

Original Research

Heterotrophic Nitrification and Aerobic Denitrification by Four Novel Isolated Bacteria

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Received: 26 November 2014

Accepted: 28 January 2015

Abstract

Heterotrophic nitrifying bacteria are attracting increasing interest due to their advantages in treatment of wastewater with high nitrogen concentration. In this work, four bacterial isolates (WS-1, WS-4, WS-6, and WS-7) with the capability of simultaneous heterotrophic nitrification and aerobic denitrification from soil, sludge, and water by a fast isolation procedure were studied. Isolates WS-1, WS-6, and WS-7 were identified as *Pseudomonas* sp. and WS-4 as *Agrobacterium* sp. based on 16S rRNA gene sequence analysis. All four isolates could utilize ammonia, nitrate, and nitrite as sole nitrogen source and the metabolic characteristics were shown to be related with the genus. In addition, *Agrobacterium* sp. WS-4 accumulated less nitrate than the three *Pseudomonas* sp. strains with ammonia as the sole nitrogen source. When nitrate or nitrite was the sole nitrogen source, *Agrobacterium* sp. did not produce ammonia, and it consumed the least nitrogen compared with the three *Pseudomonas* sp. isolates. Our studies implied that *Pseudomonas* sp. strains had great potential in treating high nitrogen in wastewater.

Keywords: *Pseudomonas*, *Agrobacterium*, nitrogen removal, wastewater treatment

Introduction

Biological nitrogen removal has commonly been used in treatment of wastewater with high nitrogen concentration. Traditionally, nitrogen removal processes were accomplished by nitrifiers and denitrifiers under alternating aerobic and anaerobic conditions. However, the different demands of two sorts of microorganisms in organic matter and oxygen made the treatment process quite complex and tedious. Organic matter was disfavored for nitrifiers while it was indispensable for denitrifiers [1]. Furthermore, the growth of nitrifiers required an aerobic condition that was

harmful to denitrifiers [2]. Due to the different requirements for organic matter and oxygen, the two sorts of microorganisms had to be separated in the wastewater treatment system during the process of wastewater treatment. This extra step increased the operational difficulties. Meanwhile, the slow growth of autotrophic nitrifiers easily caused the wastewater treatment process to be time-consuming and cost-ineffective [2].

Heterotrophic nitrogen removal refers to the simultaneous processes of heterotrophic nitrification and aerobic denitrification by a single strain [3]. The metabolic pathway has been widely accepted as heterotrophic nitrification and simultaneous aerobic denitrification. In 1972, an *Arthrobacter* sp. strain with the capability of heterotrophic

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nitrification was first isolated from the natural environment [4]. Subsequently, *Bacillus* sp., *Comamonas* sp., *Diaphorobacter* sp., and *Pseudomonas stutzeri* were identified as strains with heterotrophic abilities [3, 5, 6].

Heterotrophic nitrification had several advantages over traditional methods. Firstly, the utilization of organic substrates and the tolerance to oxygen of heterotrophic nitrifiers allowed the conduct of simultaneous nitrification and denitrification in the same space and phase. In addition, the process of denitrification could balance pH in the reactor, avoiding acidification by nitrification. Consequently, heterotrophic nitrogen removal has attracted increasing interest in wastewater treatment [2].

Generally speaking, heterotrophic microbes were isolated from wastewater [2] and membrane bioreactors [3, 7]. In fact, heterotrophic nitrification widely existed in various kinds of environments such as soil and sludge. In this work, four heterotrophic bacteria were isolated and identified from soil and sludge, as well as eutrophic water. Furthermore, their nitrogen removal percentage and transformation profiles were evaluated. Thus it lays the foundation for future wastewater treatment research.

