaminated groundwater system. Nitrogen removal and metabolic mechanisms of AN-1 were evaluated at 10 °C under aerobic conditions. The kinetics of nitrate removal was further investigated,

and the key parameters influencing nitrate removal were optimized by response surface methodology (RSM). This cold-adapted and aerobic-denitrifying bacterium may play an important role in the nitrogen cycle in groundwater environment at low temperatures and may be an alternate microbial resource for nitrogen removal treatment of wastewater at low temperatures.

Experimental

Media and screening of aerobic denitrifier

Groundwater samples for the isolation of aerobic denitrifiers were obtained from an aniline-contaminated subsurface aquifer situated at Jilin City, China. The aquifer has been contaminated with aniline for more than 8 years and much of the contaminant plume contains little oxygen and relatively high concentrations of nitrate (> 20 mg L^{-1}).

The groundwater samples (5 mL) were transferred into 250-mL Erlenmeyer flasks containing 50 mL sterile liquid broth (LB) medium and cultured at 20 °C in a shaking incubator (120 rev/min) for 48 h. The enriched cultures were sampled using gradient dilution. The diluent was streaked onto modified bromothymol blue (BTB) medium plates[14] and incubated at 10 °C until visible blue colonies (positive result) were formed. The blue colonies were transferred to a denitrification medium (DM) containing KNO_3 (0.72 g L^{-1}) as sole nitrogen source and incubated at 10 °C with shaking at a speed of 120 rev/min for 72 h. Prominently growing single colonies were harvested and cultured in 100 mL of DM containing KNO_2 (0.6 g L^{-1}) as sole nitrogen source for another 48 h under the same aerobic conditions. The predominant cold-adapted isolates with the highest NO_2^- removal efficiency were then selected.

The BTB medium with pH 7.0 to 7.3 comprised the following: 1.0 g L^{-1} KNO_3, 0.1 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $FeCl_2$, 0.2 g L^{-1} $CaCl_2{\cdot}2H_2O$, 1.0 g L^{-1} $MgSO_4{\cdot}7H_2O$, 8.5 g L^{-1} sodium citrate, 5 mL of 1% BTB in ethanol, and 2% agar. The components of trace elements in the solution at pH 7.0 were as follows: 1.0 g L^{-1} EDTA, 0.2 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.1 g L^{-1} $CaCl_2{\cdot}2H_2O$, 0.5 g L^{-1} $FeSO_4{\cdot}7H_2O$, 0.02 g L^{-1} $NaMnO_4{\cdot}2H_2O$, 0.02 g L^{-1} $CuSO_4{\cdot}5H_2O$, 0.04 g L^{-1} $CoCl_2{\cdot}6H_2O$, and 0.01 g L^{-1} $MnCl_2{\cdot}2H_2O$. The DM at pH 7.0 contains the following: 2.75 g L^{-1} glucose, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, 1.0 g L^{-1} KH_2PO_4, 3.8 g L^{-1} $Na_2HPO_4{\cdot}12H_2O$, 3.0 g L^{-1} KCl, and 1 mL of trace element solution. All the chemical reagents used were of analytical grade.

Identification of the isolates

Total DNA was prepared using a genomic DNA extraction kit (Sangong, China), following the manufacturer's instructions. The genes encoding 16S rRNA were amplified from the extracted genomic DNA using PCR with universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GATTACCTT GTTACGACTT).[15] The 1.5 kb PCR products were purified using Agarose Gel DNA Purification Kit (Takara, Dalian, China), cloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced by Sangon Co. Ltd. (Shanghai, China). The sequences were searched against GenBank database (National Center for Biotechnology Information, NCBI) using BLAST. The closest matching sequences were obtained, and a phylogenetic tree was constructed using MEGA 5.2.1 by applying the neighbor-joining method and maximum-likelihood analysis on the various data sets.

Amplification of the denitrification genes

Fragments of the *nirS* and *nosZ* genes were amplified using primer pairs *nirS*-F/*nirS*-R for *nirS* and *nosZ*-F/*nosZ*-R for *nosZ* developed by Rosch et al.[16] The primers pairs were *nirS*-F: 5′-CAC GGY GTB CTG CGC AAG GGC GC-3′, *nirS*-R: 5′-CGC CAC GCG CGG YTC SGG GTG GTA-3′, *nosZ*-F: 5′-CGY TGT TCM TCG ACA GCC AG-3′, and *nosZ*-R: 5′-CAT GTG CAG NGC RTG GCA GAA-3′. Takara Taq hot-start polymerase (Takara, China) was used for the PCR reaction. Negative controls without DNA template were performed simultaneously. The PCR products were separated by 1% agarose gel electrophoresis and stained by SYBR Safe DNA gel stain (Molecular Probes, USA). Bands were visualized by UV excitation.

Assessment of nitrogen removal performance

The strains were pre-cultured in BTB medium for 48 h at 10 °C and 120 rev/min to determine the nitrogen removal performance of the isolates. The bacterial suspension was centrifuged for 10 min at 4 °C and 8000 × g. The pellets were washed thrice with sterilized water to purify the bacterial suspension. 1 mL of preculture (optical density (OD_{600}) = 1.0) was inoculated into 100 mL of DM containing 97.48 mg L^{-1} NH_4^+-N, 92.75 mg L^{-1} NO_3^--N, and 96.18 mg L^{-1} NO_2^--N, respectively. Sample tests were performed in a rotary shaker at 10 °C and 120 rev/min to allow better gas exchange. The culture was periodically sampled to determine cell OD_{600} and centrifuged to obtain supernatants for the determination of ammonium, nitrite, and nitrate. All tests were performed in triplicate. Flasks without inoculation served as sterility controls.

