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# Heterotrophic Nitrification and Aerobic Denitrification of a Wastewater from a Chemical Company by *Alcaligenes faecalis* No.4

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## Abstract

A wastewater from a chemical company (abbreviated as WC) containing approximately 5000 mg $NH_4$-N/L ammonium and a slight amount of BOD was treated with *Alcaligenes faecalis* No.4 (No.4),which is capable of heterotrophic nitrification and aerobic denitrification. The repeated-batch operation was conducted at 30°C for 900 h at controlled dissolved oxygen concentration (DO) of 2-3 mg/L in the presence of citrate as the carbon source and inorganic nutrients. The removal efficiency of ammonium reached 90-100% within 24-30 h, and the average ammonium removal rate was 1.1 kg $NH_4$-N/$m^3$/day, which is more than 100 fold greater than that achieved with conventional nitrification-denitrification processes. The operation was shut-down for 220 h, and after that the ammonium removal activity achieved by No.4 resumed without delay, and a high removal rate was maintained. The addition of 3% NaCl to the wastewater resulted in almost the same ammonium removal rate as that without NaCl.

**Keywords:** Heterotrophic nitrification; Aerobic denitrification; High-strength ammonium; *Alcaligenes faecalis*; Wastewater containing least amount of BOD

## Introduction

The efficient removal of ammonium from receiving water body is essential for the prevention of eutrophication. Conventional ammonium removal in wastewater treatment plants consists of nitrification by autotrophic bacteria under aerobic conditions, followed by denitrification by heterotrophic bacteria under anaerobic conditions. This system is disadvantageous because the rate of nitrification is slow, and the autotrophic bacteria are vulnerable to high loads of ammonium and organic matter. Therefore, the conventional method requires a large-scale treatment plant due to the long hydraulic retention time required for nitrification [1-3].

Recently, many bacteria are known to be capable of heterotrophic nitrification and aerobic denitrification [4-10]. The removal rates of these bacteria were calculated as in the range of 0.08 to 0.6 kg $NH_4$-N/$m^3$/day for an initial ammonium concentration in the range of 30 to 300 mg $NH_4$-N/L. These experiments were conducted in flasks in laboratories under low-strength ammonium conditions.

In a previous study [11], we showed that *A. faecalis* No.4 (No.4) has the ability to carry out the following heterotrophic nitrification and aerobic denitrification, $NH_4^+ \rightarrow NH_2OH \rightarrow N_2O \rightarrow N_2$. Approximately 40% of ammonium and 60% of ammonium were converted to $N_2$ gas and cell mass, respectively. Only a few percents of $NO_2^-$ and $NO_3^-$ were produced from ammonium. No.4 removed more than 90% of high-strength ammonium and COD from crude piggery wastewater without dilution of the wastewater [12]. No.4 also exhibited an ammonium removal rate of 3 kg $NH_4$-N/$m^3$/day in the treatment of anaerobically digested sludge from a municipal wastewater plant [13]. This value is a few hundred-fold higher than that in conventional treatment method.

An anammox method has been intensively studied as an alternative to the conventional method mainly because this operation does not require the addition of a carbon source [14-17]. However, the extremely slow growth rate of anammox bacteria and the vulnerability of these bacteria to high concentrations of organic carbon and ammonium must be addressed to enable the development of a practical wastewater treatment system.

Some wastewaters from chemical companies or power-generation plants contain a high concentration of ammonium and a small amount of BOD. In this study, No.4 was applied to a wastewater from a chemical company to assess the possibility of the efficient biological treatment of high-strength ammonium under dissolved oxygen-controlled condition.

## Materials and Methods

### Strain used

The detailed characteristics of No.4 are described in previous papers [11]. Cultured cells of No.4 were mixed with a 50% glycerol solution in vials and stored at -84°C. For each pre-culture, one vial was used as the No.4 inoculums.