Material and Methods

Medium and Reagents

We used the heterotrophic nitrification medium (HNM, g/L): $(\text{NH}_4)_2\text{SO}_4$ 0.66, sodium succinate 4.72, K_2HPO_4 0.50, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, NaCl 30.00, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0025, and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.0025. Nitrate medium (NAM) and nitrite medium (NIM) were prepared by replacing $(\text{NH}_4)_2\text{SO}_4$ in HNM with 1.05 g KNO_3 and 0.886 g KNO_2 , respectively. When prepared for plating, HNM, NAM, and NIM plates contained 1.5% (w/v) agar. Nessler and Griess reagent were obtained from Sigma-Aldrich. Ferrous sulfate solution was prepared by adding 0.1 g ferrous sulfate to 100 ml distilled water.

Fast Isolation of Bacteria

The method to isolate simultaneous heterotrophic nitrifying and aerobic denitrifying bacteria were modified according to reference [7, 8]. Briefly, 1 g or ml soil, sludge, or water was added to sterile water and vortexed for 5 min. After disposition for 5 min, 1.0 ml supernatant was added to 100 ml HNM and incubated on a shaker (180 rpm) at 30°C for 24 h. One ml of the incubated mixture was collected and dripped to white cuvettes with 0.2 ml Nessler reagent. Decolorization of the mixture with Nessler Reagent was regarded as a sign of ammonia removal. Vials showing ammonia removal were selected for further study. Those culture media were plated on HNM medium at proper dilution and incubated (30°C) for 2 d. The single colonies with lighter color were presumed to have nitrification ability and purified further on NAM and NIM plates. After 2 d

incubation (30°C), drops of Griess reagent and Ferrous sulfate solution were respectively introduced on individual colonies on NAM and NIM, respectively. The colonies with lighter color were identified as able to denitrify. Both sorts of strains were isolated and stored on slants.

DNA Extraction, PCR Amplification and 16S rRNA Gene Sequence Analysis

DNA was extracted from bacterial suspensions (1 ml, $\text{OD}_{600} = 0.7$) using the EZ-10 Spin Column Genomic DNA Isolation Kit (Sangon, Shanghai). Primers F27 (GTTTGATCCTGGCTCAG) and R1492 (TACGGYTACCTTGTTACGACTT) were used to amplify the 16S rRNA gene by a PCR protocol (PTC200, Bio-Rad, USA). PCR-amplified products were purified by the EZ-10 Spin Column PCR Product Purification Kit (Sangon, Shanghai) and 16S rRNA genes were sequenced by Shanghai Invitrogen Biotech Company Ltd. (Shanghai, China). Finally, the 16S rRNA sequence of the isolate was compared with that of other microorganisms by way of BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>).

Shake Flask Experiments

Strains selected in the previous works were cultivated in HNM, NAM, or NIM for 12 h (30°C, 120 rpm) according to [3]. The treatments without any inoculation served as control and each treatment had three replicates. After centrifuging (4°C, 15 min, 5000 g), the pellets were washed three times with sterile water. Then the pellets were suspended in sterilized water to adjust cell optical density (OD_{600}) to 0.7. Then bacterial suspension (1 ml) was placed into conical flasks (250 ml) containing 100 ml HNM and cultivated at 30°C and 120 rpm. After 72 h incubation, the medium was centrifuged (4°C, 15 min, 5000 g) to obtain supernatants for the determination of ammonium, hydroxylamine, nitrite, nitrate, and total nitrogen (TN).

Analytical Methods

Ammonia, nitrite, and nitrate were analyzed according to the standard methods. The ammonia concentration was determined by Nessler's reagent assay at a wavelength of 420 nm. Nitrite was determined by N-(1-naphthyl)-ethylene diamine spectrophotometry and nitrate was determined by ultraviolet spectrophotometry under the wavelength of 220 nm and 275 nm. The amount of total nitrogen was determined by oxidation of potassium persulfate by ultraviolet spectrophotometry. Hydroxylamine was determined according to Frear and Burrell [9]. Bacterial growth was determined by monitoring the optical density at 600 nm (OD_{600}) using a spectrophotometer. Morphological characteristics of the strain were observed by scanning electron microscopy (SEM; JSM-5610LV, Jeol, Japan) after 12 h incubation.