Investigation of heterotrophic nitrification and aerobic denitrification capability

A total of 100 mL of DM containing 102 mg L^{-1} NH_4^+-N was placed in triplicate 250 mL glass head-space bottles and inoculated with 1 mL of preculture. These bottles were then fully aerated with pure oxygen gas and tightly sealed with rubber septa. Tests were then performed in a rotary shaker at 10 °C and 120 rev/min. Gas samples (20 μL) were periodically extracted using a 50 μL air-tight glass syringe to detect N_2, N_2O, and O_2. Cultures were also sampled to determine the concentration of ammonium, hydroxylamine, nitrite, nitrate, and intracellular nitrogen.

Measurements of nitrate removal kinetics with batch experiments

Batch experiments were performed in DM containing different initial NO_3^--N concentrations (9.33, 20.50, 37.03, 46.25, 56.79, 68.39, 76.25, 85.31, and 92.76 mg L^{-1}) and incubated at 10 °C on a rotating orbital shaker at 120 rev/min. Control experiments were performed by incubating the DM with NO_3^--N without an inoculum. Each experiment was performed in triplicates. Samples were taken at regular time intervals during the experiments to determine biomass production and nitrate consumption.

RSM for the optimization of nitrate removal conditions

RSM was used to investigate the effects of initial NO_3^--N, DO concentration, and pH on the overall removal efficiency of nitrate by AN-1 at 10 °C. A three-level (−1, 0, 1) Box–Behnken[17] factorial design with 17 experiments was used to evaluate the quadratic effects and two-way interactions among the three independent variables. Nitrate removal percentages of all the experimental tests were assessed in triplicates after a 36-h incubation. Results were analyzed using Design-Expert 8.0 software.

A second-order polynomial equation was fitted to correlate the relationship between variables and response to predict the optimal point. Nitrate removal efficiency was analyzed by multiple regression through the least-squares method to fit Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, k is the number of factor variables, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

Analytical methods

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) using a spectrophotometer. Ammonia, nitrite, and nitrate were determined according to the standard methods[18] of Nessler's reagent, N-(1-naphthalene)-diaminoethane, and diphenylamine spectrophotometry, respectively. Hydroxylamine was analyzed using the method described by Frear and Burrell.[19] Intracellular nitrogen content was determined according to Yao et al.[11] N_2O was assayed by gas chromatography (6890 N; Agilent, USA) using an electron capture detector according to Zhao et al.[20] N_2 and O_2 were determined by gas chromatography (6890 N; Agilent, USA) using a thermal conductivity detector, a HP-Plot 5A molecular sieve column (30 m × 0.32 mm × 25 μm), and He carrier gas at 10 mL min^{-1} flow rate. The column, injector, and detector temperatures were 50, 150, and 200 °C, respectively. DO concentration and pH of the suspensions were determined using DO and pH meters, respectively.

Results and Discussion

Isolation and phylogenetic analysis of the aerobic denitrifying bacterium AN-1

In the selective plate containing BTB, 24 strains formed blue colonies. Among these colonies, a strain designated as AN-1, which exhibits the highest nitrite removal efficiency at 10 °C after 48 h, was selected for further characterization. The strain was strictly heterotrophic and aerobic. The colonies were white and circular, with semitransparent, slabby, wet, and smooth surfaces on LB plates. The complete 16S rDNA sequences (1516 bp, accession number: KF857261) of AN-1 was determined, and a similarity search against GenBank indicated that AN-1 is 99% identical to the genus *Pseudomonas*. NJ phylogenetic tree was constructed (Fig. 1). The tree clearly showed that AN-1 was closest to *Pseudomonas migulae* (accession number: AY047218.1) based on evolutionary relationship along with the morphological and biochemical characteristics. Therefore, AN-1 is proposed to be *P. migulae*. To our knowledge, despite the numerous studies on the SND capability of the members of the genus *Pseudomonas, P. migulae* species has not been reported to exhibit aerobic denitrifying or heterotrophic nitrifying ability.

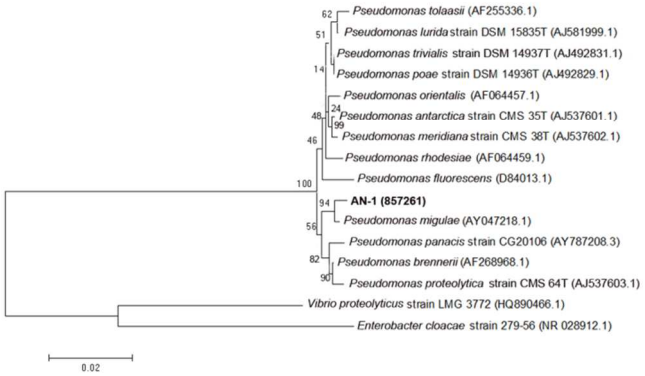

Fig. 1. NJ phylogenetic tree of 16S rDNA gene sequence of strain AN-1 (shown in bold face) and most closely related species. The GenBank accession numbers for the corresponding sequences are given in parenthesis after the strain name.

Denitrification genes

Nitrite-reductase genes are closely related to the denitrification properties of bacteria. They are often used as a functional markers to identify aerobic denitrifying bacteria. In this study, 702 and 707 bp of *nirS* and *nosZ* gene fragments, respectively, were successfully amplified from the strain AN-1 using primers designed to amplify about 700 bp of the fragment from the bacteria (Fig. 2). PCR amplification results suggest the presence of the two key enzymes, namely, NIR and NOS. NOS is involved in the aerobic conversion of nitrate to nitrite, and NIR is responsible for nitrite reduction in aerobic denitrification.[21] Therefore, the potential occurrence of heterotrophic nitrification coupled with aerobic denitrification in AN-1 can be reasonably considered.

