

## Simultaneous Removal of High-Strength Ammonium and Phosphorus by *Alcaligenes faecalis* No. 4

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### Abstract

*Alcaligenes faecalis* No. 4 (No. 4), which conducts heterotrophic nitrification and aerobic denitrification, removed 800 mg-N/l of high-strength ammonium and 100 mg-P/l of phosphorus, both in a synthetic medium and in an anaerobically digested municipal sludge solution. The estimated  $N_2$  conversion ratio from ammonium-nitrogen was approximately 50%. When the initial phosphorus concentration was reduced to approximately 30 mg-P/l in the synthetic medium, the phosphorus limited the growth of No. 4 after 8 h when more than 90% of P had been consumed, and then stopped the growth of No. 4 after that. The  $N_2$  conversion ratio from ammonium-nitrogen was nearly 100%.

**Keywords:** *Alcaligenes faecalis*; High-strength of ammonium; Phosphorus removal;  $N_2$  conversion ratio

### Introduction

Many bacteria are capable of heterotrophic nitrification and aerobic denitrification [1-5]. The use of these bacteria for ammonium removal is more advantageous than the conventional nitrogen removal process by aerobic nitrification and anaerobic denitrification because the ammonium removal occurs in one reactor using one type of bacterium under aerobic conditions. The ammonium removal rates of these bacteria are higher than those of the conventional ammonium removal process mainly because of their high growth rates and short hydraulic retention time.

*Alcaligenes faecalis* No. 4 (No. 4) is one of the bacteria that have ability to carry out heterotrophic nitrification and aerobic denitrification, and its several applications have been demonstrated. No. 4 carried out the following heterotrophic nitrification and aerobic denitrification processes,  $NH_4^+ \rightarrow NH_2OH \rightarrow N_2O \rightarrow N_2$  and approximately 40% and 60% of the ammonium-nitrogen were converted to  $N_2$  gas and cell mass, respectively. Only a small percentage of  $NO_2^-$  and  $NO_3^-$  were produced from the ammonium [6]. No. 4 removed more than 90% of the high-strength ammonium and chemical oxygen demand (COD) from crude piggery wastewater without diluting the wastewater [7]. No. 4 also removed ammonium at a rate of 3 kg- $NH_4$ -N/ $m^3$ /day in the treatment of anaerobically digested sludge from a municipal wastewater plant [8]. Wastewater from a chemical company that contained a high concentration of ammonium 5,000 mg- $NH_4$ -N/l and a small amount of BOD was treated using No. 4 and the average ammonium removal rate was 1.1 kg- $NH_4$ -N/ $m^3$ /day [9]. No. 4 was used to treat coking wastewater (CW) to remove 400 mg/l of high-strength ammonium-nitrogen and 400 mg/l of phenol from coking wastewater [10]. These removal rates were several hundred-fold higher than that of the conventional treatment method.

As No. 4 primarily uses organic acids as a carbon source, and no sugar is available in practical treatment, inexpensive production and supply of organic acids is a key for the materialization of No. 4 in ammonium treatment. We conducted anaerobic fermentation using leachate from a municipal waste dumping site as seed supplemented with sugar to

obtain a high organic acid solution, and the prepared mixture of organic acid solution was supplemented with high-ammonium and low-carbon wastewater by balancing the total organic carbon (TOC) and  $NH_4$ -N. The effectiveness of this solution was confirmed [11]. In these studies, phosphorus removal was not a focus. Some wastewaters containing high-strength ammonium also contains a high concentration of phosphorus with more than 100 mg-P/l, and simultaneous biological removal of high-strength ammonium nitrogen and phosphate is difficult. In this study, the simultaneous removal of nitrogen and phosphorus was confirmed using No. 4 in synthetic artificial wastewater and in an anaerobically digested municipal sludge solution. Under low phosphorus conditions, the  $N_2$  conversion efficiency was found to be nearly 100%.

### Materials and Methods

#### Strain

The detailed characteristics of No. 4 were described in a previous paper [6]. Cultured cells from No. 4 were mixed in vials with a 50% glycerol solution and stored at -80°C. For each pre-culture, one vial was used as the No. 4 inoculum.