Table 1. Ammonia transformation by four strains.

Strains	OD	TN		NH ₄ ⁺ -N		NO ₃ ⁻ -N		NO ₂ ⁻ -N	
		Initial concentration	Removal percentage	Final concentration	Removal percentage	Final concentration	Productive percentage ^{a)}	Final concentration	Productive percentage ^{a)}
		(mg/L)	(%)	(mg/L)	(%)	(mg/L)	(%)	(µg/L)	(%)
WS-1	1.2	146.00	72.19±0.58	3.78±0.29	97.41±0.20	17.90±1.67	12.26±1.15	6.9±0	0.005±0
WS-4	2.5	146.00	70.62±1.20	8.35±0.40	94.28±0.30	1.90±1.28	1.30±0.87	8.7±0.2	0.005±0.0001
WS-6	1.4	146.00	71.85±0.33	6.09±1.27	95.83±0.90	12.70±1.05	8.70±0.71	12.1±0.9	0.008±0.0006
WS-7	1.8	146.00	73.29±1.10	3.72±0.07	97.45±0.05	9.10±0.41	6.23±0.28	14.2±0	0.009±0

^{a)}Productive percentage represents the percent of compound accounted for the removed NO₃⁻-N.

Data are presented as mean±S.D., based on three replicates.

Table 2. Nitrate transformation by four strains.

Strains	OD	TN		NH ₄ ⁺ -N		NO ₃ ⁻ -N		NO ₂ ⁻ -N	
		Initial concentration	Removal percentage	Final concentration	Productive percentage ^{a)}	Final concentration	Removal percentage	Final concentration	Productive percentage ^{a)}
		(mg/L)	(%)	(mg/L)	(%)	(mg/L)	(%)	(µg/L)	(%)
WS-1	2.3	146.00	57.77±0.34	3.73±0.26	2.55±0.18	23.42±1.05	83.96±0.72	4.02±0.02	2.75±0.01
WS-4	1.7	146.00	25.01±0.48	0	0	81.32±4.30	44.30±2.95	2.97±0.02	2.03±0.01
WS-6	2.3	146.00	61.69±0.68	2.68±0.02	1.84±0.01	18.36±0.30	87.42±0.21	3.88±0.01	2.66±0.01
WS-7	2.7	146.00	54.49±0.34	1.18±0.07	0.81±0.05	23.82±2.45	83.68±1.68	3.90±0.01	2.67±0.01

^{a)}Productive percentage represents the percent of compound accounted for the removed NO₃⁻-N.

Data are presented as mean±S.D., based on three replicates.

Results

Isolation of the Microorganisms

Different samples including polluted soil, water, and sludge were inoculated in HNM and incubated for 24 h. One ml medium was collected and mixed with 0.2 ml Nessler reagent. The color of 6 samples became very light compared with the control, indicating that those six samples contained microbes with ammonia removal abilities. The Griess reagent and Ferrous sulfate experiments further indicated the presence of these organism colonies on the plate. These were characterized as potential heterotrophic nitrifying bacteria. Four strains were ultimately selected as aerobic nitrification microbes, and were named as WS-1, WS-4, WS-6, and WS-7.

Identification of Isolates

Gram-staining, electron microscopy, and genotypic analysis were carried out to identify the isolates. Staining showed that all four strains were Gram-negative. Electron microscopy showed that all WS-1, WS-4, WS-6, and WS-7 were rod-shaped and their sizes were (0.2-0.3) µm×(0.8-1.5) µm, (0.8-1.2) µm×(1.5-2.0), (0.2-0.6)

µm×(0.8-1.2) µm, and (0.3-0.8) µm×(1.0-1.4) µm, respectively.

The nucleotide sequences of their 16S rRNA genes alignment revealed that WS-1, WS-6, and WS-7 were members of the genus *Pseudomonas* sp., while WS-4 was a member of the genus *Agrobacterium* sp. (Fig. 1).