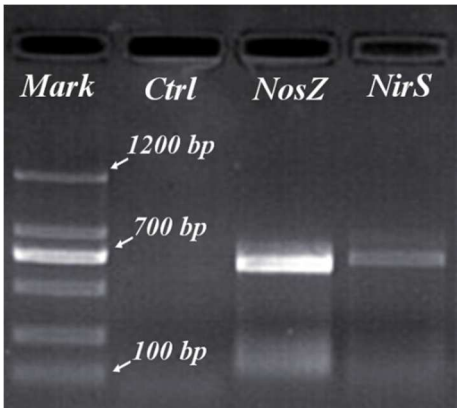

Fig. 2. Amplification profiles of *nirS* and *nosZ* genes from *P. migulae* AN-1.

Nitrogen removal performance of AN-1 at low temperatures

The ability of heterotrophic organisms to utilize ammonium is generally associated with aerobic denitrification. Fig. 3a illustrates the cell growth and removal of ammonium by *P. migulae* AN-1 at 10 °C in shaken cultures. Ammonium (97.48 ± 0.23 mg L^{-1} initial NH_4^+-N) was completely removed within approximately 72 h. Simultaneously, cell OD_{600} increased from 0.01 to 2.0. Moreover, the average specific $NH4^+$-N removal rate of the strain was

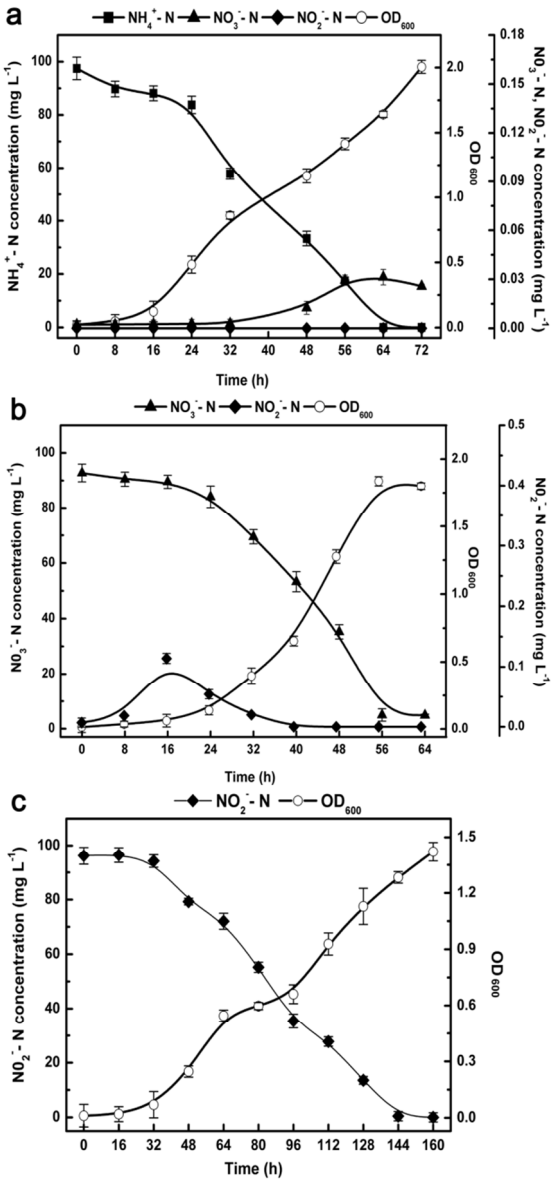

Fig. 3. Growth of *P. migulae* AN-1 and changes in nitrogen compound at 10 °C. (a) ammonium (b) nitrate and (c) nitrite.

calculated at 1.56 mg L^{-1} h^{-1}. This value is similar to the 1.48 mg L^{-1} h^{-1} removal rate obtained from *Pseudomonas stutzeri* YZN-001 under the same temperature of 10 °C.[22] Results indicated that heterotrophic nitrification by AN-1 was efficient at 10 °C with relatively high NH_4^+-N removal at low temperatures.

NO_3^--N and NO_2^--N were used as nitrogen sources in the DM to clarify the denitrifying ability of *P. migulae*. Fig. 3b shows the growth and nitrate reduction of AN-1 at 10 °C under aerobic conditions. A significant decrease in NO_3^--N by AN-1 was observed. During the first 16 h, the growth of AN-1 slowly increased, but the removal of NO_3^--N was slow. AN-1 then entered the rapid growth stage (16 h to 64 h), during which NO_3^--N was reduced from 92.75 mg L^{-1} to 4.9 mg L^{-1} and the NO_3^--N removal ratio was 1.57 mg L^{-1} NO_3^--N h^{-1}. For nitrite reduction, approximately 30% of the NO_2^--N with initial concentration 96.18 mg L^{-1} was removed after 64 h (Fig. 3c). At 166 h of cultivation, NO_2^--N was undetectable, which was well correlated with the rate of cell growth. The NO_2^--N removal rate was 0.69 mg L^{-1} NO_2^--N h^{-1} at 10 °C. A similar result was obtained in previous studies of *Pseudomonas fluorescens*.[23] The observed denitrification rate was 1.0 mg L^{-1} NO_3^--N h^{-1} at 10 °C. Kim et al.[24] reported that pure cultures of *Janthinobacterium lividum* strain 47 remove nitrate at low temperatures (10 °C) with a denitrification rate of 2.38 mg L^{-1} NO_3^--N h^{-1}. However, the ability of *J. lividum* strain 47 to grow and remove nitrite at low temperatures (10 °C) has not been described. Results showed that both nitrite and nitrate could be utilized by strain AN-1 at low temperatures, which suggests the existence of aerobic denitrification from nitrite or nitrate to nitrogenous gas in AN-1. Moreover, higher growth rate and cell yield were obtained with nitrate as nitrogen source compared with nitrite. This result may be attributed to the inhibitory effect of relatively high nitrite concentration (96.18 mg L^{-1}) on strain AN-1. The phenomena are also reported in *Pseudomonas* sp. yy7,[25] which grows poorly at an initial nitrite concentration of 50 mg L^{-1}.