### Medium used

A synthetic medium containing (all in units of g per liter) 14$K_2HPO_4$, 6$KH_2PO_4$, 15 trisodium citrate dihydrate, 2$(NH_4)_2SO_4$, and 0.2$MgSO_4 \cdot 7H_2O$, in 2 mL of trace mineral solution was used for the pre-culture of No.4. The trace mineral solution contained the following components (g/liter): 57.1 EDTA (2,2',2",2'"-(ethane-1,2-diyldinitrilo) tetra acetic acid) • 2Na, 3.9$ZnSO_4 \cdot 7H_2O$, 7$CaCl_2 \cdot 2H_2O$, 5.1$MnCl_2 \cdot 4H_2O$, 5.0$FeSO_4 \cdot 7H_2O$,1.1$(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 1.6$CuSO_4 \cdot 5H_2O$, and 1.6$CoCl_2 \cdot 6H_2O$. This medium was sterilized for the use of pre-culture, but in fed-batch treatment culture, no sterilization was conducted.

### Wastewater used

The wastewater (WC), which was supplied by a Japanese chemical company, was produced from the chemical processes conducted by the company. The main characteristics of the WC are as follows: pH 10.6, total COD concentration of 2280 mg/L, total BOD concentration of less than 2 mg/L, total-nitrogen concentration of 4840 mg/L, and ammonium-nitrogen concentration of 4800 mg/L. In each experiment, the pH of the original WC was adjusted to approximately 7.5 by 5N $H_2SO_4$, and the ammonium concentration of the pH-adjusted WC was diluted to approximately 1000 mg/L unless specifically described.

### Reactors used

In preliminary experiments, 500 mL shaking flasks (working volume of 100 mL) were used to confirm the growth of No.4 and the removal of ammonium from WC by No.4 at 30°C and a shaking speed of 100 strokes per min (spm). A small-scale jar fermenter (total volume of 1 liter, working volume of 300 mL; BMJ-01PI, Able Corp., Tokyo, Japan) was then used. The dissolved oxygen (DO) concentrations and pH values were monitored with a DO sensor (SDOC-12F, Able Corp., Tokyo, Japan) and a pH sensor (Easyferm Plus 225, Hamilton Bonaduz AG, Bonaduz, Switzerland) inserted into the fermenter. The temperature was maintained at 30°C. The DO concentration was controlled at 2-3 mg/L by changing the agitation speeds with a constant air supply rate of 30 mL/min.

### Preliminary experiments using shaking flasks

The pH-adjusted and ten-fold-diluted WC solution was mixed with a culture of No.4 cells, trisodium citrate dehydrate and inorganic compounds excluding $(NH_4)_2SO_4$ in a shaking flask, and the removal of ammonium was measured. As a control, No.4 was grown in the synthetic medium.

### Experimental procedure in a jar fermenter

The No.4 cells were pre-cultivated in 100 mL of synthetic medium in a 500 mL shaking flask at 30°C with a shaking speed of 100 spm for two days, and the culture was used as inoculums.

A repeated-batch culture was conducted. In the first repeated-batch experiment, 50 mL of the pre-culture of No.4, 250 mL of diluted WC, 20 g of trisodium citrate dehydrate and inorganic nutrients excluding $(NH_4)_2SO_4$ were mixed in the fermenter, and the ammonium treatment was then monitored. The inorganic nutrients were fortified because the content of inorganic components in the original WC was unknown. Twenty grams of trisodium citrate dehydrate was used to avoid limited carbon supply to No.4. One milliliter of the culture was sampled periodically, and the concentration of ammonium was determined. After the ammonium concentration was confirmed to be reduced by more than 90% of the initial concentration, 10-50 mL of the culture was used as the inoculums for the subsequent treatment with fresh 250-290 mL of four times diluted WC and trisodium citrated hydrate and inorganic nutrients. The cell numbers of No.4 were determined at the start and end of each cycle of batch cultivation.

### Analytical method

The ammonium concentration was determined using an ammonium sensor (SNH-10, Able Corp., Tokyo, Japan). Nitrite and nitrate concentrations after several repeated-batch operation were determined by the ferrous sulfate method and the dimethylphenol method (HACH Company, Loveland, Colorado, USA), respectively. The ammonium exhausted from the reactor by aeration was trapped in the 0.1N $H_2SO_4$ solutions and the accumulated ammonium was determined. To determine the number of No.4 cells, the sampled culture was diluted and plated on synthetic agar plates containing the synthetic medium and 1.5% agar, and the plates were then incubated at 30°C for two days. Because it was previously confirmed that No.4 grew on the plates significantly faster than other cells indigenous to the WC samples and that No.4 exhibited characteristic morphological features, the colonies that appeared on the plates after two days were counted as No.4 cells, and the cell concentration was expressed as cells/mL.