#### Synthetic medium

A synthetic medium containing (in g per liter) 14  $K_2HPO_4$ , 6  $KH_2PO_4$ , 12.5 sodium lactate, 2  $(NH_4)_2SO_4$ , 0.2  $MgSO_4 \cdot 7H_2O$ , and 2 ml of trace mineral solution was used for the pre-culture of No. 4. The tracemineral solution contained the following components (in g per liter): 57.1 EDTA (2,2',2'',2'''-(ethane-1,2-diyl)dinitrilo)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  $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$ , 1.6  $CuSO_4 \cdot 5H_2O$ , and 1.6  $CoCl_2 \cdot 6H_2O$ .

#### Wastewater used

The anaerobically digested sludge was supplied by the Yokohama Municipal Sewage Treatment Center (Yokohama, Japan) where the excess municipal dehydrated activated sludge was digested at 37°C in a 6,000 ton-scale anaerobic digester. The main characteristics of the digested

sludge were as follows: pH 7.3, volatile fatty acids concentration 24 mg/l, ammonium-nitrogen concentration 1000 mg/l, and phosphorus concentration 100 mg/l.

Artificial wastewater that contained approximately 800 mg-N/l, 100~40 mg-P/l and 10 g-C of lactate/l was prepared based on the synthetic medium described above.

### Reactors used

A small-scale jar fermenter (total volume of 1 liter, working volume of 300 ml; BMJ-01PI, Able Corp., Tokyo, Japan) was 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 agitation speed was controlled at 650 rpm with a constant air supply rate of 30 ml/min to guarantee the DO concentration remained at greater than 2 mg/l.

### Experimental procedure

The pre-culture that was prepared using the synthetic medium was used as inoculum for the following experiments.

The two artificial wastewater samples based on the synthetic medium were prepared. The first contained approximately 800 mg-N/l and 100 mg-P/l, which simulated the contents of ammonium and phosphorus in the anaerobically digested sludge. The second contained approximately 800 mg-N/l and 30 mg-P/l. Both wastewater samples were mixed with approximately 10 g/l of lactate and a pre-culture of No. 4, and the change in N, P and C was monitored in a jar fermenter.

Two hundred thirty milliliters of the anaerobically digested sludge solution, 50 ml of lactate solution, and 20 ml pre-culture of the No. 4 were put into the jar fermenter and the removal of N, P and C concentrations was also monitored.

### Analytical method

Ammonium concentration was determined using an ammonium sensor (SNH-10, Able Corp., Tokyo). Concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were determined by the ferrous sulfate method (TNT840) and the dimethylphenol method (TNT835) of the HACH Company (Colorado, USA).

Samples of 20~50 ml were centrifuged at 10,000 rpm at 4°C, and the precipitated cell mass was rinsed with sterile distilled water and dried at 100°C for 2 days after centrifugation of the rinsed cell mass. The dried cell mass weight of No. 4 was measured, and the elemental analysis of the dried cell mass was determined at KURITASU Analyzing CO., Ltd., (Tukuba, Japan).

For determining the cell number of No. 4, the sample culture was diluted and plated on synthetic agar plates that contained the synthetic medium and 1.5% agar, and the plates were incubated at 30°C for 2 days. Since it was previously confirmed that No. 4 grew on the plates significantly faster than other cells indigenous to the anaerobically digested sludge, and the colonies that appeared on the plates after 2 days, which also exhibited the characteristic morphological features of No. 4, were counted as No. 4. The cell concentration was expressed as cells/ml.

The ammonium exhausted from the jar fermenter by aeration was trapped in the 0.1 N  $\text{H}_2\text{SO}_4$  solution, and the accumulated ammonium was determined. The lactate concentration was determined with the biosensor, BF-7S/D (Model BioFlow STAT, Oji Scientific Instruments Co., Ltd., Hyogo, Japan). The phosphorus concentration was determined by JIS (Japan Industrial Standard) K0102.4601.

