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Heterotrophic Nitrification and Aerobic Denitrification by *Alcaligenes faecalis* No. 4

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/68052>

Abstract

Alcaligenes faecalis No. 4 (No. 4) has the ability to carry out the following heterotrophic nitrification and aerobic denitrification, $\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$. Approximately, 40 and 60% of ammonium were converted to N_2 gas and cell mass, respectively. Only a few percent of NO_2^- and NO_3^- were produced from ammonium. After brief explanation of significant properties of No. 4, several examples of application of No. 4 to treat ammonium, especially high-strength ammonium in several wastewaters were presented. The ammonium removal rates in these examples showed several hundredfold higher than those in conventional ammonium treatment method. In wastewater treatment plants, the selection of handling of excess sludge after treatment is a problem to be solved. Some possibilities of utilization of the excess cells of No. 4 in agriculture or in cattle farming were also presented.

Keywords: heterotrophic nitrification, aerobic denitrification, high-strength ammonium, ammonium removal rates, utilization of organic acids, *Alcaligenes faecalis* No. 4

1. Introduction

1.1. Brief review of conventional ammonium removal by autotrophic nitrification and anaerobic denitrification

The oxidation of the ammonium to nitrogen gas is achieved with two step reactions, namely aerobic nitrification and anaerobic denitrification. The most common bacteria responsible for the aerobic nitrification are the autotrophic organisms, such as *Nitrosomonas* and *Nitrobacter*. They obtain energy from the oxidation of ammonia, obtain carbon from CO_2 and use oxygen as the electron acceptor. Many different heterotrophs are responsible for anaerobic denitrification. They use carbon from complex organic compounds, prefer low to zero dissolved oxygen, and use nitrate as the electron acceptor.

Biological removal of ammonium in a conventional treatment system has been conducted using the two reactions. However, this system faces several problems including: (1) an extremely slow nitrification reaction, (2) deterioration of activity against overloading of ammonium and organic matter, (3) strong sensitivity to oxygen limitation, and (4) requirement of two separate reactors for an aerobic process in nitrification and an anaerobic process in denitrification. The low nitrification rates in this process result in the need for long hydraulic retention times or large reactor volumes to accomplish complete NH_4^+ removal. Consequently, conventional treatment demands multiple and larger reactors and high capital and operation costs.

Over the past two decades, several new bioprocesses for ammonium removal from municipal and domestic wastewaters have been developed, including: simultaneous nitrification and denitrification, shortcut nitrification and denitrification, aerobic deammonification, complete autotrophic nitrogen removal over nitrite (CANON), oxygen-limited nitrification and denitrification (OLAND), advanced treatments using combination of these process including membrane bioreactors, and cell-immobilization systems. However, these processes also have some potential problems or limitations especially for high-strength ammonium treatment [1].

1.2. Brief review of anammox method

Anammox (anaerobic ammonium oxidation) is a recent understanding on the nitrogen cycle. *Candidatus "Brocadia anammoxidans"* and *Candidatus "Kuenenia stuttgartiensis"* are representative anammox bacteria.

Anammox method consists of partial aerobic nitrification and anaerobic denitrification.



The advantages of this method are: (1) very little sludge production, (2) reduced oxygen supply, and (3) no need to supplement organic carbons, which are related with operating cost problems in conventional ammonium treatment.

Concerning the ammonium removal rates in anammox method, the relatively higher removal rates, 0.96 kg-N/m³/day in SHARON-anammox process [2], 2.3 kg-N/m³/day in fluidized bed using synthetic medium [3], and more than 4 kg-N/m³/day of gel pellets of anammox biomass [4], were reported. The problems of the method are that: (1) sufficient amount of biomass production is time-consuming, (2) the long time for stabilization of the system, (3) difficult quick recovery of the system when the inefficient removal occurred, (4) NO_3^- accumulation, and (5) slow phosphate removal rate.

1.3. Heterotrophic nitrification and aerobic denitrification

Recently, many microorganisms have been found to conduct heterotrophic nitrification and aerobic denitrification. **Table 1** shows representative microorganisms published previously and their removal abilities. These microorganisms have advantages such as (1) procedural simplicity, where nitrification and denitrification can take place simultaneously, (2) less acclimation problems, (3) lesser buffer quantity needed because alkalinity generated during denitrification can partly compensate for the alkalinity consumption in nitrification.

Strains	Initial NH ₄ ⁺ -N concentrations (mg/l)	NH ₄ ⁺ -N removal (%)	Temperature (°C)	N ₂ production (%)	Carbon sources	Removal rate (NH ₄ ⁺ -N kg/m ³ /day)	Ref. no.
<i>Pseudomonas alcaligenes</i> AS-1	35	100	30	60	Acetate	0.015	[14]
<i>Pseudomonas</i> sp. 3-7	75	86	30	n. r.	Succinate	0.75	[13]
<i>Pseudomonas</i> sp. <i>Rhodoferax ferrireducens</i>	80	38	10	n. r.	Acetate	0.06	[9]
<i>Agrobacterium</i> sp.LAD9	97	90	30	50	Succinate	0.088	[6]
<i>Rhodococcus</i> sp. CPZ 24	100	85	30	48	Succinate	0.08	[5]
<i>Bacillus subtilis</i> A1	104	65	28	20	Acetate	0.08	[8]
<i>Pseudomonas stutzeri</i> YZN-001	110	95	30	39	Succinate	0.13	[10]
<i>Acinetobacter calcoaceticus</i> HNR	120	96	30	40.2	Glucose	0.46	[12]
<i>Bacillus methylotrophicus</i> L7	140	70	37	n. r.	Succinate	0.05	[11]
<i>Diaphorobacter</i> sp.	212	100	30	n. r.	Citrate	0.05	[7]
<i>Acinetobacter</i> sp.Y1	110	99	30	54	Citrate	0.25	[15]
<i>Acinetobacter junii</i> YB	100	100	37	51	Succinate	0.59	[16]
<i>Marinobacter</i> sp.	242	48	30	n. r.	Succinate	0.24	[17]
n.r.: not reported.							

Table 1. Reported strains which have ability of heterotrophic nitrification and aerobic denitrification.

The two mechanisms for heterotrophic nitrification and aerobic denitrification are reported.



Both reactions occur simultaneously [6, 16].



Almost no nitrite or nitrate was produced and neither nitrite nor nitrate was utilized as electron acceptors [12, 18].

This kind of bacteria may have the potential to overcome the problems inherent in the conventional nitrogen removal process and to realize one-stage nitrogen removal under aerobic conditions.

In **Table 1**, low-strength ammonium in synthetic medium was used and main carbon sources are organic acids. The use of practical wastewater is scarcely reported. *Alcaligenes faecalis* No. 4 (No. 4) we isolated is one of these microorganisms, and No. 4 showed efficient removal ability for high-strength ammonium and significantly higher removal rate. The following sections present the results when No. 4 was applied to practical wastewaters.

2. Characteristics of *Alcaligenes faecalis* No. 4 (No. 4)

2.1. Basic features of No. 4 [18]

2.1.1. Materials and methods

Strain used: *A. faecalis* No. 4 (No. 4) was isolated from sewage sludge as an antagonistic microorganism to plant pathogens [19].

Synthetic medium used: A synthetic medium containing (in units of g/L) 14 K_2HPO_4 , 6 KH_2PO_4 , 17 trisodium citrate dihydrate, 2 $(\text{NH}_4)_2\text{SO}_4$, 0.2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 mL of trace mineral solution was used for the preculture of No. 4. The trace mineral solution contained the following components (in g/L): 57.1 EDTA (2,2',2'',2'''-(ethane-1,2-diyl)dinitrilo)tetra acetic acid)·2Na, 3.9 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 7 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.1 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 5.0 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.1 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1.6 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 1.6 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$.

Method: Available carbon sources and available nitrogen sources were surveyed using various carbon and nitrogen materials. Then, the initial ammonium concentration of $(\text{NH}_4)_2\text{SO}_4$ was fixed and carbon content of citrate was change from C/N ratio 5–20 and optimal C/N ratio was determined. Optimal temperature and pH were determined using synthetic medium. Nitrogen balance was obtained using NO_x analyzer to detect NO and NO_2 in exhaust gas. All experiments were conducted using shaking flasks (100 ml working volume in 500 ml nominal volume of flask).