## Results and Discussion

### Flask experiments

In preliminary experiments, shaking flasks were used to assess the removal of ammonium in the WC sample by No.4. The result is shown in Figure 1. Because the ammonium removal pattern obtained for the WC sample was similar to that obtained for the synthetic medium, the use of the WC sample as the nitrogen source appeared to have no adverse effect on the activity of No.4. The final cell numbers for the two samples were almost identical (data not shown). The ammonium removal rates for the two samples were 0.1-0.2 kg $NH_4$-N/$m^3$/day and the lower values are presumably due to the limited oxygen supply.

### Ammonium removal in the repeated-batch experiment

Figure 2 shows the change in the ammonium concentration over times in a repeated-batch experiment at 30°C, and Figure 3 shows the change in the number of No.4 cells during the same experiment. More than 90% of ammonium was removed within 24-30 h, and the number of No.4 cells varied between $10^8$ and $10^{10}$ cells/mL. The average ammonium removal rate during the experimental period was 1.1 kg $NH_4$-N/$m^3$/day. This value is significantly higher than that obtained with conventional nitrification-denitrification processes, 0.01 kg-N/$m^3$/day [1] and similar to that obtained with an efficient anammox process, 1-2 kg-N/$m^3$/day [16-17]. Between 620 and 800 h, the operation was stopped, and the jar fermenter was maintained static at room temperature (average 10°C). When the operation was resumed, ammonium removal was observed without any delay, indicating that the interruption in the operation exerted no adverse effect on the activity of No.4.In these experiments, the pH values were fluctuated between 7 and 8 which is within the optimal pH range of No.4 (data not shown). Total amounts of nitrite, nitrate and exhausted ammonium from the reactors were less than 2% of inlet nitrogen and thus the majority of inlet ammonium was converted into $N_2$ gas and the cellular nitrogenous compounds.

The aeration of No.4 free samples that contained WC, and inorganic nutrients with or without trisodium citrate dihydrate for five days at an agitation speed of 600 rpm and an aeration speed of 300 mL/min in a jar

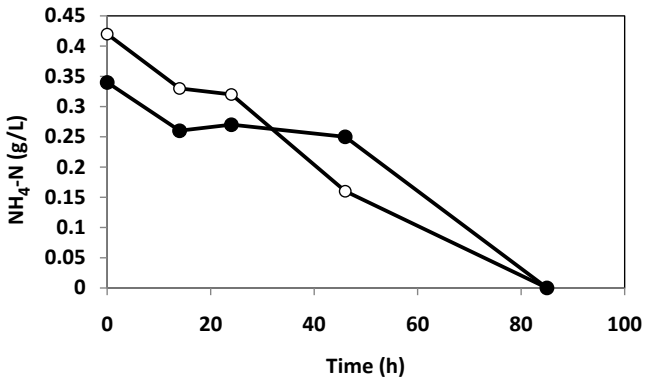


**Figure 1:** Ammonium removal by flask cultivation: shaking flask containing synthetic medium plus No.4 pre-culture (○), and ten-fold-diluted WC plus synthetic medium excluding $(NH_4)_2SO_4$ and No.4 pre-culture(●).

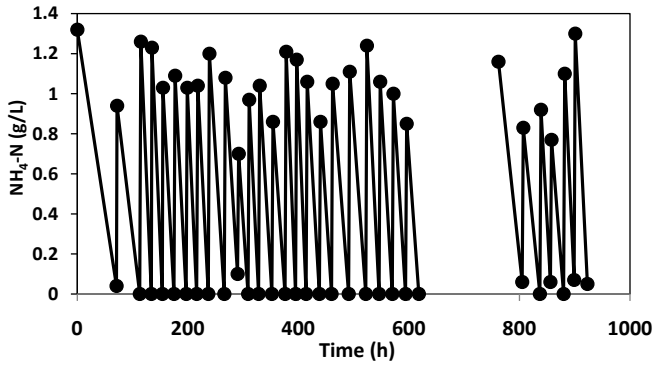


**Figure 2:** Ammonium concentration in the WC during repeated-batch treatment with No.4 at 30°C and at controlled DO concentration of 2-3 mg/L.