## Results and Discussion

### Synthetic medium with phosphorus concentration of 100 mg/l

The artificial wastewater that was based on the synthetic medium with 750 mg-N/l and 125 mg-P/l, of which the values were similar to the contents of the anaerobically digested sludge wastewater, was prepared, and the removal of N, P and C by No. 4 was monitored as shown in Figure 1. The removed patterns of  $\text{NH}_4\text{-N}$  and phosphorus (P) were similar, and both were simultaneously exhausted after 22 h. When  $\text{NH}_4\text{-N}$  and phosphorus (P) became low enough to stop the growth of No. 4, the DO concentration began to increase. The consumption of lactate proceeded during this time and the change in pH value was minimal.

**Nitrogen balance:** Nitrogen balance in the reactor was as follows.

$$N(\text{input}) = N(\text{residual}) + N(\text{synthesized into cells}) + \text{NH}_3(\text{evaporated}) + \text{NO}_2^-(\text{produced}) + \text{NO}_3^-(\text{produced}) + \text{N}_2(\text{converted from NH}_4\text{-N}). \quad (1)$$

Therefore,

$$\text{N}_2(\text{converted from NH}_4\text{-N}) = N(\text{input}) - N(\text{residual}) - N(\text{synthesized into cells}) - \text{NH}_3(\text{evaporated}) - \text{NO}_2^-(\text{produced}) - \text{NO}_3^-(\text{produced}). \quad (2)$$

The values of each term measured after 22 h, were introduced into the equation (2).

$$N(\text{input}) - N(\text{residual}) = 750 \text{ mg/l} - 0 \text{ mg/l} \quad (3)$$

The increase in dried cell mass after 22 h was 5.39 g/l. (4)

From the elemental analysis of the dried cell mass, the N content was 7.9%. (5)

$$\text{Therefore, intracellular N content} = 5.39 \times 0.079 = 426 \text{ mg/l}. \quad (6)$$

The measured values of  $\text{NH}_3$ (evaporated),  $\text{NO}_2^-$ (produced) and  $\text{NO}_3^-$ (produced) were 5.0 mg/l, 1.3 mg/l and 2.8 mg/l, respectively.

$$\text{Therefore, N}_2(\text{converted from NH}_4\text{-N}) = 750 - 426 - 5 - 1.3 - 2.8 = 315 \text{ mg/l}. \quad (7)$$

$$\text{The N}_2 \text{ conversion ratio from NH}_4\text{-N} = (315/750) \times 100 = 42\%. \quad (8)$$

This value was similar to the values reported in the previous paper [6].

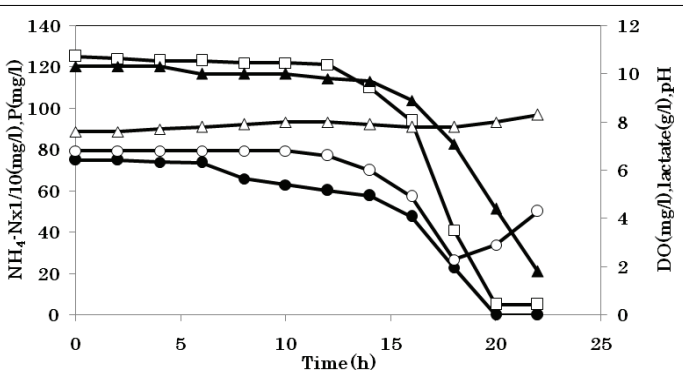
**Carbon consumption:** The consumed lactate = initial concentration - concentration after 22 h = 10.3 - 4.4 = 6.2 g/l. The carbon content of lactate =  $(6.2 \times 12 \times 3)/90 = 2.48$  g/l. Then, the ratio of consumed C/ consumed N = 2.48/0.75 = 3.3.

In our previous paper [6], we demonstrated that the optimal C/N ratio needed to ensure simultaneous exhaustion of carbon and nitrogen was 10 in the synthetic medium with the phosphorus concentration set at 100 times higher than the present experimental value in order to control the pH value. This indicates that the lower phosphorus concentration is more economical in the treatment of high-strength ammonium in terms of the carbon demand.