2.1.2. Results

The following results were obtained.

Available carbon sources: Organic acids (oxalate, citrate, lactate, formate, acetate, propionate, *iso*-butyrate, *n*-butyrate), amino acids, and phenol. No sugars were available.

Available nitrogen sources: Inorganic ammonium salts, peptone, yeast extract, and hydroxylamine. Neither nitrate nor nitrite was utilized.

Optimal C/N ratio: Optimal C/N ratio was 10 when the NH_4^+ -N removal rate was the highest and citrate and ammonium were exhausted simultaneously.

Temperature range: 15–37°C. Optimal temperature was 30°C

Initial pH: In the range of 6–8, ammonium removal rate was almost the same.

Nitrogen balance: Nitrogen balance at the initial 1122 mg-N/l is shown in **Table 2**.

The emitted NO was less than 3% of removed NH_4^+ -N.

2.2. Verification of N_2 production directly from ammonium by No. 4 [18]

A ^{15}N tracer experiment using $(^{15}\text{NH}_4)_2\text{SO}_4$ (50% by atomic fraction, Nippon Sanso Co., Ltd.) was carried out to confirm the production of N_2 by No. 4 in an aerated batch culture in the basic medium under $\text{C/N} = 10$ at 30°C. The exhaust gas was directly introduced into the GC/MS (GC 6850, Agilent Technologies, Japan, Ltd.). The change in nitrogen isotope ratio was measured and N_2 production by No. 4 was calculated from the difference between output $^{29}\text{N}_2$ and input $^{29}\text{N}_2$.

Figure 1 shows temporal changes in N_2 and N_2O concentrations. It was confirmed that No. 4 can convert NH_4^+ -N to N_2 gas and that N_2 production ratio among denitrified products was about 90%. In conventional denitrification, 20–30% of influent nitrogen was estimated to be emitted as N_2O under high-strength ammonium conditions. In this system, N_2O production was less than 10% of removed ammonium.

2.3. Ammonium removal under high salt condition by No. 4 [20]

No. 4 exhibited the unique feature of removing ammonium under high salt conditions. **Figure 2** 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 1 day at 3% NaCl and 5 days at 6% NaCl and the ammonium removal rates were similar to those found in the presence of 0% NaCl. Although No. 4 is not osmophilic, the cells

NH ₄ ⁺ -N concentration		Nitrification products			Intracellular N (ratio)	Stripped NH ₃ -N (ratio)	NO-N	Denitrified products (ratio)
Initial	Final	NH ₂ OH-N	NO ₂ ⁻ -N	NO ₃ ⁻ -N				
1122	0	0.03	16	13	510 (46%)	41 (4%)	36	584 (52%)

Table 2. Nitrogen balance (units: mg/l) of ammonium removal in shaken flask experiment by *A. faecalis* No. 4 after 93h cultivation [18].

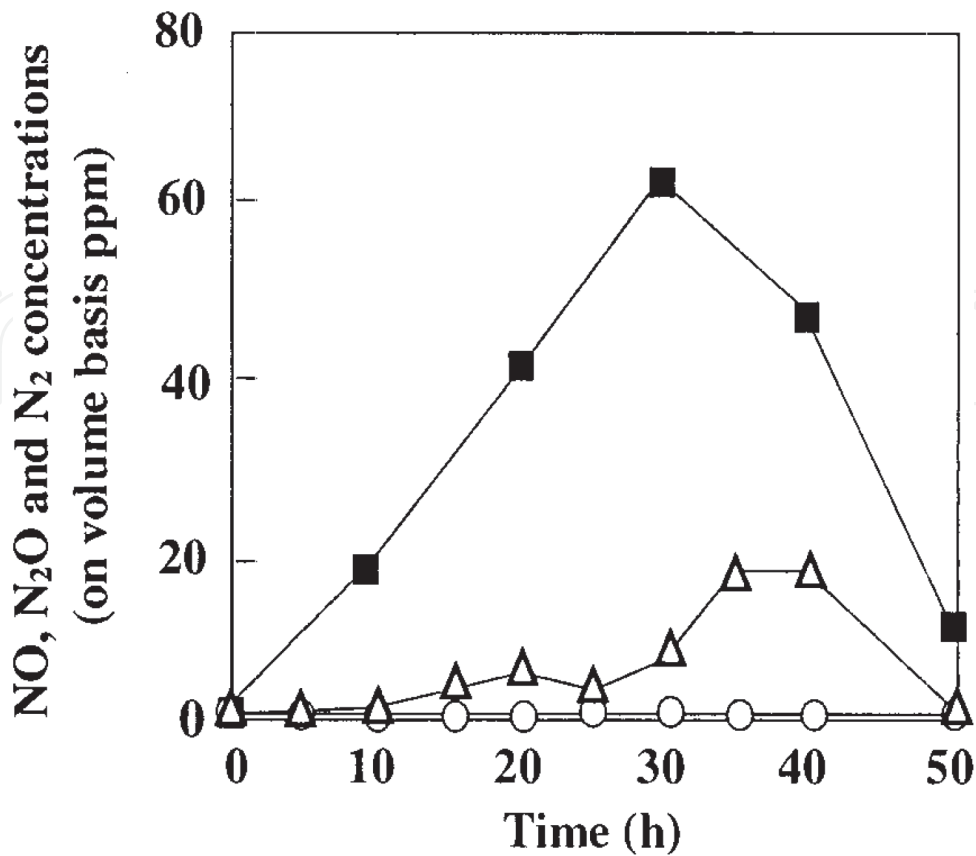


Figure 1. Denitrification characteristics of No. 4 detected by using $(^{15}\text{NH}_4)_2\text{SO}_4$. Symbols: NO (○), N₂O (△) and N₂ (■) [18].

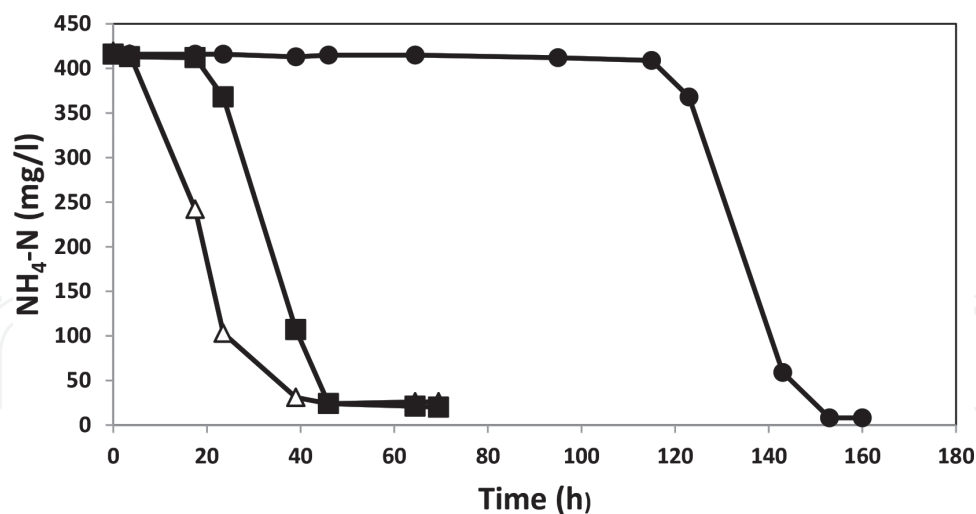


Figure 2. Ammonium removal by No. 4 under 0% NaCl (△), 3% NaCl (■) and 6% NaCl (●) conditions in shaking flasks containing 100 ml of synthetic medium at 30°C [20].

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. 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, the No. 4 system can remove high-strength ammonium from marine aqua-culture wastewater or fishery processing wastewater.

In the following sections, examples of removal of high-strength ammonium from practical wastewaters are presented.

3. Application of No. 4 to removal of high strength ammonium in various wastewaters

3.1. Crude piggery wastewater [21]

3.1.1. Introduction

Piggery wastewater contains not only high concentrations of nitrogen compounds but also high concentrations of carbon materials. The ammonium concentration reaches up to 1000–3000 mg/l, which is 50–100 times higher than in municipal wastewater. The C/N ratio of the mixture of urine and feces in piggery wastewater is usually in the range of 5–20. Therefore, conventional nitrification using autotrophic bacteria is difficult to apply to such wastewater because nitrification by autotrophic bacteria requires a long retention time of flowing wastewater in the reactor due to the slow growth rates of these autotrophic bacteria. Thus, No. 4 was applied to batch and continuous cultures using solids-free wastewater (referred to as SFW) either alone or supplemented with additional carbon sources such as citrate or feces.