Denitrification of gaseous nitrogen products and nitrogen balance

Gases were detected during the heterotrophic nitrification-aerobic denitrification process to understand the mechanism involved in the nitrogen removal of AN-1 at low temperatures. Oxygen was continuously detected during the whole process by gas chromatography qualitative analysis, indicating that aerobic conditions were maintained throughout the experiment (data not shown). Meanwhile, an increase in N_2 was observed, but N_2O, a greenhouse gas responsible for the depletion of ozone layer, was not detected (Fig. 4). The production of N_2 further confirmed the occurrence of heterotrophic nitrification and aerobic denitrification during the removal of ammonium by AN-1. Many known denitrifiers convert ammonium to denitrification products, which are mainly N_2O or equivalent amounts of N_2 and N_2O under aerobic conditions.[26] Therefore, AN-1 predominantly generates N_2 rather than N_2O, which could be valuable in actual wastewater treatments.

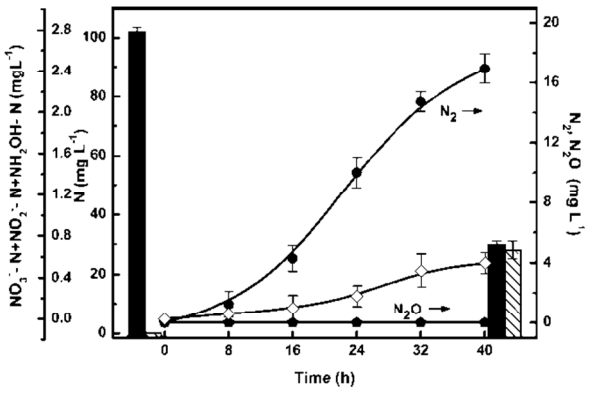

Fig. 4. Gas production during the heterotrophic nitrification and aerobic denitrification process. Symbols: closed circles, N_2; closed regular pentagon, N_2O; open diamonds NH_4OH-N + NO_3^--N + NO_2^--N; closed columns, NH_4^+-N; columns with rightward streak, intracellular nitrogen.

Table 1 Nitrogen balance during the heterotrophic nitrification-aerobic denitrification by AN-1 at 10 °C.

Initial TN (mg L^{-1})	Final N formations (mg L^{-1})						N lost [a] (%)
	NH_4^+-N	NO_2^--N	NO_3^--N	NH_4OH-N	Intracellular nitrogen	Nitrogen gas	
102.00	30.10	5.43	0	0	29.45	16.94	4.73

[a] % N lost = 100 × (initial TN - final N formations) / initial TN.

Furthermore, 71.90 mg L^{-1} (70.49% of initial TN 102 mg L^{-1}) of nitrogen in the medium disappeared after 40 h of inoculation by strain AN-1 through nitrogen balance analysis (Table 1). Minor intermediates (nitrites; 5.32% of initial TN) also were detected. The amount of N_2 measured was 16.94 mg L^{-1} (33.22% of initial TN). About 4.73% of nitrogen source could not be accounted for, which could be due to measurement errors caused by using different methods.[11, 20] Determination of intracellular nitrogen via assimilation revealed that 28.87% of nitrogen removed was converted to biomass. In other heterotrophic nitrification-aerobic denitrification bacteria under moderate temperature (28 °C to 30 °C), sequestration was 24.8 % for *Bacillus* strains,[1] 52.1% for *A. calcoaceticus* HNR,[27] and for 49.7% for *Paracoccus versutus* LYM.[28] These observations indicated that assimilation significantly contributes to heterotrophic nitrogen removal. As a consequence, the ammonium removal pathway in AN-1 may be proposed to come from two dominant processes. One is via assimilation into intracellular material, and the other is via nitrite, that is, $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$, as reported in several previous studies.[11, 29]

Effect of initial nitrate concentration and kinetics analysis

Co-respiration is generally speculated to be an important mechanism of denitrification in aerobic conditions. It involves the simultaneous use of oxygen and nitrate as electron acceptors.[30] Therefore, the concentration of nitrate in the system could greatly influence the overall performance of nitrogen removal in the bacteria. Fig. 5a shows time-course variation in NO_3^--N concentration for different initial NO_3^--N contents in the DM using AN-1 at 10 °C. *P. migulae* AN-1 could reduce NO_3^--N to low concentrations, and high initial concentrations of NO_3^--N took more time to be removed completely. For instance, 9.33 mg L^{-1} of NO_3^--N took about 32 h to remove, but 92.76 mg L^{-1} of NO_3^--N took 88 h. The results also revealed that the time taken by the *P. migulae* AN-1 to remove the NO_3^--N at low temperatures depends on its initial concentration.

Fig. 5b shows that the highest initial specific denitrification rate was observed at the initial concentration of 68.39 mg L^{-1}, and the curve of the initial specific denitrification rate could be divided into two parts. When the initial concentration of NO_3^--N was below 68.39 mg L^{-1}, the initial specific denitrification rate gradually increased as the NO_3^--N concentration decreased. By contrast, the initial specific denitrification rate apparently declined as the NO_3^--N concentration increased more than 68.39 mg L^{-1}. Thus, concentrations > 68.39 mg L^{-1} were inhibitory.