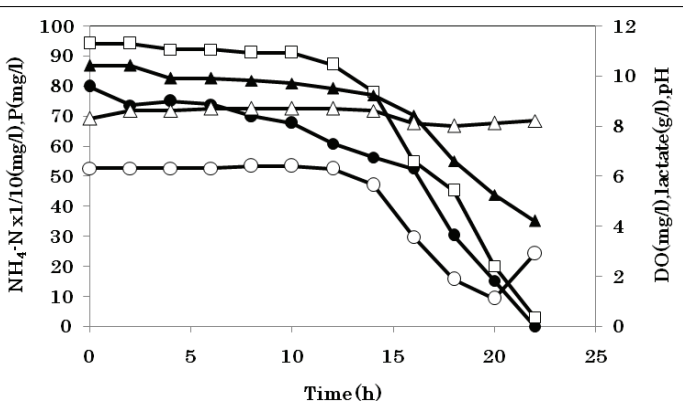
For the removed phosphorus,  $P = 125 - 5 = 120$  mg/l. Therefore, the accumulated P in the cells was  $(120/5390) \times 100 = 2.2\%$ . This value is similar to the intracellular phosphorus content in common bacteria.

### Anaerobically digested sludge wastewater

The crude anaerobically digested sludge wastewater from Yokohama City was used to investigate the removal of N and P under similar operational conditions as those described above for the synthetic medium. The result is shown in Figure 2. The removal pattern shown in Figure 2 is similar to that of Figure 1.  $\text{NH}_4\text{-N}$  and phosphorus in the wastewater were simultaneously removed almost completely after 22 h.



**Figure 1:** Removal of ammonium-nitrogen ( $\text{NH}_4\text{-N}$ ) (●), phosphorus (P) (□) and lactate (▲) by No. 4 in the artificial wastewater that contained 750 mg-N/l and 125 mg-P/l. Dissolved oxygen (DO) concentration (○) and pH (△) are also shown. The original ammonium-nitrogen ( $\text{NH}_4\text{-N}$ ) values were divided by 10 to make the figure clear.



**Figure 2:** Removal of ammonium-nitrogen ( $\text{NH}_4\text{-N}$ ) (●), phosphorus (P) (□) and lactate (▲) by No. 4 in the anaerobically digested sludge that contained 797 mg-N/l and 95 mg-P/l. Dissolved oxygen (DO) concentration (○) and pH (△) are also shown. The original ammonium-nitrogen ( $\text{NH}_4\text{-N}$ ) values were divided by 10 to make the figure clear.

The nitrogen balance was calculated by the method described above:  $\text{N}(\text{input}) - \text{N}(\text{residual}) = 797 \text{ mg/l}$ .

The increase in cell mass was 4.57 g/l.

From the elemental analysis of the dried cell mass, the N content was 7.9%.

Therefore, intracellular nitrogen content =  $4.57 \times 0.079 = 361 \text{ mg/l}$ .

Measured values of  $\text{NH}_3$  (evaporated),  $\text{NO}_2^-$  (produced) and  $\text{NO}_3^-$  (produced) were 8.0 mg/l, 9.2 mg/l and 1.4 mg/l, respectively. Therefore,  $\text{N}_2$  (converted from  $\text{NH}_4\text{-N}$ ) =  $797 - 361 - 8 - 9.2 - 1.4 = 417 \text{ mg/l}$ . The  $\text{N}_2$  conversion ratio from  $\text{NH}_4\text{-N}$  =  $(417/797) \times 100 = 52\%$ .

**Carbon consumption:** The consumed lactate =  $10.4 - 4.21 = 6.19 \text{ g/l}$ . The carbon content of the lactate = 2.48 g/l. Then, the ratio of consumed C/ consumed N =  $2.48/0.797 = 3.1$ . These results were similar to those in the synthesized data described above. In this experimental condition, the removal of P was 91 mg/l. Therefore, when the initial concentration is 100 mg-P/l in the anaerobically digested sludge solution, the  $\text{NH}_4\text{-N}$  concentration of 876 mg/l will be enough for the complete removal of phosphorus. Thus, nitrogen and phosphorus can be removed almost simultaneously from the crude wastewater by adding No. 4 because the crude solution contained approximately 1000 mg-N/l.