3.1.2. Materials and methods

Microorganisms: The cells of No. 4 were stored in a 25% glycerol solution in vials at -80°C and each vial was used for preculture.

Medium: The synthetic medium described above was used as a preculture. In continuous culture, 500 ml of the preculture was prepared and put into the reactor.

Piggery wastewater: Piggery wastewater was provided by the Kanagawa Prefectural Livestock Industry Research, Kanagawa, Japan. Solids-free wastewater (SFW) was obtained by separating the solids from the raw wastewater containing urine and washing water and feces by centrifugation at 1000 rpm. **Table 3** shows the characteristics of the SFW and mixed wastewater comprised of SFW supplemented with feces (3:1 on a weight basis) (referred to as MW).

Continuous experiments: Continuous treatment of SFW and MW was conducted in a 2.3 l aeration tank at room temperature at the airflow rate of 2.5 l/min. A total of 500 ml of No. 4 culture was mixed with wastewater and open continuous experiments were started by supplying SFW.

	NH ₄ ⁺ -N	NO ₂ ⁻ -N, NO ₃ ⁻ -N	COD _{Cr}	C/N ratio
*SFW	830–1250	Less than 1	4150–5300	4–5
**MW	1850–1960	Less than 1	13800–14650	7–9

*SFW: Solid-free wastewater (units, mg/l).

**MW: Mixed wastewater (units, mg/l).

Table 3. Characteristics of piggery wastewaters used [21].

3.1.3. Results

3.1.3.1. Batch experiments

SFW or MW was treated with No. 4 in shaking flasks and the removal of NH₄⁺-N was measured. In SFW, the addition of citrate was needed mainly due to small amount of carbon in SFW. In MW without addition of carbon, the ammonium removal proceeded smoothly and the maximum ammonium removal rates in SFW with supply of citrate and in MW were 0.7 and 0.66 kg-N/m³/day, respectively.

3.1.3.2. Continuous experiments

Figure 3 shows the results of SFW and MW treatment in continuous treatment in for 80 days. Initial 10 days, only SFW was supplied and the ammonium removal ratio declined mainly because of lacking of carbon source. Then, citric acid was added and the hydraulic retention time (HRT) was reduced. Consequently, ammonium removal was stabilized to about 80%. Then, the inlet ammonium concentration and citric acid increased gradually and the removal ratio reached almost 100%. From 52 days, instead of supplying citrate, influent NH₄⁺-N concentration was increased to 2000 mg/l with addition of feces and HRT was set at 60 h. After 52 days, ammonium removal was high at 100% and outlet of ammonium was less than 20 mg/l. The pH was maintained at 7.4.8 after supply of MW and stripped ammonia from reactor was 2–5% of inlet ammonium concentration. The system was in a steady state. The cells number of No. 4 was measured with L agar plates. The data are summarized in **Table 4**. The nitrogen and carbon balances in the experimental periods are also shown in **Table 4**. After 52 days, feces containing MW were added directly. The denitrified N calculated from inlet ammonium minus the nitrogenous items was 73% and striking result was the high removal of COD. The estimated cell number of No. 4 reached up to 97% of total cells in the samples. The ammonium removal rate, 33 mg-N/l/h corresponded to 0.79 kg-N/m³/day, which was a few hundred times higher than conventional treatment methods. In diluted and digested piggery wastewater at C/N = 1, 64 mg/l/h removal rate was reported [22]. However, in the present study, No. 4 provided suitable to treat undiluted piggery wastewater with C/N ratio of 10, yielding removal rate of 33 mg-N/l/h (0.79 kg-N/m³/day).

3.2. Anaerobically digested sludge [20]

3.2.1. Introduction

Due to recent trends of limiting fossil energy consumption, sustainable methods of energy production including methane production in anaerobic digestion or bioethanol production have

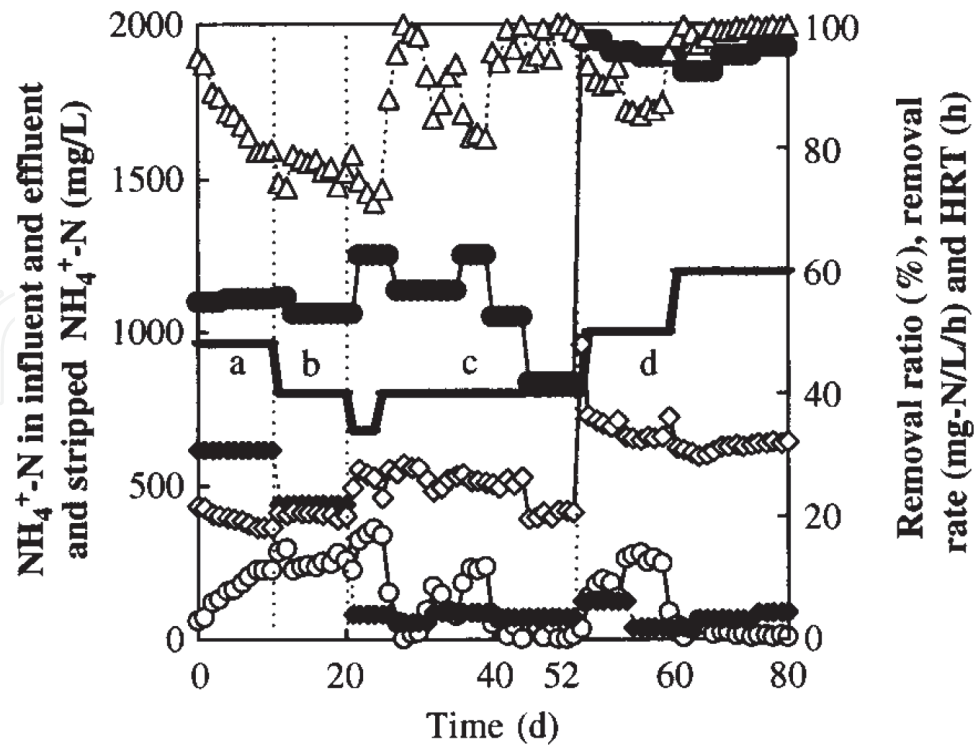


Figure 3. Ammonium concentration in influent (●) and effluent (○), stripped ammonia (◆), removal rate of ammonium (◇), removal ratio of ammonium (△) and hydraulic retention time (HRT) (solid line) in the continuous treatment of solid-free piggy wastewater (before 52 days) and mixed wastewater (after 52 days) by No. 4 [21].

Items	Operation periods	
	21–51 days	52–80days
Load of $\text{NH}_4^+\text{-N}$ (mg/l/day)	670	837
Influent $\text{NH}_4^+\text{-N}$ (mg/l)	1084	1901
Effluent $\text{NH}_4^+\text{-N}$ (mg/l)	116	97
Removed $\text{NH}_4^+\text{-N}$ (mg/l)	968	1804
Intracellular N ^a (mg/l)(%) ^b	232(24)	419(23)
Stripped $\text{NH}_3\text{-N}$ (mg/l)	74	73
Denitrified N (mg/l)(%) ^b	662(68)	1312(73)
Ammonium removal rate (kg-N/m ³ /day)	0.6	0.79
Influent COD _{Cr-C} (mg/l)	13,491	12,762
Effluent COD _{Cr-C} (mg/l)	1679	342
COD removal ratio (%)	87	97
Cell number of No. 4 (cfu/ml)	1.7×10^9	8.8×10^9

^aCalculated by elementary analysis of the dry sludge.

^bPercentage against removed ammonium nitrogen.

Table 4. The nitrogen balance and carbon change in continuous experiment using No. 4 for treatment of solid free piggy wastewater and mixed wastewater All data were average values in the operation periods [21].

been attracting increasing attention. In anaerobic digestion, livestock waste, municipal garbage, and waste from the food industry are used for the digestion, leading to the production of wastewater containing a high concentration of ammonium. Therefore, the development of an effective method of the wastewater treatment is a crucial factor enabling the production of methane.