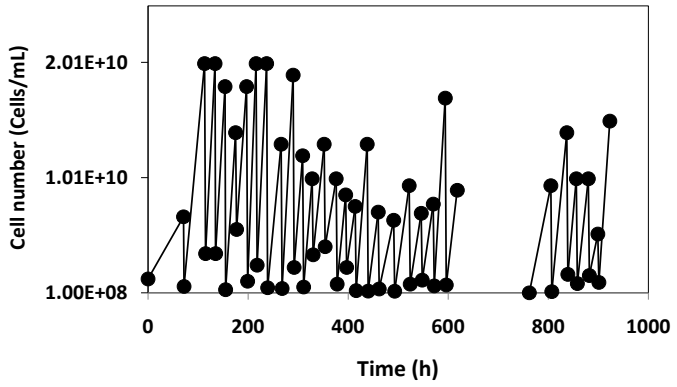


**Figure 3:** Change in the number of No.4 cells in the same experiment shown in Figure 2.

fermenter, did not result in decrease in the ammonium concentrations and the DO (data not shown). This finding indicated that the contribution of introduced air-borne microorganisms to the removal of ammonium was negligible, and the removal of ammonium from WC sample observed in this study was thus primarily achieved by No.4.

Because the WC used in this research contained the least amount of BOD for No.4, the supply of carbon sources is essential. The experimental protocol used in this study included excessive 20 g of trisodium citrate dehydrate to avoid carbon-limiting in the activity of No.4. In general, the C/N ratio of the intracellular components in microorganisms is 10, indicating that 10 units of carbon are used to synthesize cellular materials when 1 unit of N is consumed. In previous isotope experiments, 40% of ammonium was converted to nitrogen gas by No.4 [11]. Assuming a similar level of denitrification in this study in the presence of 1 g-N/L, 0.6 g-N/L was used for the cell synthesis, indicating that at least 6 g-N/L is required. Twenty grams of trisodium citrate dihydrate per 300 mL contained 16 g-C/L in the reactor. Actually, when the excessive citrate was decreased to 10 g, complete removal of ammonium was still observed (data not shown). The 10 g of trisodium citrate dehydrate contained 2.5 g carbon. Thus, for the complete removal of the initial 0.3 g of $NH_4$-N in this 300 ml reactor, approximately 3 g of carbon based on C/N ratio 10 in general metabolism was necessary. In this regard, we assumed that 10 g may be minimum amount of carbon to be added.

The power requirement in wastewater treatment is an important factor in the operation. In other experiment in which the air supply rate was decreased from 30 mL/min to 3 mL/min and DO concentration was less than 0.5 mg/L, significantly slow ammonium removal was observed. Thus, DO concentration should be more than 0.5 mg/L and the control of the DO level through the maintenance of lower agitation speeds will minimize the power requirement.

When the carbon requirement of No.4 was compared with that in the conventional denitrification process using methanol, the amount of carbon necessary for the No.4 system was two to three-fold higher [13]. In this respect, the No.4 process is disadvantageous. However, as a total system, the No.4 process is advantageous over the conventional process because no dilution of high-strength wastewater is required and thus, only a single compact-size reactor is needed. It is also easy to cultivate No.4 cells in synthetic medium with a doubling time of 2-3 h, and immobilization of the cells on different immobilization carriers is possible [13]. This high activity of cultured No.4 cells is maintained for several months of storage at 4°C, and the cells remained tolerant to high osmotic pressure (see below).

A significantly high removal rate is possible when less expensive carbon sources from waste or unused resources are available mainly because these materials contain a mixture of unused carbon materials and least amount of sugars. These materials are favorable for the activity of No.4 because No.4 utilizes fatty acids or some kinds of solvents which are less degradable for common bacteria.