### Synthetic Medium when the Initial P Concentration was 38 mg/L

A unique phenomenon was discovered when the initial phosphorus level was reduced to approximately 30 mg-P/l. The experimental procedures were similar to those above except that the initial P concentration was reduced to 30 mg/l. The result is shown in Figure 3. Phosphorus decreased from 38 mg/l to 3 mg/l after 8 h, and this concentration of P limited the growth of No. 4. The decrease in  $\text{NH}_4\text{-N}$  and lactate continued after 8h. Therefore, the analysis was divided into two parts, 0~8 h and after 8 h.

#### Analysis during 0~8h

$\text{N}(\text{input}) - \text{N}(\text{residual}) = 936 - 440 = 496 \text{ mg/l}$ .

The increase in dried cell mass at 8h was 2.66 g/l.

The N content determined by elemental analysis of the dried cell mass was 9%.

The intracellular nitrogen content =  $2.66 \times 0.09 = 239 \text{ mg/l}$ .

The measured values of  $\text{NH}_3$  (evaporated),  $\text{NO}_2^-$  (produced) and  $\text{NO}_3^-$  (produced) were 0.4 mg/l, 6 mg/l and 7 mg/l, respectively.

Therefore,  $\text{N}_2$  (converted from  $\text{NH}_4\text{-N}$ ) =  $496 - 239 - 0.4 - 6 - 7 = 244 \text{ mg/l}$ .

The  $\text{N}_2$  conversion ratio from  $\text{NH}_4\text{-N}$  =  $(244/496) \times 100 = 49\%$ .

This value was similar to those in the two experiments shown above.

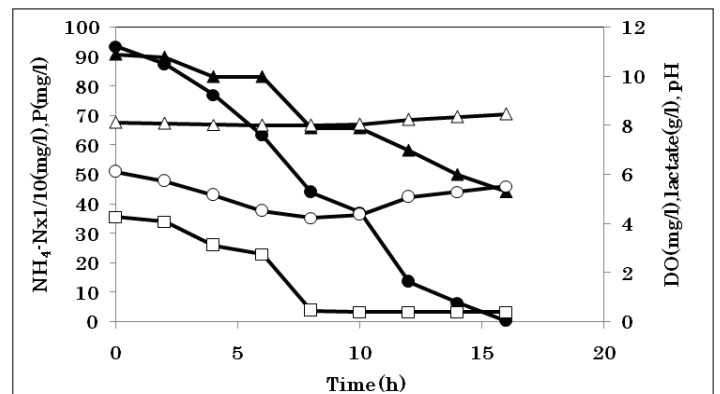
**Carbon consumption:** The consumed lactate =  $10.9 - 7.9 = 3 \text{ (g/l)}$ . The carbon content of lactate = 1.2 g/l. Consumed C/ consumed N =  $1.2/0.496 = 2.4$ . This value indicates that the carbon demand during this stage was one-fourth that of the previously reported value [6].

#### Analysis after 8h

As shown in Figure 3, after 8h, the phosphorus concentration was lowered to limit the growth of No. 4, primarily because the DO concentration stopped decreasing and gradually began to increase. Using the change in concentrations of  $\text{NH}_4\text{-N}$  and lactate, a similar analysis was conducted.

$\text{N}(\text{input}) - \text{N}(\text{residual}) = 440 \text{ mg/l}$ . The increase in cell mass after 8h was 0.96 g/l. The N content determined by elemental analysis of the dried cell mass was 9.4%. The intracellular nitrogen =  $0.96 \times 0.094 = 90 \text{ mg/l}$ .

The measured values of  $\text{NH}_3$  (evaporated),  $\text{NO}_2^-$  (produced) and  $\text{NO}_3^-$  (produced) were 0.4, 0.4 and 0.7 mg/l, respectively.