In this section, No. 4 was applied to remove high-strength ammonium from digested sludge generated in a municipal anaerobic digestion plant to assess the possibility of efficient biological treatment of the wastewater.

3.2.2. Materials and methods

The reactor used the reactions that were carried out in a small-scale jar fermenter (total volume 1 l, working volume 300 ml). Dissolved oxygen (DO) concentrations and pH values were monitored with a DO sensor and pH sensor inserted into the fermenter. The temperature was controlled at 20 or 30°C. The oxygen transfer coefficients, k_La , were varied by changing the agitation speed from 300 to 700 rpm at a constant air supply rate of 300 ml/min.

Experimental material: The digested sludge was supplied by Yokohama Municipal Sewage Center, Yokohama, Japan, where the excess municipal dehydrated activated sludge was digested at 37°C in a 6000 ton-scale anaerobic digester. The main characteristics of the digested sludge are as follows: pH 7.3, 24 mg/l volatile fatty acids, 2700 mg/l total nitrogen, 1200 mg/l ammonium-nitrogen, 150 mg/l soluble BOD, 1000 mg/l total BOD, 900 mg/l soluble COD, and 20,000 mg/l total COD.

Experimental procedure: The strain No. 4 cells were precultivated in 100 ml synthetic medium in a 500 ml shaking flask at 30°C. The ammonium removal was confirmed in the mixture of 250 ml of digested sludge, 50 ml of strain No. 4 preculture, and 20 g of trisodium citrate dihydrate in the jar fermenter operated at 30°C at an airflow rate of 300 ml/min and at an agitation speed of 700 rpm.

In repeated batch experiments, 50 ml of the preculture of No. 4, 250 ml of the digested sludge, and 20 g of trisodium citrate dihydrate were mixed in the fermenter, and the treatment of the ammonium was conducted. After the ammonium concentration was confirmed to be reduced by more than 90% of the initial concentration, 50 ml of the culture was used for the subsequent treatment by adding a fresh 250 ml of digested sludge and 20 g of trisodium citrate dihydrate.

The optimal C/N ratio for No. 4 was 10, indicating that at this ratio, nitrogen and carbon sources were simultaneously consumed. Based on the ratio, 1 g-N and 10 g-C was balanced and thus 10 g-C corresponded to 38 g of trisodium citrate dihydrate. If no other carbon source existed in the sludge, 38 g of trisodium citrate should be added. In this experiment, 20 g of trisodium citrate dihydrate was arbitrarily chosen by expecting existence of some carbon sources in the sludge.

Analytical method: The ammonium concentration was determined using an ammonium sensor. To determine the number of cells No. 4, the sampled culture was diluted and plated

on synthetic agar plates containing the synthetic medium and 1.5% agar, and then the plates were incubated at 30°C for 2 days. As No. 4 grew on the plates significantly faster than other cells indigenous to the digested sludge and that No. 4 exhibited characteristic morphological features, the colonies that appeared on the plates after 2 days were counted as No. 4 cells and the cell concentration was expressed as cells/ml.

3.2.3. Results

3.2.3.1. Ammonium removal in the repeated batch experiment

Figure 4 shows the change in ammonium concentration over times in a repeated batch experiment at 30°C, and **Figure 5** shows the change in the number of No. 4 cells during the same experiment. More than 90% of ammonium was removed within 10–20 h, and the number of No. 4 cells varied between 10^8 and 10^9 cells/ml. The average ammonium removal rate during the experimental period was 2.9 kg-N/m³/day. This value is significantly higher than that in conventional nitrification-denitrification processes and similar to that in an efficient anammox process [3, 4]. Between 169 and 221 h, the operation was stopped and the jar fermenter was left statically at room temperature. When the operation resumed, ammonium removal was observed without any delay, indicating that interrupted operation causes no adverse effect on the activity of No. 4. At 20°C, the average ammonium removal rate decreased to 1.5 kg-N/m³/day.

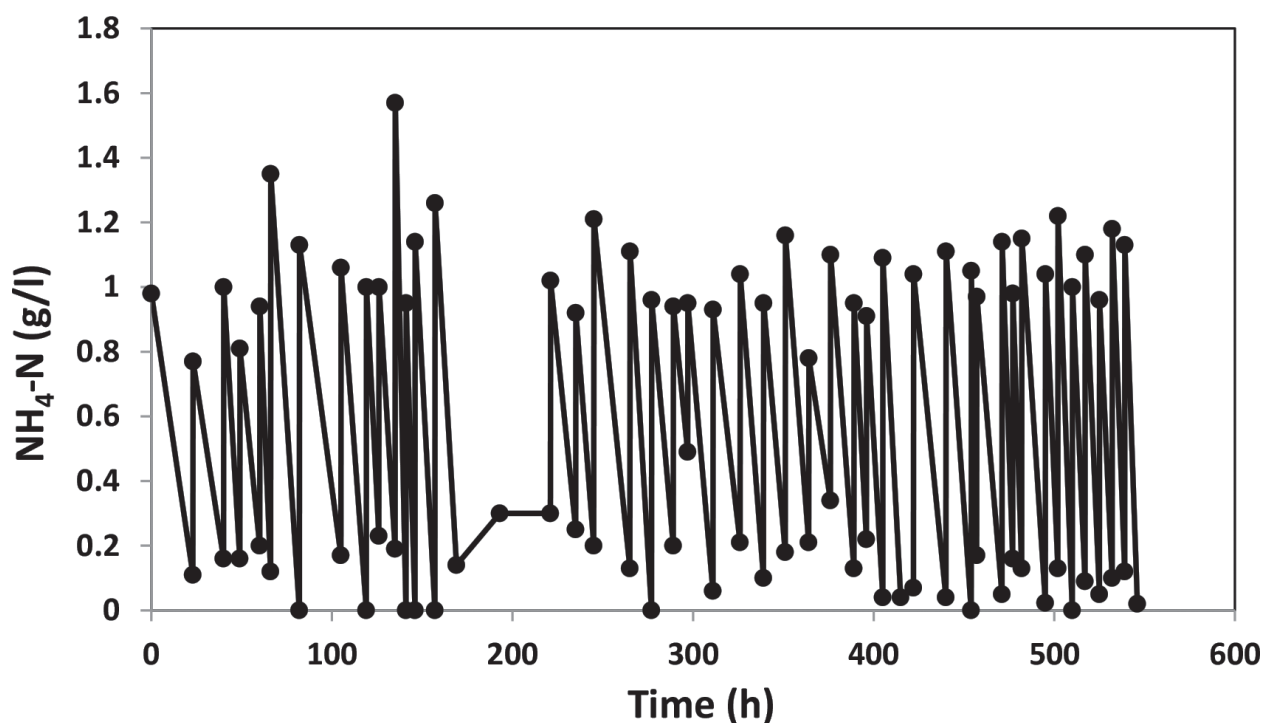


Figure 4. Change in ammonium concentrations of the digested sludge in repeated batch treatment by No. 4. Operation was conducted at 30°C at agitation speed of 700 rpm in a jar fermenter [20].

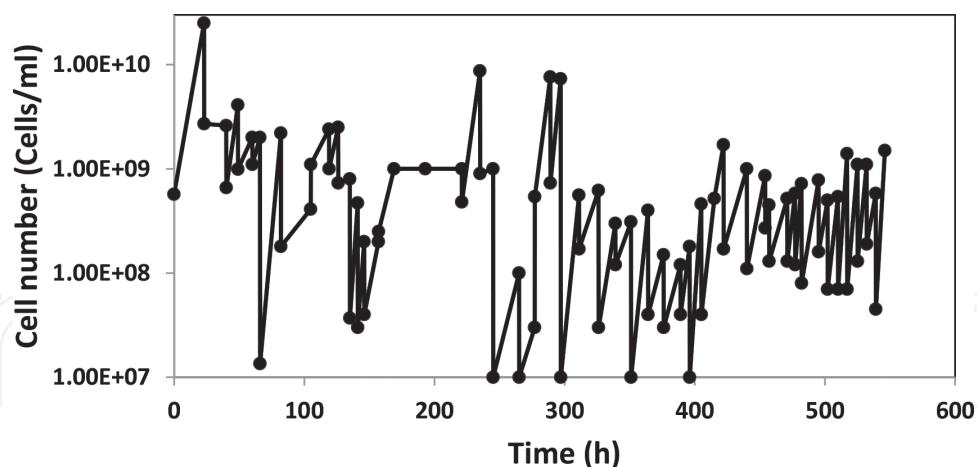


Figure 5. Change in the cell number of No. 4 in the same experiment as shown in **Figure 4** [20].