Ammonia Transformation by the Isolates

We evaluated the efficiency of heterotrophic nitrification by four strains with ammonia as the sole nitrogen and sodium succinate as the organic carbon source (Table 1). After 48 h incubation, more than 70% of total nitrogen was removed by all four strains, and the maximal nitrogen removal percentage was obtained by WS-7, which was up to 73.29%. All these strains removed more than 90% of ammonia, and the maximal nitrogen removal percentage was also obtained by WS-7, which was up to 97.45%. Nitrate and nitrite but not hydroxylamine were detected in the culture. WS-1 produced the most NO₃⁻-N (17.90 mg/L), which occupied 12.26% of initial NH₄⁺-N. Concentration of nitrate in culture of WS-4 was minimal and only occupied 1.3% of initial NH₄⁺-N. The accumulation of nitrite by four strains was only detected on the level of µg/L.

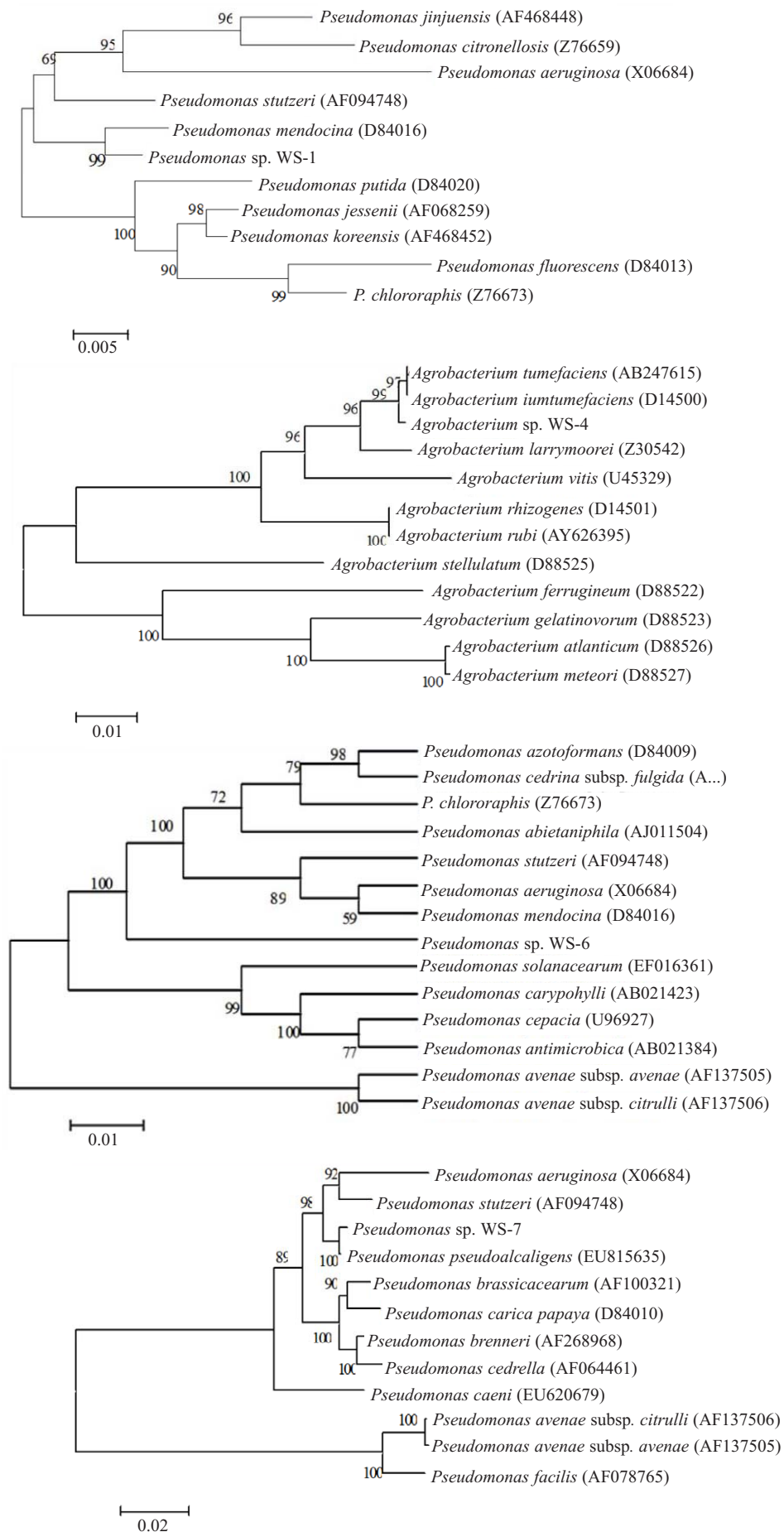


Fig. 1. Phylogenetic tree of strain WS-1, WS-4, WS-6, and WS-7 based on 16S rDNA.

Table 3. Nitrite transformation by four strains.

Strains	OD	TN		NH ₄ ⁺ -N		NO ₃ ⁻ -N		NO ₂ ⁻ -N	
		Initial concentration	Removal percentage	Final concentration	Productive percentage ^{a)}	Final concentration	Productive percentage ^{a)}	Final concentration	Removal percentage
		(mg/L)	(%)	(mg/L)	(%)	(mg/L)	(%)	(μg/L)	(%)
WS-1	0.8	146.00	56.54±1.58	4.73±0.57	3.24±0.39	29.07±1.30	19.91±0.89	19.73±0.17	86.49±0.12
WS-4	0.1	146.00	4.03±0.34	0	0	116.60±0.05	79.86±0.03	21.25±0.01	85.46±0.01
WS-6	0.7	146.00	56.13±2.26	2.96±0.19	2.03±0.13	30.08±2.91	20.60±1.99	20.00±0.04	86.30±0.03
WS-7	1.4	146.00	63.26±1.58	2.26±0.28	1.55±0.19	16.31±1.75	11.17±1.20	15.19±0.03	89.59±0.02

^{a)}Productive percentage represents the percent of compound accounted for the removed NO₃⁻-N.

Data are presented as mean±S.D., based on three replicates.