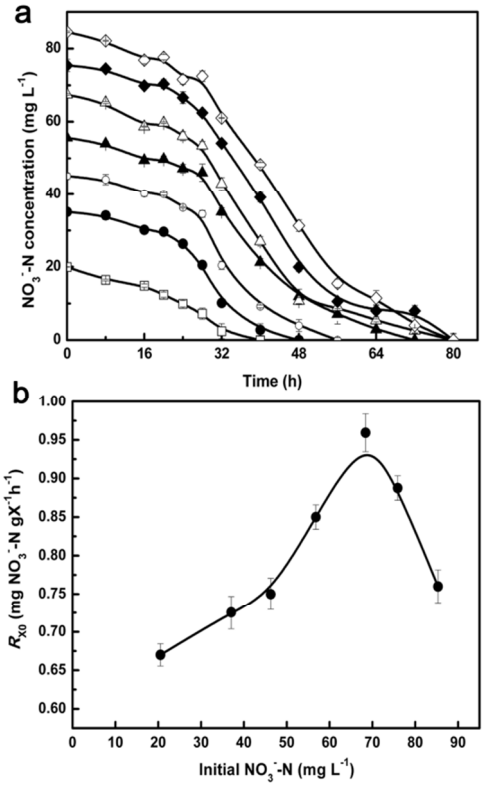

Fig. 5. Time course of variation of nitrate for *P.migulae* AN-1 at different initial nitrate concentrations (a) and variation of initial specific rates of nitrate removal (R_{X0}) with the initial nitrate concentration (b). Temperature was 10 °C. Error bars mean ± SD of three replicates.

For the inhibitory effect of nitrate on biodenitrification, the following non-competitive substrate inhibition kinetics was fit for nitrate removal by AN-1 based on the initial rates.

$$R_{S0} = \frac{kX_0S_0}{K_S + S_0}\frac{K_{SI}}{K_{SI} + S_0} = \frac{kX_0}{(1 + K_S / S_0)(1 + S_0 / K_{SI})} \quad (2)$$

$$R_{X0} = \frac{R_{S0}}{X_0} = \frac{kS_0}{K_S + S_0}\frac{K_{SI}}{K_{SI} + S_0} \quad (3)$$

These parameters were described by Pamukoglu and Kargi,[31] and the initial biomass concentration X_0 was 0.608 g L^{-1}. The first term in Eq. (2) represents the rate of nitrate removed by the bacteria in the form of Monod rate expression. Eq. (3) represents the non-competitive substrate inhibition by nitrate. At low initial concentrations of NO_3^--N (< 68.39 mg L^{-1}), the inhibition term could be neglected (S_0 « K_{SI}). Thus, Eqs. (2) and (3) (in double reciprocal form) can be written as

$$R_{S0} = \frac{kX_0S_0}{K_S + S_0} = \frac{R_{m0}S_0}{K_S + S_0} \quad (4)$$

$$\frac{1}{R_{X0}} = \frac{1}{k} + \frac{K_S}{k}\frac{1}{S_0} \quad (5)$$

where R_{m0} is the maximum initial rate of nitrate removal (mg L^{-1} NO_3^--N h^{-1}).

Experimental data were plotted in the form of $1/R_{x0}$ versus $1/S_0$ for NO_3^--N < 68.39 mg L^{-1}. From the slope and intercept of the best-fit line, the following values were found:

k = 270.27 mg NO_3^--N g X^{-1} h^{-1} and K_S = 43.65 mg L^{-1}
(R^2 = 0.9962)

For high initial concentrations of NO_3^--N (> 68.39 mg L^{-1}), the first term in Eq. (2) can be neglected ($S \gg K_S$) and the inhibition term becomes the rate-determining factor. Therefore, Eqs. (2) and (3) (in double reciprocal form) can be written as

$$R_{S0} == kX_0\frac{K_{SI}}{K_{SI} + S_0} \quad (6)$$

$$\frac{1}{R_{X0}} = \frac{1}{k} + \frac{S_0}{kK_{SI}} \quad (7)$$

Experimental data were plotted in the form of $1/R_{x0}$ versus $1/S_0$ for NO_3^--N > 68.39 mg L^{-1}. Therefore, the rate in Eq. (2) takes the following form:

$$R_{S0} = \frac{270.27X_0}{(1 + 43.65/S_0)(1 + S_0/185)} \quad (8)$$

$$R_{X0} = \frac{R_{S0}}{X_0} = \frac{270.27S_0}{43.65 + S_0}\frac{185}{185 + S_0} \quad (9)$$

From the slope [$1/(kK_{SI})$] and the intercept ($1/k$) of the best-fit line, the following values were found:

k = 270.27 mg NO_3^--N g X^{-1} h^{-1} and K_{SI} = 185 mg L^{-1}
(R^2 = 0.9911)

Aerobic denitrification is known to be catalyzed by constitutive nitrifying and denitrifying enzymes, activities of which are most sensitive to variations in temperature and oxygen. Nitrate, along with the other intermediates of the pathway, can also control the expression of the individual enzymes.[32] Several studies demonstrated that adding NO_3^- either initiates or increases the production of nitrate reductase; high NO_3^- concentrations can decrease the permeability of the cell membrane to NO_3^-, which can affect its transport into the cell.[33, 34] In the current study, experiments were conducted to investigate the denitrifying kinetics of AN-1 using NO_3^- as substrate under aerobic conditions at 10 °C. Although a combination of effects of low temperatures and NO_3^- may expand the incapacity of AN-1 to denitrify, the results indicate that AN-1 grew well with high concentrations of NO_3^--N (92.76 mg L^{-1}). This finding suggests that AN-1 could also exhibit efficient aerobic denitrification when high concentrations of NO_3^--N were used as substrate at low temperatures. The rate decreased to one-half of the maximum level when NO_3^--N was 185 mg L^{-1}, which indicates that the AN-1 was less sensitive to substrate inhibition and had a larger K_{SI} value for NO_3^--N.