3.2.3.2. Effect of DO concentration on ammonium removal

In practical operation, DO concentration is related to energy consumption, agitation, air supply, and the activity of No. 4. The effects of changes in the oxygen supply rate on ammonium removal were studied by changing the agitation speed from 700 to 300 rpm. At 700 rpm, the DO concentration was maintained at more than 2 mg/l during the operation, and the ammonium was completely removed within 10 h. However, when the agitation speed was decreased to 400 or 300 rpm, the DO concentration decreased below 1 mg/l, reducing the ammonium removal rate, indicating that the oxygen supply is an important factor for efficient ammonium removal.

3.2.3.3. Ammonium removal under high salt conditions

Strain No. 4 exhibited the unique feature of removal ammonium under high salt conditions as shown in Sections 2 and 3. NaCl was added to the digested sludge to 3%, and repeated batch treatment was conducted at 30°C in a jar fermenter. The results of this experiment are shown in **Figure 6**. The ammonium removal rate reached 3 kg-N/m³/day at the sixth repeated batch operation after the gradual acclimation of No. 4 to the saline medium.

3.2.3.4. Carbon requirement by No. 4

The experiment performed here included 20 g of trisodium citrate dihydrate. Generally, the C/N ratio of the intracellular components in microorganisms is 10, indicating that 10 units of carbon are used when 1 unit of N is consumed with the C mainly to synthesize cellular materials. In previous experiments, 30–40% of ammonium was reduced to nitrogen gas by No. 4. Assuming a similar level of denitrification in these experiments, 0.6–0.7 g-N/l was used for cell synthesis, indicating that 6–7 g-C/l was required. When 20–30% of carbon is available from the digested sludge, approximately 5 g-C/l should be supplied from outside. As 20 g of trisodium citrate dihydrate contained 16 g-C/l, 6 g of trisodium citrate dihydrate is sufficient to enable the complete removal of 1 g-N/l.

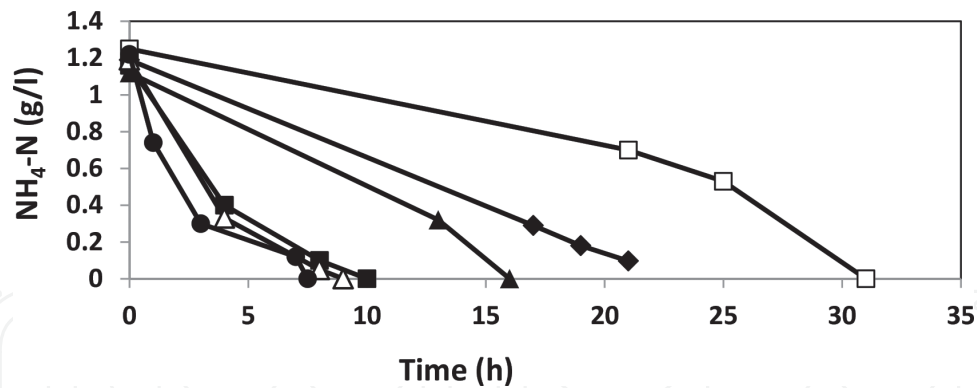


Figure 6. Ammonium removal by No. 4 in the digested sludge containing 3% NaCl by repeated batch experiment. Symbols: 1st (□), 2nd (◆), 3rd (▲), 4th (■), 5th (△) and 6th (●) [20].

Concerning carbon requirement in strain No. 4, the conventional denitrification process using methanol is compared by using the following reaction.



The C/N ratio in this reaction is 2. No. 4 process demanded C/N ratio 10. In this point, strain No. 4 process is disadvantageous. However, as a total system, No. 4 process will be advantageous over the conventional process in that no dilution of high strength of wastewater is required, only single reactor with compact size is needed and significantly high removal rate is possible when less expensive carbon sources from waste or unused resources are available. Under these conditions, this system can achieve efficient ammonium removal.

Higher ammonium removal rates have been reported using the anammox method as described in Sections 1 and 2. On the other hand, it is easy to cultivate strain No. 4 in a synthetic medium with a doubling time of 2–3 h. When cultured strain No. 4 cells were stored at 4°C, high activity was maintained for several months, and the cells remained tolerant to high osmotic pressure. The comparison of three methods of ammonium treatment is shown in **Table 8** of Section 5.

In relatively small-scale reactors like this jar fermenter, oxygen supply capacity is lower than those large-scale reactors. The power requirement in wastewater treatment is one of the important factors considered to be in operation. Thus, DO level in large scale reactors can be maintained at lower agitation speeds and the power requirement for strain No. 4 for high-strength ammonium treatment will be almost equivalent to that for low-strength ammonium treatment in conventional aerobic nitrification process.

3.3. Wastewater from a chemical company [23]

3.3.1. Introduction

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

3.3.2. Materials and methods

3.3.2.1. Wastewater used

The wastewater (WC) was supplied by a Japanese chemical 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 concentrations of pH-adjusted WC was diluted to approximately 1000 mg/l unless specifically described.

The experimental procedures in this section were similar to those in Section 3.2.

3.3.3. Results

3.3.3.1. Ammonium removal in the repeated-batch experiment

Figure 7 shows the change in the ammonium concentration over times in a repeated-batch experiment at 30°C, and **Figure 8** 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-N/m³/day. 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 are within the optimal pH range of No. 4. 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.

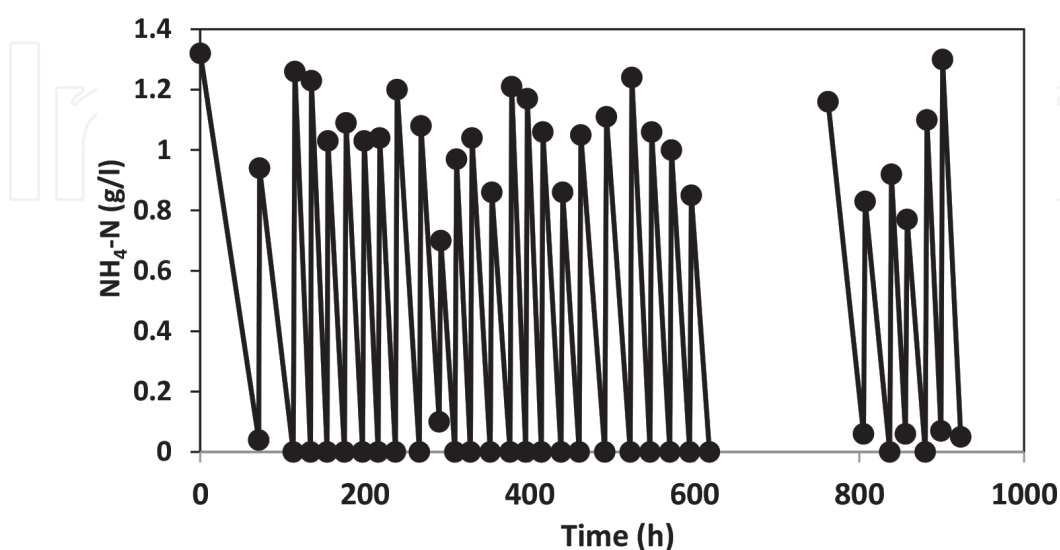


Figure 7. Ammonium concentration in the wastewater from a chemical company during repeated-batch treatment with No. 4 at 30°C [23].