Nitrate Transformation by the Isolates under Aerobic Conditions

The efficiency of aerobic denitrification was evaluated by four strains with nitrate as the sole nitrogen source (Table 2). The results showed that all four strains could utilize nitrate. The three *Pseudomonas* sp. strains (WS-1, WS-6, and WS-7) removed 54.49~61.69% and 83.68~87.42% of total nitrogen and nitrate, respectively, which was much more than that of WS-4. All four strains accumulated NO₂⁻-N on the similar level of 2.97~4.02 mg/L. The three *Pseudomonas* sp. strains produced a small amount of NH₄⁺-N, ranging from 1.18 to 3.73 mg/L (except *Agrobacterium* sp. WS-4).

Nitrite Transformation by the Isolates under Aerobic Conditions

Aerobic denitrification efficiency by four strains with nitrite as the sole nitrogen source was evaluated (Table 3). The results showed that all four strains could utilize nitrite. Three *Pseudomonas* sp. strains (WS-1, WS-6, and WS-7) removed 56.13~63.26% of total nitrogen, which was much more than that of WS-4 (4.03%). All four strains consumed approximately similar amounts of NO₂⁻-N (86.49~89.59%). The strain WS-4 produced maximal amount of NO₃⁻-N (116.60 mg/L), which was greater than that of the other strains (16.31~30.08 mg/L). The three *Pseudomonas* sp. strains produced a small amount of NH₄⁺-N, ranging from 2.26 to 4.73 mg/L (except *Agrobacterium* sp. WS-4).

Discussion

Traditionally, heterotrophic nitrifying strains were usually obtained from wastewater [2, 10] or membrane bioreactors [3, 7]. In our works, various environment samples, such as soil, sludge, and water, were screened for microorganisms with heterotrophic nitrification ability. As a result, four strains were finally isolated from three different environments. This indicates that heterotrophic nitrifying bacteria were rich in water, soil, and sludge environments.