Optimization of nitrate removal conditions by strain AN-1

All the aforementioned results showed that *P. migulae* AN-1 carried out SND under aerobic conditions at 10 °C and exhibited application potential for nitrogen removal from wastewater. However, the operation conditions must be further optimized, particularly for large-scale applications. RSM is a time-saving and effective approach for developing, improving, and optimizing a process. From single-factor experiments, the three main variable factors for finding the optimum values in nitrate removal were decided. An experimental matrix for Box–Behnken design and the response of dependent variable for nitrate removal are presented in Table S1. A quadratic polynomial function was fitted to the experimental values, resulting in the following regression equation:

$$\begin{aligned} Y = {} & 48.03 - 5.42X_1 - 15.97X_2 - 0.52X_3 \\ & + 5.77X_1X_2 - 1.19X_1X_3 - 0.23X_2X_3 \\ & - 9.25X_1^2 - 16.53X_2^2 - 4.38X_3^2 \end{aligned} \quad (10)$$

where Y is the predicted response and X_1, X_2, and X_3 are the initial NO_3^--N concentration, pH, and DO, respectively.

Table 2 shows results of the second-order response surface model fitting in the form of ANOVA. The model F-value of 47.85 and low probability (p) value (< 0.0001) indicated that the model was significant. The adequacy of the model was indicated by the determination coefficient (R^2 = 0.9840), which explained 98.40% of the response variability, suggesting that the predicted values of the model agreed well with the experimental values. The high value of the adjusted R^2 (0.9652), which is close to 1, further supported the accuracy of the model.[35] Both the lack of fit value which was not significant (p-value > 0.05) and the low coefficient of variation (CV = 9.41%) demonstrated that the model was precise and reliable to predict and optimize the nitrate removal conditions within the limits of the chosen factors. Table 2 presents model coefficients estimated by regression analysis and corresponding p-values for the model of nitrate removal. The p-values were used to verify the significance of each coefficient. Small p-values indicate a high level of significance for the corresponding coefficient. The results of the regression parameter estimate revealed that except for the linear term of X_3 and the cross-product of X_1 X_2 and X_2 X_3 (p-value > 0.05), others were significant (p-value < 0.05).

Table 2 presents model coefficients estimated by regression analysis and corresponding p-values for the model of nitrate removal. The p-values were used to verify the significance of each coefficient. Small p-values indicate a high level of significance for the corresponding coefficient. The results of the regression parameter estimate revealed that except for the linear term of X_3 and the cross-product of X_1 X_2 and X_2 X_3 (p-value > 0.05), others were significant (p-value < 0.05).

3D graphical representations of the responses were generated to visualize the combined effects of the three variable factors on nitrate removal (Fig. 6). Fig. 6a shows that leaning alkalinity was stronger and thus more disadvantageous to denitrification. By contrast, DO

Table 2 Regression analysis and ANOVA of the fitted quadratic polynomial model for nitrate removal by AN-1 at 10 °C [a].

Source	Coefficient	Sum of squares	df	Mean squares	*F*-value	*p*-value
X_1 [b]	-5.24	234.64	1	234.64	24.40	0.0017
X_2 [c]	-15.97	2040.36	1	2040.36	212.25	<0.0001
X_3 [d]	-0.52	2.16	1	2.16	0.22	0.6502
$X_1 X_2$	5.77	133.11	1	133.11	13.84	0.0075
$X_1 X_3$	-1.19	5.70	1	5.70	0.59	0.4665
$X_2 X_3$	-0.23	0.21	1	0.21	0.022	0.8858
X_1^2	-9.25	360.20	1	360.20	37.45	0.0005
X_2^2	-16.53	1150.32	1	1150.32	119.61	<0.0001
X_3^2	-4.38	80.83	1	80.83	8.4	0.0230
Model	—	4141.99	9	460.22	47.85	<0.0001
Residual	—	67.32	7	9.62	—	—
Lack of Fit	—	7.00	3	2.33	0.15	0.9215
Pure Error	—	60.33	4	15.08	—	—
Total	—	4209.32	16	—	—	—

[a] $R^2 = 0.9840$; adjusted $R^2 = 0.9652$； CV = 9.17%

[b] refers to NO_3^--N concentration: -1 (60 mg L^{-1}), 0 (70 mg L^{-1}), +1 (80 mg L^{-1});

[c] refers to initial pH: -1 (6), 0 (7.5), +1 (9);

[d] refers to initial DO concentration: -1 (6 mg L^{-1}), 0 (7.5 mg L^{-1}), +1 (9 mg L^{-1}).