3.3.3.2. Ammonium removal at initial ammonium concentrations of 1000, 2000, and 5000 mg $\text{NH}_4^+\text{-N/l}$

Figure 9 shows the ammonium removal obtained with initial ammonium concentrations of approximately 5000 mg $\text{NH}_4^+\text{-N/l}$, 2000 mg $\text{NH}_4^+\text{-N/l}$ and 1000 mg $\text{NH}_4^+\text{-N/l}$. For concentrations of 5000 mg $\text{NH}_4^+\text{-N/l}$ and 2000 mg $\text{NH}_4^+\text{-N/l}$, an intermittent supply of 20 g of trisodium citrate dihydrate was introduced, as indicated by the arrows in **Figure 9**. The average ammonium removal rates for 1000, 2000, and 5000 mg $\text{NH}_4^+\text{-N/l}$ were 0.63, 0.96, and 0.92 kg-N/ m^3 /day, respectively. This indicates that even ammonium concentrations higher than 1000 mg $\text{NH}_4^+\text{-N/l}$ were removed efficiently by supplying a sufficient amount of the carbon source.

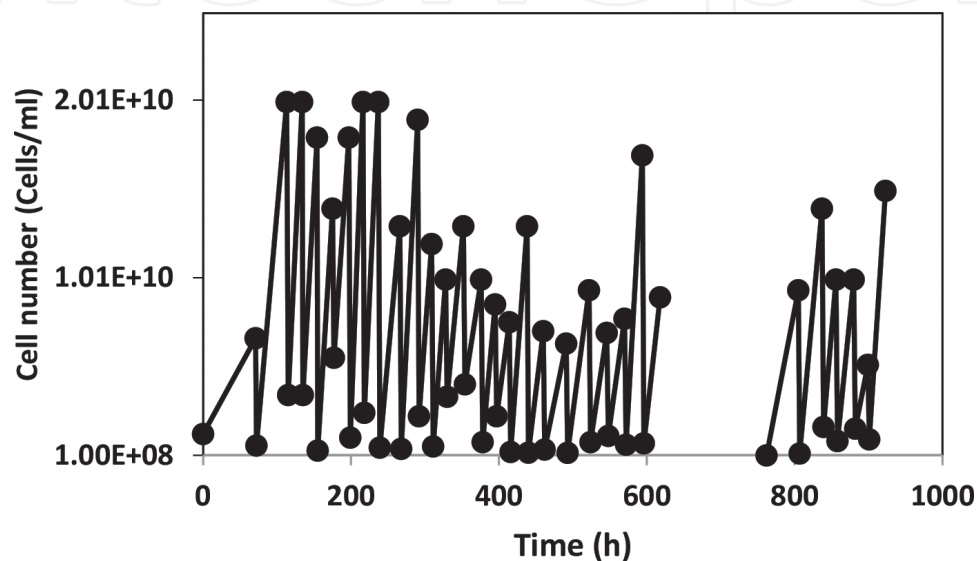


Figure 8. Change in the number of No. 4 cells in the same experiment shown in **Figure 7** [23].

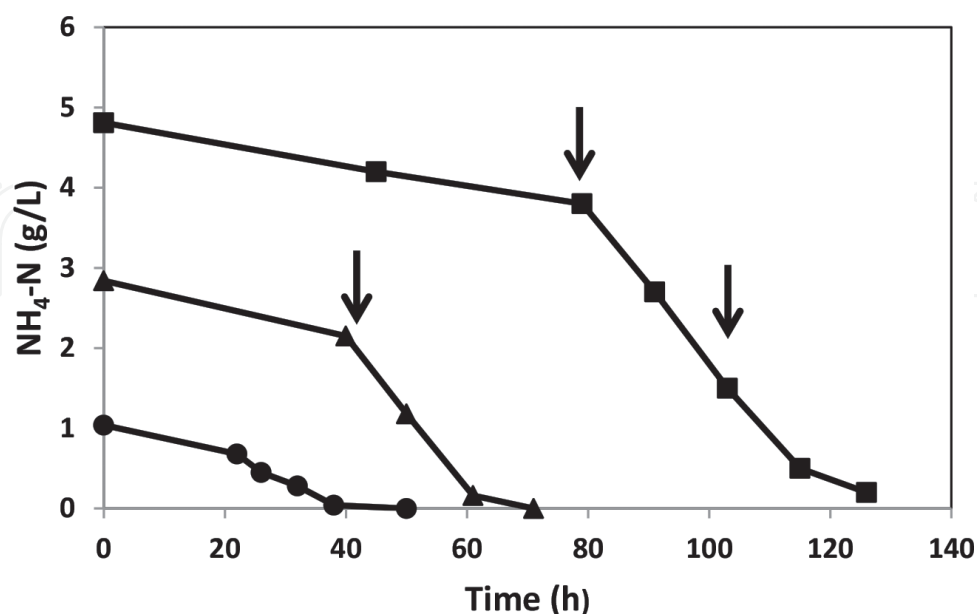


Figure 9. Change in the initial ammonium concentrations when 5000 mg- $\text{NH}_4^+\text{-N/l}$ (■), 2000 mg- $\text{NH}_4^+\text{-N/l}$ (▲), and 1000 mg- $\text{NH}_4^+\text{-N/l}$ (●) of wastewater from a chemical company were used in a batch culture. The arrows indicate the times at which citrate was added [23].

3.3.3.3. Ammonium removal under high salt conditions

NaCl was then added to the WC to a final concentration of 3%, and repeated-batch treatment was conducted using protocol similar to that described in Section 3.2. The result was similar to that in Section 3.2. The ammonium removal rate reached 1.0 kg-N/m³/day with the four-batch operation after the gradual acclimation of No. 4 to the saline medium.

3.4. Coking wastewater [24]

3.4.1. Introduction

Coking wastewater (CW), which originates from the process of destructive distillation of coal at high temperatures in the absence of air, has been a severe problem. Phenols are the major constituents of the coking wastewater and seriously inhibit various biological reactions, especially the nitrification reaction. Conventional biological treatment for CW is difficult mainly due to refractory substances. When high-strength ammonium is involved in CW, BOD in the wastewater is not sufficient to complete the removal of ammonium. In this section, first, phenol-degradation ability by No. 4 was confirmed, and No. 4 was applied to a coking wastewater supplied by a chemical company to assess the effects of biological treatment of high-strength ammonium and phenol using a 1-l jar fermenter.

3.4.2. Materials and methods

Medium: A synthetic medium described above was used in a preculture, using lactate as a carbon source.

3.4.2.1. Wastewater used

The coking wastewater (CW) was supplied by a Japanese chemical company. The primary characteristics of the CW are as follows: pH 8.5, total COD concentration of 5200 mg/l, ammonium-nitrogen concentration of 800 mg/l, and phenol concentration of 820 mg/l. In each experiment, the pH of the original CW was adjusted to approximately 7.5 by 5N H₂SO₄, and the pH-adjusted CW was diluted arbitrarily.

3.4.2.2. Experimental procedure

The synthetic medium was prepared in the preculture of No. 4 containing phenol (0.2 g/l) only as a carbon source and No. 4 was precultivated for 3 days and the preculture was centrifuged at 10,000 rpm for 10 min. The collected cells of No. 4 were washed with 0.1 M phosphate buffer two times and the cells were inoculated into the synthetic medium, which was devoid of lactate and contained only phenol as a carbon source and utilization of phenol by No. 4 was tested.

In CW treatment, precultured No. 4 cells were introduced into different dilution CW and the growth of No. 4 was confirmed at 50% dilution in shaking flasks. The diluted CW was added with lactate and No. 4 culture in a jar fermenter and ammonium removal test was conducted.

3.4.2.3. Analytical method

For phenol concentration determination, the chemical analysis kit for phenol (LR-PNL, Kyoritsu Chemical-Check Lab., Corp., Tokyo, Japan) was used. The initial and final values of TOC in the prepared solution were determined. The crude coking wastewater was streaked on the LB medium and the synthetic agar medium and no colonies appeared after 3 days of incubation. Therefore, indigenous microorganisms in the crude coke-production wastewater were negligible in number.

The air was supplied to the CW sample containing lactate in the jar fermenter for 4 days and neither the removal of lactate nor ammonium was observed, and thus the air-borne microorganisms were neglected.

3.4.3. Results

3.4.3.1. Availability of phenol by No. 4

Complete removal of ammonium and phenol in the synthetic medium were confirmed when phenol was added as a sole carbon source and the growth of No. 4 (data not shown). The ammonium removal rates using phenol as a carbon source were 0.098–0.12 kg-N/m³/day, which is approximately one-tenth of the rate when organic acids were used as a carbon source [20]. However, these data were approximately 10-fold higher than the rate in conventional nitrification-denitrification method. When the initial phenol concentration was 600–700 mg/l, this includes 459–535 mg/l of carbon. If the C/N ratio of cell synthesis was 10, consumption of 600–700 mg/l of phenol corresponded with the consumption of only approximately 50–60 mg/l of ammonium-nitrogen. This suggests that for complete removal of high-strength of ammonium in CW, addition of available carbon for No. 4 is needed.

