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Ammonium removal by *Agrobacterium* sp. LAD9 capable of heterotrophic nitrification–aerobic denitrification

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Characteristics of ammonium removal by a newly isolated heterotrophic nitrification–aerobic denitrification bacterium *Agrobacterium* sp. LAD9 were systematically investigated. Succinate and acetate were found to be the most favorable carbon sources for LAD9. Response surface methodology (RSM) analysis demonstrated that maximum removal of ammonium occurred under the conditions with an initial pH of 8.46, C/N ratio of 8.28, temperature of 27.9°C and shaking speed of 150 rpm, where temperature and shaking speed produced the largest effect. Further nitrogen balance analysis revealed that 50.1% of nitrogen was removed as gas products and 40.8% was converted to the biomass. Moreover, the occurrence of aerobic denitrification was evidenced by the utilization of nitrite and nitrate as nitrogen sources, and the successful amplifications of membrane bound nitrate reductase and cytochrome *cd*$_1$ nitrite reductase genes from strain LAD9. Thus, the nitrogen removal in strain LAD9 was speculated to comply with the mechanism of heterotrophic nitrification coupled with aerobic denitrification (NH_4^+–NH_2OH–NO_2^-–N_2O–N_2), in which also accompanied with the mutual transformation of nitrite and nitrate. The findings can help in applying appropriate controls over operational parameters in systems involving the use of this kind of strain.

[**Key words:** Nitrogen removal; *Agrobacterium* sp.; Heterotrophic nitrification; Aerobic denitrification; Nitrogen balance analysis; NAP; *nir*S]

The conventional system for ammonium removal consists of two steps: nitrification by autotrophs under aerobic conditions and denitrification by heterotrophs under anaerobic conditions. However, this type of system is difficult to operate due to the low rate of nitrification and the complexity of separating nitrification and denitrification reactors. Recently, bacteria capable of combined heterotrophic nitrification and aerobic denitrification have been investigated as potential microorganisms in biological nitrogen removal systems (1–3). Certain groups of bacteria, such as *Thiosphaera pantotropha*, *Alcaligenes faecalis*, *Bacillus* sp., *Diaphorobacteria* sp. and *Acinetobacter calcoaceticus*, have been isolated from soils, and wastewater treatment systems (4–8). These microorganisms, due to their high growth rate and ability to convert ammonia nitrogen to nitrogenous gas aerobically, have a great number of advantages as applied for the nitrogen removal: (i) procedural simplicity, where nitrification and denitrification can take place simultaneously; (ii) less acclimation problems; (iii) lesser buffer quantity needed because alkalinity generated during denitrification can partly compensate for the alkalinity consumption in nitrification (9).

Carbon source, C/N ratio, temperature, initial pH and dissolved oxygen (DO) concentration are the major factors affecting the heterotrophic nitrification–aerobic denitrification process. Previous studies have shown that different species of heterotrophic nitrification–aerobic denitrification bacteria have distinct response to these factors (10–12). However, systematically evaluating the influence strength of these parameters and their interactions on the heterotrophic nitrification–aerobic denitrification process are limited, especially for novel strains.

Furthermore, it is indicated that there may be two nitrogen removal pathways for the process of heterotrophic nitrification–aerobic denitrification. The difference between them is concentrated on the aerobic denitrification route (13). One exhibits a fully nitrification and denitrification pathway, representative as *T. pantotropha* (7), while the other demonstrates that denitrification via hydroxylamine rather than nitrite or nitrate, representative as *A. faecalis* (12). However, little attention has been paid to novel strains, and it is still difficult to generalize the biochemical mechanisms due to the limitation of the number of tested species.

A novel heterotrophic nitrification–aerobic denitrification bacterium *Agrobacterium* sp. LAD9 was isolated from the landfill leachate treatment system in our lab (14). However, neither ammonium removal characteristics nor the nitrogen removal pathway of *Agrobacterium* genus is available up to now. Herein, factors affecting the performance of *Agrobacterium* sp. LAD9 were comprehensively evaluated based on the response surface methodology (RSM) analysis, and the possible nitrogen removal pathway was explored

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based on the analysis of nitrogen balance and the confirmation of aerobic denitrification.