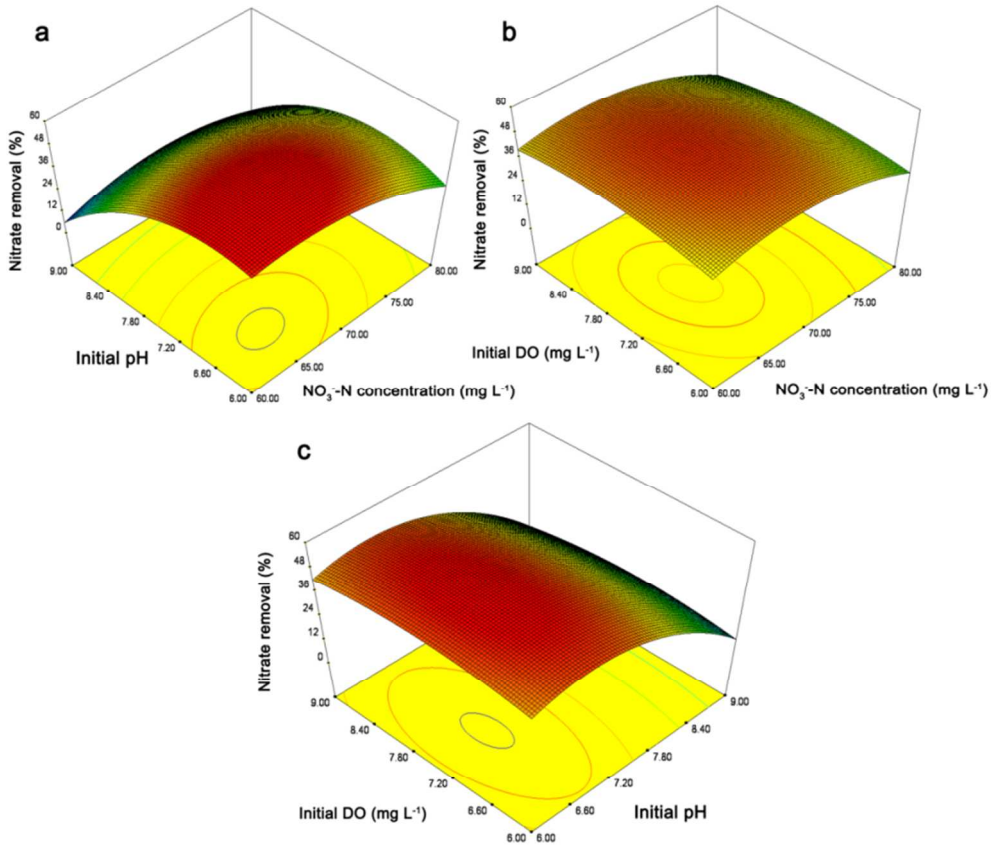

Fig. 6. Three-dimensional (3D) response surface for three variables (a) initial pH and the concentration of NO_3^--N: initial DO = 7.5 mg L^{-1} (b) initial DO and the concentration of NO_3^--N: initial pH = 7.5 (c) initial DO and initial pH: the concentration of NO_3^--N = 70 mg L^{-1}.

had minimal effect on nitrate removal (Fig. 6b). As shown in Fig. 6c, nitrate removal rate reached the maximum values at 7.5 mg L^{-1} DO (X_2) and pH (X_3) 7.5.

Based on the RSM results, the nitrate removal rate at each NO_3^--N concentration was determined under optimal operation conditions. Three repeated experiments were performed, and the results were compared for model validation. The optimum DO concentration and pH were determined at each NO_3^--N concentration, which were 7.64 mg L^{-1} DO and pH 6.51, 7.43 mg L^{-1} DO and pH 6.78, and 7.22 mg L^{-1} DO and pH 7.04 for 60 mg L^{-1}, 70 mg L^{-1}, and 80 mg L^{-1}, respectively. Under the corresponding optimized conditions, the nitrate removal percentages were 49.96%, 50.87%, and 34.94% after a 24 h inoculation with strain AN-1, which agree with the predicted values (51.38%, 51.89%, and 35.09%). These results indicate that these models are reliable for predicting the aerobic denitrification rate by AN-1 and could be used for process optimization of nitrate removal at low temperatures.

Conclusions

P. migulae AN-1, a novel cold-adapted bacterium capable of heterotrophic nitrification and aerobic denitrification at low temperatures, was isolated from a groundwater system. The strain could remove ammonium, nitrate, and nitrite at 1.56, 1.57, and 0.69 mg L^{-1} h^{-1} at 10 °C under aerobic conditions, respectively. It converted ammonium to gaseous denitrification products, such as N_2, and was less sensitive to substrate inhibition of nitrate at 10 °C. Process optimization via RSM was a reliable method for predicting the aerobic denitrification rate of *P. migulae* AN-1. These results indicate that AN-1 is a promising candidate for nitrogen removal from wastewater at low temperatures.

Acknowledgements

This work was partly supported by the National Natural Science Foundation of China (Grant No. 41101226), Specialized Research Fund for the Doctoral Program of Higher Education of China (Grant No. 20110061120076), and Fundamental Research Funds for the Central Universities of Jilin University (Grant No. 2013ZY02).

Notes and references

[a] Key Laboratory of Groundwater Resources and Environment of the Ministry of Education, College of Environment and Resources, Jilin University, 2519 Jiefang Road, Changchun, 130021, P. R. China.
Tel: +86-431-88502606; Email: renhejun@jlu.edu.cn
[b] Engineering Research Center of Bioreactor and Pharmaceutical Development of the Ministry of Education, College of Traditional Chinese Medicine, Jilin Agriculture University, 2888 Xincheng Road, Changchun, 130118, P. R. China.

Table S1 Box-Behnken design matrix and the responses of the dependent variables nitrate removal efficiency

Run order	Independent variables, coded levels			Dependent variables, NO_3^--N removal efficiency (%)	
	X_1 [a]	X_2 [b]	X_3 [c]	Predicted	Experimental
1	0	1	-1	0.1109	0.1215±0.012
2	0	0	0	0.4803	0.5071±0.046
3	-1	-1	0	0.494	0.5050±0.041
4	0	1	1	0.104	0.1050±0.016
5	0	-1	1	0.428	0.4254±0.052
6	1	-1	0	0.2703	0.2630±0.072
7	0	0	0	0.4803	0.4574±0.050
8	1	1	0	0.0663	0.0553±0.089
9	1	0	1	0.2727	0.2825±0.018
10	1	0	-1	0.3069	0.3153±0.018
11	0	0	0	0.4803	0.4223±0.058
12	0	-1	-1	0.4337	0.4326±0.043
13	-1	0	1	0.4049	0.3964±0.021
14	0	0	0	0.4803	0.5071±0.046
15	-1	1	0	0.0592	0.0665±0.020
16	0	0	0	0.4803	0.5071±0.046
17	-1	0	-1	0.3914	0.3815±0.047

[a] refers to NO_3^--N concentration: -1 (60 mg L^{-1}), 0 (70 mg L^{-1}), +1 (80 mg L^{-1});
[b] refers to initial pH: -1 (6), 0 (7.5), +1 (9);
[c] refers to initial DO concentration: -1 (6 mg L^{-1}), 0 (7.5 mg L^{-1}), +1 (9 mg L^{-1}).