The No. 4 culture was directly mixed with crude-coking wastewater with fortified lactate in a jar fermenter, but removal rates of ammonium and lactate were significantly decreased, presumably toxic substances in coking wastewater inhibited the activity of No. 4. When CW was diluted, the normal growth of No. 4 was observed at 50% dilution. Then, 50% of dilution CW wastewater was mixed with No. 4 preculture and 4 g/l of lactate. The result is shown in **Figure 10**. The initial ammonium-nitrogen concentration, phenol concentration, and lactate concentration were 420 mg/l, 380 mg/l, and 4 g/l, respectively. The ammonium removal rate was 1.8 kg-N/m³/day and phenol removal rate was 0.7 kg/m³/day. Phenol removal rate 0.7 kg/m³/day was two times larger than that in the previous report [25].

COD in the initial wastewater containing lactate was 12,000 mg/l, and after the treatment, this value decreased to 2830 mg/l. The COD of 50% diluted CW was 2130 mg/l. Thus, this ammonium treatment was primarily undertaken by No. 4 by consumption of added lactate and indigenous phenol. As coking wastewater contained some other carbon substances not available for No. 4, further treatment may be needed for complete treatment of COD after this system.

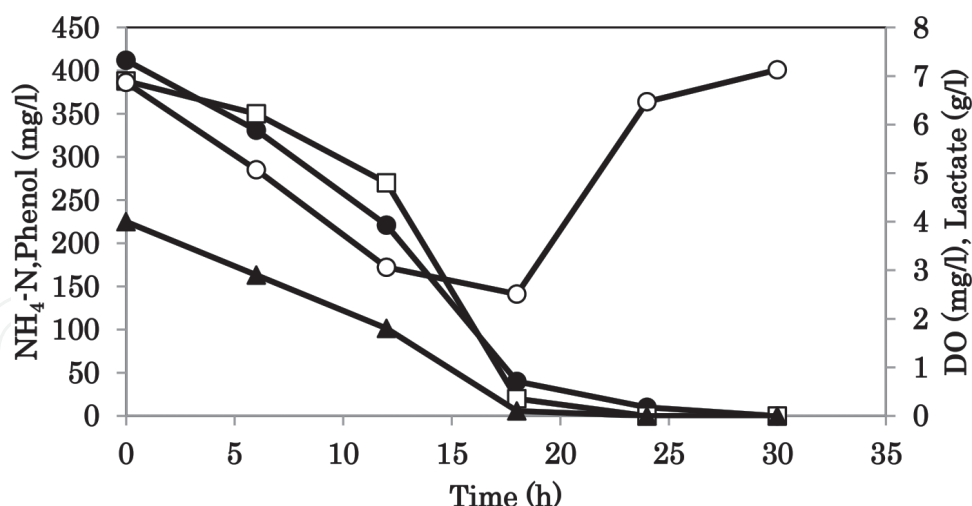


Figure 10. Removal of ammonium, phenol, and lactate in coking wastewater by No. 4. Symbols: $\text{NH}_4^+\text{-N}$ concentration (●), phenol concentration (□), lactate concentration (▲), and dissolved oxygen (DO) concentration (○) [24].

3.5. Preparation of organic acid solution for No. 4 [26]

3.5.1. Introduction

As No. 4 utilizes primarily organic acids as a carbon source and no sugar is available, chemical agents of citrate or lactate were used as a carbon source in the previous sections. In practical treatment, inexpensive production and supply of organic acids is a key for the materialization of No. 4 in ammonium treatment. In this section, anaerobic fermentation was conducted and then a mixture of high organic acid solution was prepared and this mixture was supplemented with two high-ammonium and low-carbon wastewaters by balancing C/N ratio around to 10 and the effectiveness of the prepared organic acid solution was confirmed.

3.5.2. Materials and methods

3.5.2.1. Wastewaters

The leachate wastewater from a landfill area in B city where the city garbage was land filled was used for ammonium treatment. The total organic carbon (TOC) and ammonium concentration were 4310 mg/l and 880 mg $\text{NH}_4^+\text{-N/l}$, respectively.

For a sample containing high $\text{NH}_4^+\text{-N}$ concentration and the least amount of carbon, anaerobically digested sludge wastewater used in Section 3.2 was used. This contained approximately 900 mg $\text{NH}_4^+\text{-N/l}$ and almost no BOD was used for ammonium treatment.

3.5.2.2. Preparation of the organic acid solution

Forty milliliter leachate wastewater and 20 g of glucose were mixed in a 1-l plastic container and statically incubate at 30°C for 2 weeks. The TOC and concentrations of eight organic acids in the prepared solution were determined. The volume of organic acid solution was determined so as to be C/N 10.

Experiment 1: Ammonium treatment of the leachate wastewater using No. 4 culture and organic acid solution 230 ml of leachate wastewater, 30 ml of No. 4 culture, and 40 ml of organic acid solution were mixed and the ammonium treatment was conducted in a jar fermenter.

Experiment 2: Ammonium treatment of anaerobically digested sludge using No. 4 culture and organic acid solution 180 ml of the wastewater, 30 ml of No. 4 culture, and 90 ml of organic acid solution were mixed and the ammonium treatment was carried out.

3.5.3. Results

3.5.3.1. Prepared highly concentrated organic acid solution

After 2- week anaerobic incubation of the leachate wastewater, the resulting organic acid solution contained 20,049 mg/l of TOC and 52,103 mg/l of total organic acid content of eight types, as shown in **Table 5**. The estimated carbon content from the organic acid data was 20,754 mg/l, as shown in **Table 5**. As the carbon contents in the TOC and organic acid solution were almost similar, TOC was used as an indicator to adjust to the necessary carbon content required to treat ammonium completely by balancing C/N ratio 10.

3.5.3.2. Experiment 1

The result is shown in **Figure 11**. The initial TOC was 7017 mg/l and the initial $\text{NH}_4^+\text{-N}$ concentration was 659 mg/l. The initial value of eight kinds of organic acids was 17,750 mg/l in which the estimated carbon content was 6500 mg/l (**Table 6**). The final value of TOC was 900 mg/l and the final carbon value of eight kinds of organic acids was 817 mg/l, as shown in **Table 6**. Complete ammonium removal was observed and thus the effectiveness of the use of organic acid solution and the use of TOC as an index to determine C/N ratio was confirmed. The ammonium removal rate was 1.1 kg-N/m³/day.

	Content (mg/l)	Carbon content (mg/l)
Oxalate	250	64
Citrate	250	94
Lactate	41202	16481
Formate	479	125
Acetate	4750	1900
Propionate	2402	613
iso-Butyrate	250	102
n-Butyrate	2520	1375
Total	52,103	20,754

Table 5. Organic acid distribution and carbon content in the prepared organic acid solution [26].

3.5.3.3. Experiment 2

In Section 3.2, the high-strength ammonium from anaerobically digested sludge was removed using No. 4 with addition of citrate. A similar sample that contained 900 mg $\text{NH}_4^+\text{-N/l}$ and 20 mg/l of organic content indicated that the available carbon for No. 4 is scarce, and supplementation of the organic acid solution is essential for complete removal of ammonium. For the initial 180 ml sludge sample, 90 ml of organic acid solution and 30 ml of No. 4 culture were mixed, and the ammonium removal was measured. The results are shown in **Figure 12** and **Table 7**. Similarly, the initial $\text{NH}_4^+\text{-N}$ concentration 635 mg/l was completely removed and the ammonium removal rate was 0.8 kg N/m³/day.