### Ammonium removal at initial ammonium concentrations of 1000, 2000 and 5000 mg $NH_4$-N/L

Figure 4 shows the ammonium removal obtained with initial ammonium concentrations of approximately 5000 mg $NH_4$-N/L, 2000 mg $NH_4$-N/L and 1000 mg/L. For concentrations of 5000 mg $NH_4$-N/L and 2000 mg $NH_4$-N/L, an intermittent supply of 20 g of trisodium citrate dihydrate was introduced, as indicated by the arrows in Figure 4. The average ammonium removal rates for 1000, 2000 and 5000 mg $NH_4$-N/L were 0.63, 0.96 and 0.92 kg $NH_4$-N/$m^3$/day, respectively. This indicates that even ammonium concentrations higher than 1000 mg $NH_4$-N/L were removed efficiently by supplying a sufficient amount of the carbon source. In these experiments, inorganic nutrients were added at the start of the experiment. The increase in ammonium removal at 100 h was not clear after addition of carbon. The reason for it is not clear, but presumably some ingredients might be limited after more than 3000 mg-N was removed.

The BOD value in this wastewater was low and it is thus necessary to add an external carbon source to the treatment process. Other available

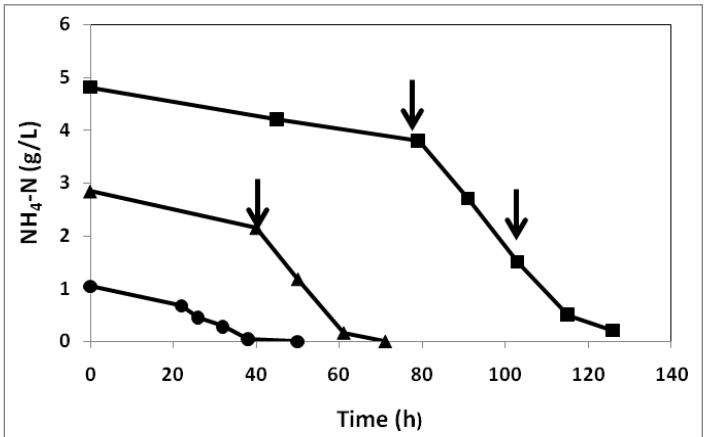


**Figure 4:** Change in the initial ammonium concentrations when 5000 mg $NH_4$-N/L (■), 2000 mg $NH_4$ N/L (▲) and 1000 mg $NH_4$-N/L (●) of WC were used in a batch culture. The arrows indicate the times at which citrate was added.

carbon sources for No.4 are known to be low- and high-molecular-weight fatty acids, phenol, methanol, some solvents and amino acids [13]. Inexpensive carbon sources containing organic fatty acids can be obtained as wastes from food industry or acid production stage in an anaerobic digestion process. The application of alkaline hydrolysis or thermal treatment of sludge is one possible approach to supply inexpensive and convenient volatile fatty acids (VAFs) [18-20]. The combination of the sludge treatment system with the No.4 system may be a promising method.

The main reason for using citrate was because all basic data in the first manuscript were obtained using citrate as a carbon source and a huge amount of citrate-containing waste was produced in citrus farming in Japan. As different carbon sources are available for No.4, selection of carbon sources will depend on the waste-producing areas.

In this study, the only temperature used was 30°C. The ammonium removal achieved by No.4 was observed at the temperature range between 15°C and 37°C, although the optimal ammonium removal was obtained at 30°C. In a previous study [13], we showed that the ammonium removal rate at 20°C was half of that obtained at 30°C, but the rate was still significantly high.

### Ammonium removal under high salt conditions

No.4 exhibited the unique feature of removing ammonium under high salt conditions. Figure 5 shows change in the ammonium concentration in the cultivation of No.4 in synthetic medium containing 0, 3 and 6% NaCl in shaking flasks. Ammonium removal began after induction periods of one day at 3% NaCl and five days at 6% NaCl, and the ammonium removal rates were similar to those found in the presence of 0% NaCl. NaCl was then added to the WC to a final concentration of 3%, and repeated-batch treatment was conducted using a protocol similar to that described above. The result of this experiment is shown in Figure 6. The ammonium removal rate reached 1.0 kg $NH_4$-N/$m^3$/day with the four-batch operation after the gradual acclimation of No.4 to the saline medium.