MATERIALS AND METHODS

Microorganism and culture medium *Agrobacterium* sp. LAD9 (CGMCC No. 2962) was stocked in 20% glycerol solution at −80°C.

The ingredients of synthetic mineral medium (SMM, g/l) were as follows: NH_4Cl; sodium succinate; $FeSO_4 \cdot 7H_2O$ of 0.006; $CaCl_2 \cdot 2H_2O$ of 0.2; $MgSO_4 \cdot 7H_2O$ of 0.5; KH_2PO_4 of 1.0 and pH of 7.0–7.2. The concentrations of NH_4Cl and sodium succinate were adjusted according to the experimental requirement.

The strain was first incubated in 1 l Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.04% NH_4Cl) for 24 h. Enriched culture was harvested by centrifugation at 6000 rpm for 10 min and washed twice with sterile distilled water. The pellet was resuspended in the SMM for inoculation.

Carbon sources Five carbon compounds, glucose, sodium acetate, sodium citrate, potassium sodium tartrate and sucrose, were used as carbon sources instead of sodium succinate in the SMM. The amount of each carbon source was determined by fixing the C/N ratio (w/w) at 8 accompanied with a constant ammonia nitrogen concentration of 110 mg/l. 2 ml of the enriched culture was transferred to 98 ml of SMM and cultured at 30°C, 160 rpm. All tests were performed in triplicate. Aliquots of 2 ml were collected periodically for chemical analysis and cell density.

Box–Behnken design for optimizing the environmental factors RSM was used to investigate the effects of initial pH, C/N ratio, temperature and shaking speed on the activity of heterotrophic nitrification–aerobic denitrification by the strain LAD9. 2 ml of the enriched culture was transferred to 98 ml of SMM in 250 ml flasks. The amount of sodium succinate was changed to adjust the C/N ratios in the SMM by maintaining a constant ammonia nitrogen concentration at 110 mg/l.

The levels of four independent variables were defined according to the Box–Behnken design, and 27 experiments were required for the procedure (Table 1). A genuine replicate of the whole matrix was done to estimate the experimental error. The statistical experimental designs and graphical analysis were performed using MINITAB program (version 15, Minitab Inc., USA).

Nitrogen balance analysis Nitrogen balance was tested in flasks of 1 l. 10 ml of the enriched culture was transferred to 490 ml of SMM, containing (g/l): NH_4Cl of 0.38, sodium succinate of 4.78, KH_2PO_4 of 1.0, $FeSO_4 \cdot 7H_2O$ of 0.006, $CaCl_2 \cdot 2H_2O$ of 0.2, $MgSO_4 \cdot 7H_2O$ of 0.5. The optimized condition obtained in the RSM analysis was used for the incubation. Sterile control was also employed in the experiments. Aliquots of 2 ml were collected periodically for the detection of COD, ammonia nitrogen, hydroxylamine, nitrite and nitrate.

Utilizations of nitrite and nitrate by *Agrobacterium* sp. LAD9 To observe the nitrogen removal pathway of strain LAD9, NO_3 (150 mg/l) and NO_2 (70 mg/l) were added to the SMM instead of NH_4Cl as the nitrogen sources. 2 ml of enriched culture was added to the 98 ml SMM containing KNO_3 and incubated at 30°C, 160 rpm. 100 ml of the LAD9 culture was added to the 4 l SMM containing $NaNO_2$ in a 5 l fermentor and the pH, DO concentration was monitored online.

TABLE 1. The Box–Behnken experimental design along with the corresponding responses.

Runs	Initial pH	C/N ratio	Temperature	Shaking speed	Response (ν_N)
1	8	4	30	200	0.720
2	6	8	20	150	0.626
3	10	8	40	150	0.273
4	6	8	40	150	0.001
5	8	12	30	200	0.954
6	10	8	20	150	1.053
7	8	8	30	150	2.163
8	8	12	30	100	1.021
9	8	4	30	100	0.911
10	10	12	30	150	1.665
11	8	8	20	200	1.499
12	8	8	20	100	0.481
13	8	8	40	200	0.075
14	8	8	40	100	0.458
15	10	4	30	150	1.572
16	6	12	30	150	2.150
17	8	8	30	150	1.535
18	6	4	30	150	0.627
19	8	4	40	150	0.501
20	6	8	30	100	0.450
21	8	4	20	150	1.015
22	8	8	30	150	2.106
23	10	8	30	200	1.051
24	6	8	30	200	0.721
25	8	12	40	150	0.217
26	10	8	30	100	0.823
27	8	12	20	150	1.434