**Figure 3:** Removal of ammonium-nitrogen ( $\text{NH}_4\text{-N}$ ) (●), phosphorus (P) (□) and lactate (▲) by No. 4 in the artificial wastewater which contained 936 mg-N/l and 38 mg-P/l. Dissolved oxygen (DO) concentration (○) and pH (△) are also shown. The original ammonium-nitrogen ( $\text{NH}_4\text{-N}$ ) values were divided by 10 to make the figure clear.

Therefore,  $N_2$  (converted from  $NH_4^+-N$ )= $440-90-0.4-0.4-0.7=349$  mg/l.

The  $N_2$  conversion ratio from  $NH_4^+-N$ =( $349/440$ )  $\times$  100=79%.

During this time, the cell numbers at 8 h and 16 h were  $1.10 \times 10^9$  and  $1.12 \times 10^9$  cells/ml, respectively. This indicates that both the increase in cell number and the nitrogen incorporated into the cell synthesis were negligible.

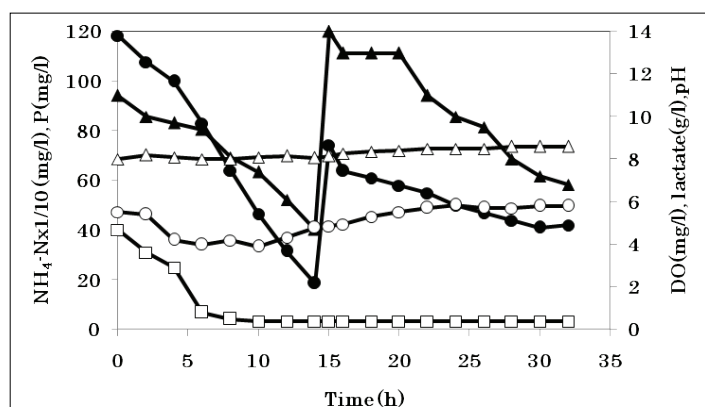
Therefore, the conversion ratio to  $N_2$ =( $(440-0.4-0.4-0.7)/440$ )  $\times$  100=99.6%.

As the weight of the cell mass increased, it was assumed that No. 4 accumulated residual carbon into the intracellular materials. We have shown that this bacterium conducts the synthesis of some intracellular substances under adverse environmental conditions. For example, under the high osmotic pressure conditions where no growth occurred, No. 4 synthesized the osmoprotectant, hydroxyectoine [7]. Under this P-limited condition, the cells color turned pink, indicating the synthesis of colored substances. The intracellular carbon content after 8 h was 45.1%, compared with the carbon content before 8 h, which was 36.8%. This supports the idea that carbon content increases intracellularly.

### The Possibility of Continued $N_2$ Production by Adding $NH_4^+-N$ and Lactate

Figure 3 suggests that  $N_2$  production continued without cell growth of the cell in No. 4. The possibility of continued  $N_2$  production after a 14 h operation was tested by adding more  $NH_4^+-N$  and lactate. Figure 4 shows the result when 14 g/l of lactate and 700 mg/l of N were added. Although the ammonium-nitrogen removal rate was decreased by 1/4 of that of the previous stage, the removal of N and carbon persisted. The decline in removal rates may be due to stress from long phosphorus starvation or the lack of other minor elements.

Concerning the biological removal of phosphorus, several reports have been published. One popular process is an enhanced biological phosphorus removal system that uses separated tanks for anaerobic and anaerobic conditions [12]. One of authors published an article on a high phosphate-accumulating bacterium, in which the intracellular



**Figure 4:** Removal of ammonium-nitrogen ( $NH_4^+-N$ )(●) and lactate (▲) after 12 h in the experiment similar to that shown in Figure 3 by the extra addition of ammonium-nitrogen ( $NH_4^+-N$ ) and lactate in 14 h. The original ammonium-nitrogen ( $NH_4^+-N$ ) values were divided by 10 to make the figure clear.

P-content reached 30%. However, this bacterium had no  $N_2$  production ability [13]. Some denitrifying bacteria have a phosphorus-accumulating ability [14,15]. Their P-removal rates are in the range of 1 mg/l/h. In No. 4 system, the P-removal rate was more than 10 times larger, and the high-strength ammonium and a fairly high concentration of phosphorus were simultaneously removed by this simple system.

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