The four newly isolated strains belonged to two genus, namely *Pseudomonas* sp. and *Agrobacterium* sp. (Fig. 1). *Pseudomonas* sp. was a very common genus with the ability of heterotrophic nitrification [2, 10]. The genus could be classified into three groups based on their modes of denitrification [6]. The first group reduces nitrate to nitrogen gas directly without accumulating nitrite. The second group possesses a two-phase denitrification process, and the remaining isolates accumulate nitrite at low concentration, just like the four strain we isolated (Table 1). Similarly, another *Pseudomonas* sp. strain was isolated from piggery wastewaters treatment system which removed 35 mg/L NH₄⁺-N with few NO₂⁻-N accumulation [10].

Heterotrophic nitrification by *Agrobacterium* sp. was not reported until 2011 with a strain was isolated from landfill leachate treatment system. The strain could remove about 65 mg/L NH₄⁺-N within 12 h and without nitrite and nitrate accumulation [11]. In our work, the newly isolated *Agrobacterium* sp. WS-4 removed 94.28% of NH₄⁺-N (146 mg/L) and only 1.30% and 0.005% of it was transformed to NO₃⁻-N and NO₂⁻-N respectively, indicating the potential of the strain in nitrogen removal. However, *Agrobacterium* sp. was usually described as aerobic microorganisms that could inhabit plant tissues causing tumors [12]. Therefore, most of strains in the genus are plant pathogens, implying the limitation of their application.

The nitrogen removal profiles were found to be relative to the type of genus. When ammonia was the sole nitrogen source, *Agrobacterium* sp. WS-4 accumulated less nitrate than three *Pseudomonas* sp. strains. When nitrate was the sole nitrogen source, the three *Pseudomonas* sp. strains removed much more nitrate than that of the remaining *Agrobacterium* sp. WS-4. Additionally, *Agrobacterium* sp. WS-4 did not produce ammonia while *Pseudomonas* sp. strains generated a small amount of ammonia (Table 2). To our knowledge, transformation of nitrate to ammonium was firstly reported in the liquid medium; however, the similar phenomena were observed in the soil [13]. Though nitrite removal percentage was approximately similar (85.46~89.59%) with nitrite as the nitrogen source for the

four strains (Table 3), the total nitrogen removal percentage of *Agrobacterium* sp. WS-4 was only 4.03%, which was much less than that of *Pseudomonas* sp. strains (56.13~63.26%). No ammonia was detected in the *Agrobacterium* sp. WS-4 culture while a small amount was in three *Pseudomonas* sp. strains'. *Agrobacterium* sp. WS-4 produced the maximal nitrate compared to levels generated by *Pseudomonas* sp. strains. These results suggested that it was a further nitrifying reaction, but not an aerobic denitrification for strain *Agrobacterium* sp. WS-4, when nitrite was employed as substrate.

Conclusions

All the four newly isolated strains could grow with ammonia, nitrate, and nitrite as the sole nitrogen source. The results demonstrated that not only water and sludge, but also soil, contained plenty of heterotrophic nitrifying microorganisms and they could all be applied in screening new strains. Based on the 16S rRNA sequences analysis, the strains WS-1, WS-6, and WS-7 were identified as *Pseudomonas* sp. and strain WS-4 was identified as *Agrobacterium* sp. The latter was an unfamiliar genus with heterotrophic nitrification ability. Nitrogen transformation profiles seemed to be related to the genus. *Agrobacterium* sp. WS-4 accumulated less nitrate than *Pseudomonas* sp. strains with ammonia as the nitrogen source. When nitrate or nitrite was the nitrogen source, *Agrobacterium* sp. WS-4 did not produce ammonia, and consumed the least nitrogen comparing to *Pseudomonas* sp. strains. Our study implied *Pseudomonas* sp. bacteria showed strong potential in treatment of high nitrogen wastewater.

Acknowledgements

The work was financial supported by Zhejiang Provincial Natural Science Foundation of China (Y5110147).

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