PCR amplification of nitrite and nitrate reductase genes Total bacterial DNA was extracted with a genomic DNA extraction kit (TianGen, China) following the manufacturer's instructions. The primers for nitrite (15) and nitrate (16) reductase genes described previously were shown in Table S1. For direct PCR amplification of the Cu-type (*nir*K) and cd_1-type (*nir*S) nitrite reductase genes, the cycling was carried out under the following conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 45 s and elongation at 72°C for 45 s; cycling was completed by a final elongation step of 72°C for 10 min. For the amplification of the periplasmic nitrate reductase (NAP) gene, annealing temperature was changed to 60°C. PCR amplification was performed with different primers in a total volume of 50 μl containing 2 μl of DNA template, 2 μl dNTP (10 mmol/l), 5 μl 10× PCR Buffer, 2.5 U Tag DNA polymerase, 1 μl of each primer (10 μmol/l) and sterile water.

Analytical measurements Cell growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer (Bio-Rad, USA). The cell density was converted to cell weight concentration via appropriate calibrations. The nitrogen content in the dry biomass was obtained using an element analyzer (Vario EL, Germany). COD, ammonia nitrogen, nitrite and nitrate were analyzed according to the standard methods. Hydroxylamine was determined according to Frear and Burrell (17). The amount of total nitrogen (TN) was calculated by adding concentrations of ammonia nitrogen, hydroxylamine, nitrate and nitrite.

Specific growth rate (μ) was determined by linear regression to the linear phase of the growth curve, plotted as OD as a function of time. Specific heterotrophic nitrification–aerobic denitrification rates (ν_N) under different conditions were determined as the overall nitrogen removal activities because denitrification of the nitrification products is unavoidable, and it is difficult to examine these activities separately. The equation was shown as follows:

$$\nu_N = -\frac{dS/dt}{X} \quad (1)$$

where S was the concentration of TN, mg/l; X was the microbial concentration, mg/l; t was the reaction time, d.

Nucleotide sequence accession number The sequence data of strain LAD9 has been submitted to the GenBank database under accession no. FJ639330.

RESULTS

Effect of carbon sources As depicted in Fig. 1, the use of different carbon compounds as carbon sources considerably affected the growth and the rate of heterotrophic nitrification–aerobic denitrification by LAD9. Among the six carbon sources tested, the growth was not detected in the case of tartrate and sucrose, indicating they could not be utilized by this bacterium. It was interesting to note that although the estimated specific growth rates for the systems operated on acetate ($0.14\ h^{-1}$), succinate ($0.16\ h^{-1}$) and citrate ($0.14\ h^{-1}$) were fairly similar, ν_N differed significantly. Succinate ($0.44\ d^{-1}$) and acetate ($0.32\ d^{-1}$) exhibited higher values, implying they were more advantageous as carbon sources for LAD9. On the contrary, the ν_N in the citrate ($0.14\ d^{-1}$) was much lower. It was probable that citrate had a higher biomass yield, and consequently the calculated ν_N would be lower. With the same type of reasoning, it was logical that despite the specific growth rate in glucose was very low

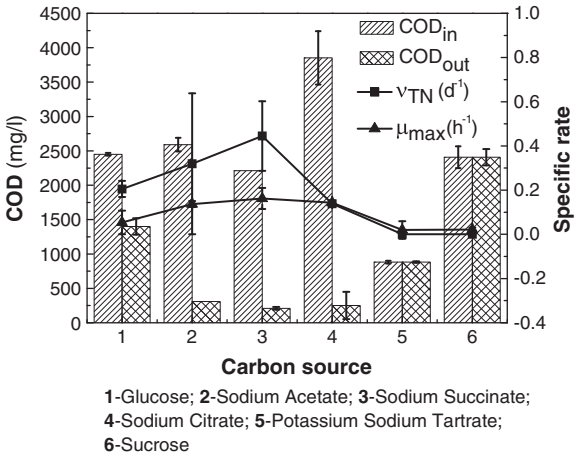

FIG. 1. Effect of different carbon sources on the activity of strain LAD9.