Although No.4 is not osmophilic, the cells were able to achieve ammonium removal under high saline conditions. In our basic experiment, No.4 was found to synthesize the osmoprotectant, hydroxyectoine during the lag time when the cells were exposed to high salt concentrations [13]. Because most microorganisms are vulnerable to wastewater with high saline concentrations or high-strength solvents due to the resulting high osmotic pressure, No.4 is able to effectively remove ammonium under such conditions after a certain acclimation period. Thus, these new systems can remove high-strength ammonium from marine aqua-culture wastewater or fishery processing wastewater.

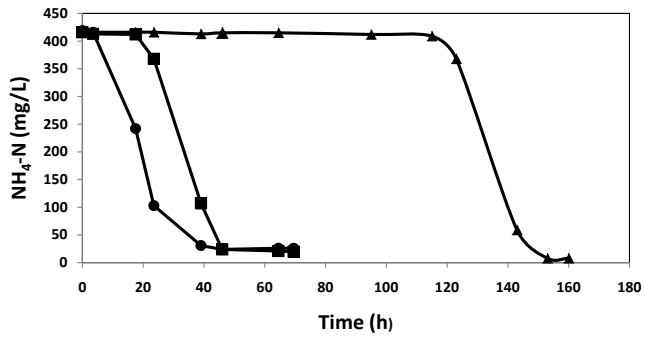


**Figure 5:** Ammonium removal by No.4 in shaking flasks containing 100 mL of synthetic medium at 30°C with 0% NaCl (●), 3% NaCl (■) and 6% NaCl (▲).

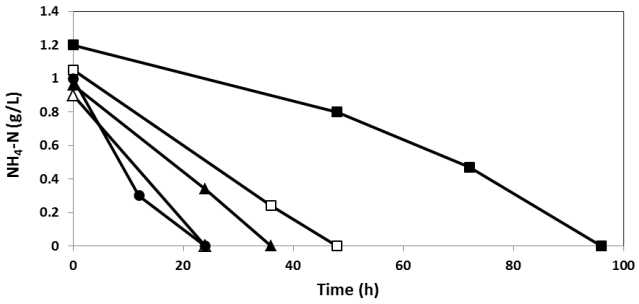


**Figure 6:** Ammonium removal by No.4 from WC containing 3% NaCl in a repeated- batch experiment. Symbols: 1st (■), 2nd (□), 3rd (▲), 4th (□), and 5th cycles (●).

Another advantage of No.4 is that No.4 cells can suppress the growth of plant pathogens, as previously reported [21,22]. This finding indicates that the excess sludge of No.4 generated in the treatment facility can be reutilized in farmland to protect against plant diseases. The cells of No.4 were also found to contribute to reduction of methane production from rumen [23].

Concerning the potential implication of wastewaters having high COD and extremely low BOD on biological treatment operation, especially in chemical companies, they also produce several biodegradable chemicals with high BOD. Therefore, mixing of biodegradable compounds into these wastewaters with high COD and low BOD will lead to biological treatment of ammonium.

## Conclusion

*A. faecalis* No.4 which is capable of heterotrophic nitrification and aerobic denitrification, effectively removed 1000-5000 mg $NH_4$-N/L from a wastewater containing a small amount of BOD. Citrate was used as a carbon source and a significantly high removal rate of ammonium was achieved. The average cell density of $10^9$ cells/mL was maintained.

Under high osmotic pressure, No.4 exhibited almost the same removal rate for ammonium as that obtained under non-osmotic pressure, resulting in an additional use of No.4. No.4 is easy to cultivate, the growth rate is higher, and a simple reactor is required and thus a simpler ammonium treatment may be possible.

As the cells of No.4 have potential to be utilized in other agricultural areas, the excessive sludge problems associated with wastewater treatment system will be solved. Thus, an efficient ammonium removal system using No.4 will allow the development of an advanced treatment system for ammonium removal.

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