1. J. K. Kim, K. J. Park, K. S. Cho, S.-W. Nam, T.-J. Park and R. Bajpai, *Bioresource Technology*, 2005, **96**, 1897-1906.
2. Q.-L. Zhang, Y. Liu, G.-M. Ai, L.-L. Miao, H.-Y. Zheng and Z.-P. Liu, *Bioresource technology*, 2012, **108**, 35-44.
3. Y.-C. Chiu, L.-L. Lee, C.-N. Chang and A. C. Chao, *International biodeterioration & biodegradation*, 2007, **59**, 1-7.
4. A. A. Khardenavis, A. Kapley and H. J. Purohit, *Applied microbiology and biotechnology*, 2007, **77**, 403-409.
5. D. Kulikowska, T. Jóźwiak, P. Kowal and S. Ciesielski, *Bioresource technology*, 2010, **101**, 3400-3405.
6. T. Khin and A. P. Annachhatre, *Biotechnology advances*, 2004, **22**, 519-532.
7. K. Third, B. Gibbs, M. Newland and R. Cord-Ruwisch, *Water Research*, 2005, **39**, 3523-3530.
8. C. Marazioti, M. Kornaros and G. Lyberatos, *Water Research*, 2003, **37**, 1239-1251.
9. D. J. Scala and L. J. Kerkhof, *FEMS Microbiology Letters*, 1998, **162**, 61-68.
10. Y. Bai, Q. Sun, C. Zhao, D. Wen and X. Tang, *Biodegradation*, 2010, **21**, 335-344.

11. S. Yao, J. Ni, Q. Chen and A. G. Borthwick, *Bioresource technology*, 2013, **127**, 151-157.
12. D. Zhang, W. Li, X. Huang, W. Qin and M. Liu, *Bioresource technology*, 2013, **137**, 147-152.
13. X. Huang, W. Li, D. Zhang and W. Qin, *Bioresource technology*, 2013, **146**, 44-50.
14. N. Takaya, M. A. B. Catalan-Sakairi, Y. Sakaguchi, I. Kato, Z. Zhou and H. Shoun, *Applied and environmental microbiology*, 2003, **69**, 3152-3157.
15. H. Heuer, M. Krsek, P. Baker, K. Smalla and E. Wellington, *Applied and environmental microbiology*, 1997, **63**, 3233-3241.
16. C. Rösch, A. Mergel and H. Bothe, *Applied and Environmental Microbiology*, 2002, **68**, 3818-3829.
17. R. H. Myers, D. C. Montgomery and C. M. Anderson-Cook, *Response surface methodology: process and product optimization using designed experiments*, John Wiley & Sons, 2009.
18. A. Apha, *Standard methods for the examination of water and wastewater. 20th ed. Washington DC: American Public Health Association, American Water Work Association, Water Environment federation*, 1998, **252**.
19. D. Frear and R. Burrell, *Analytical Chemistry*, 1955, **27**, 1664-1665.
20. B. Zhao, Q. An, Y. L. He and J. S. Guo, *Bioresource technology*, 2012, **116**, 379-385.
21. M. D. Wallenstein, D. D. Myrold, M. Firestone and M. Voytek, *Ecological Applications*, 2006, **16**, 2143-2152.
22. J. Zhang, P. Wu, B. Hao and Z. Yu, *Bioresource technology*, 2011, **102**, 9866-9869.
23. T. Nakajima-Kambe, N. Okada, M. Takeda, Y. Akutsu-Shigeno, M. Matsumura, N. Nomura and H. Uchiyama, *Journal of bioscience and bioengineering*, 2005, **99**, 429-433.
24. Y.-S. Kim, F. Nayve, K. Nakano and M. Matsumura, *Environmental technology*, 2002, **23**, 1017-1026.
25. C. Wan, X. Yang, D.-J. Lee, M. Du, F. Wan and C. Chen, *Bioresource technology*, 2011, **102**, 7244-7248.
26. E. Matsuzaka, N. Nomura, T. Nakajima-Kambe, N. Okada and T. Nakahara, *Journal of bioscience and bioengineering*, 2003, **95**, 409-411.
27. B. Zhao, Y. L. He and X. F. Zhang, *Environmental technology*, 2010, **31**, 409-416.
28. Z. Shi, Y. Zhang, J. Zhou, M. Chen and X. Wang, *Bioresource technology*, 2013, **148**, 144-148.
29. Q. Chen and J. Ni, *Journal of bioscience and bioengineering*, 2012, **113**, 619-623.
30. H. K. Huang and S. Tseng, *Applied microbiology and biotechnology*, 2001, **55**, 90-94.
31. M. Y. Pamukoglu and F. Kargi, *Enzyme and Microbial Technology*, 2008, **43**, 43-47.
32. K. L. Thomas, D. Lloyd and L. Boddy, *FEMS microbiology letters*, 1994, **118**, 181-186.
33. A. Blackmer and J. Bremner, *Soil Biology and Biochemistry*, 1978, **10**, 187-191.
34. D. Hernandez, F. M. Dias and J. J. Rowe, *Archives of biochemistry and biophysics*, 1991, **286**, 159-163.
35. S. A. Sayyad, B. P. Panda, S. Javed and M. Ali, *Applied Microbiology and Biotechnology*, 2007, **73**, 1054-1058.

Graphical Abstract

A novel groundwater origin cold-adapted aerobic denitrifying strain AN-1was isolated. Its nitrifying-denitrifying capability and nitrogen balance was determined. Nitrate removal of the strain was described by Monod kinetics with a non-competitive substrate inhibition and optimized.