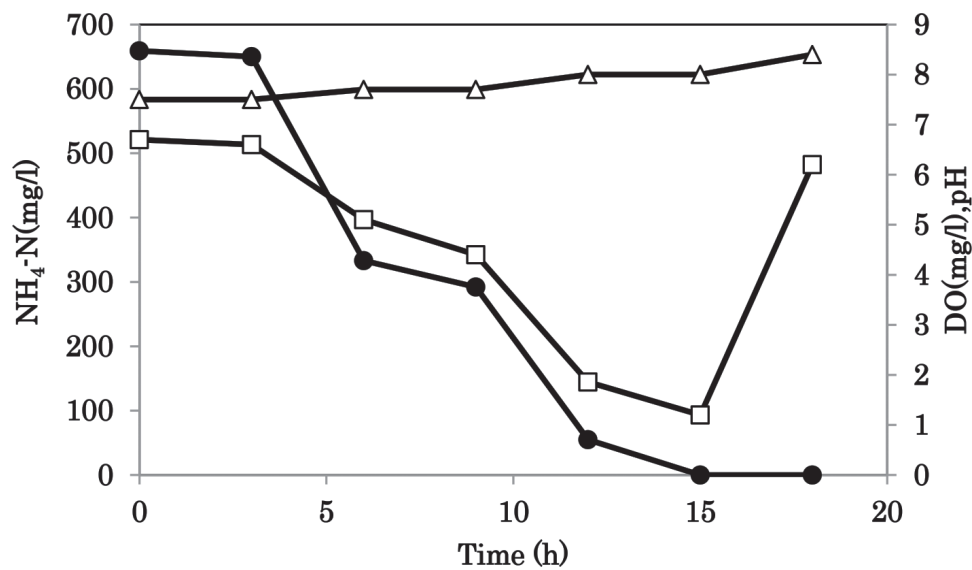


Figure 11. Ammonium removal when leachate wastewater was treated with No. 4 culture and organic acid solution. Symbols: $\text{NH}_4^+\text{-N}$ (●), dissolved oxygen concentration (DO) (□), and pH (△) [26].

	Initial carbon content (mg/l)	Final carbon content (mg/l)
Oxalate	64	64
Citrate	94	94
Lactate	3600	100
Formate	65	65
Acetate	1680	100
Propionate	842	156
iso-Butyrate	102	102
n-Butyrate	136	136
Total	6500	817

Table 6. Change in the initial and final carbon contents of organic acids in **Figure 11** [26].

	Initial carbon content (mg/l)	Final carbon content (mg/l)
Oxalate	6.4	6.4
Citrate	9.4	9.4
Lactate	6000	100
Formate	22	6.5
Acetate	720	138
Propionate	181	64
<i>iso</i> -Butyrate	10.2	10.2
<i>n</i> -Butyrate	365	13.6
Total	7314	348

Table 7. Change in the initial and final carbon contents of organic acids in **Figure 12** [26].

Factors	No. 4 method	Conventional method	Anammox method
Number of reactor	1	2	2
Reactor cost	Low	High	High
Addition of O ₂	Essential	Essential	Essential
Organic matter	Essential	Essential	No use
Maintenance of microorganisms	Easy	Difficult	Difficult
Activity persistence	Long	Short	Short
Application to high C/N waste	Applicable	Pre-treatment essential	Pre-treatment essential
N ₂ production speed	High	Low	High
System control	Easy	Difficult	Difficult

Table 8. Comparison of No. 4, anammox, and conventional methods.

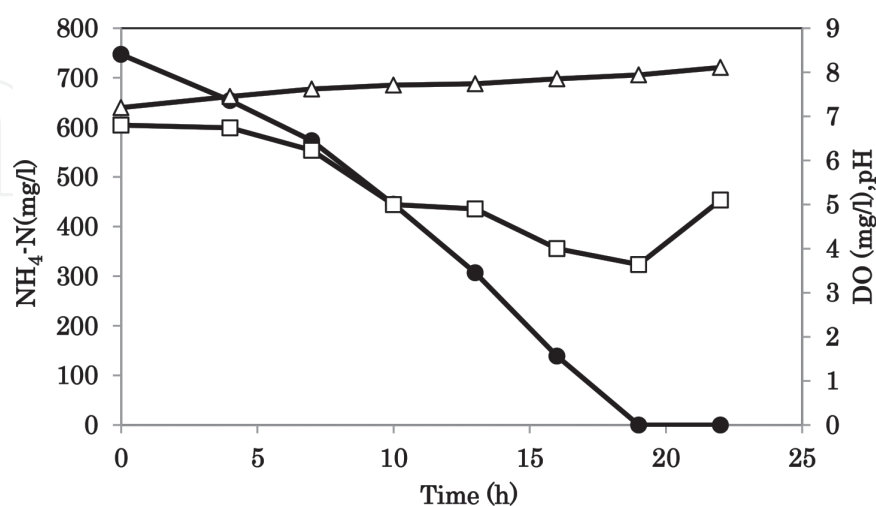


Figure 12. Ammonium removal when anaerobically digested sludge was treated with No. 4 culture and organic acid solution. Symbols: NH₄⁺-N (●), dissolved oxygen concentration (DO) (□), and pH (△) [26].

4. Additional characteristics of No. 4

In wastewater treatment, the excess sludge is inevitably produced, and new treatment possibilities of excess sludge containing No. 4 are presented in the following sections.

4.1. Suppression of growth of plant pathogens by No. 4 [19]

4.1.1. Introduction

In this section, it was presented that the *A. faecalis* No. 4 (No. 4) exhibited a suppressive effect on the damping-off caused by the plant pathogen *Rhizoctonia solani* on soil [27].

4.1.2. Materials and methods

4.1.2.1. Preparation of culture broth and cell suspension

Fifty milliliters of culture broth of No. 4 after cultivation in L medium was mixed with 150 g of soil in a pot. For the treatment consisting of only the cell suspension, the cells of No. 4 were collected by centrifugation, and the sedimented cells were suspended in sterile distilled water and 50 ml of the cell suspension was mixed with 150 g of soil.

4.1.2.2. Soil treatments and inoculation of soil with *R. solani*

These procedures were described in detail in a previous section [19].

4.1.2.3. Plant growth

For each treatment, three pots were prepared. Tomato (*Lycopersicum esculentum*) seeds were germinated on 2% agar plates at 30°C for 2 days, and nine germinated seeds were planted in each pot and incubated in a grown chamber.

4.1.3. Results

The result of the effect of No. 4 culture broth on the damping-off of tomato seedlings caused by *R. solani* in soils is shown in **Figure 13**. In a pot that was not infested with *R. solani*, all the seedlings grew normally and no disease was apparent. In a pot infested with only *R. solani*, the percentage of diseased plants was 78–82% and the shoot weight and leaf length were markedly decreased in soil. However when the culture broth of No. 4 was introduced into the soil, the percentage of diseased plants was reduced to 17%. When the cell suspension of No. 4 was applied to soil, the percentage of diseased plants in soil was similar to that soil treated with No. 4 culture broth (data not shown).

The finding suggests that the treatment with No. 4 cells is effective for plant disease control.



Figure 13. *A. faecalis* No. 4 exhibits suppressive effect on plant pathogens. Pot ③: Tomato seedlings with plant pathogens. Pot ①: Tomato seedling without plant pathogens. Pot ②: *A. faecalis* No. 4 was introduced to pot ③.

4.2. Reduction of methane production from rumen of cows [28]

4.2.1. Introduction

Enteric methane (CH_4) production from livestock is a significant source of greenhouse gas. It is reported that nitrate (NO_3) suppresses enteric CH_4 production. However, the reduction of NO_3 to nitrite (NO_2) in the rumen results in the accumulation in NO_2 , which is toxic to livestock.

A denitrifying bacterium, *A. faecalis* No. 4, was coincubated with a low concentration of NO_3 (2 mmol/l) and the *in vitro* CH_4 production was tested.

4.2.2. Materials and methods

4.2.2.1. Rumen liquid

The rumen liquid which was collected from Holstein cows and No. 4 cells obtained after centrifugation of culture broth prepared in synthetic medium were mixed with 2 mmol/l NO_3 . The mixture was placed in a 1 l jar fermenter and CH_4 production was monitored under anaerobic condition.

4.2.3. Result

The methane production from rumens is shown in **Figure 14**. When No. 4 and 2 mmol/l NO_3 were mixed, methane production showed a significant decrease without causing an adverse impact on anaerobic fermentation in rumens. This suggests a possibility of re-use of No. 4, which was produced as excess sludge after treatment of high-strength ammonium.

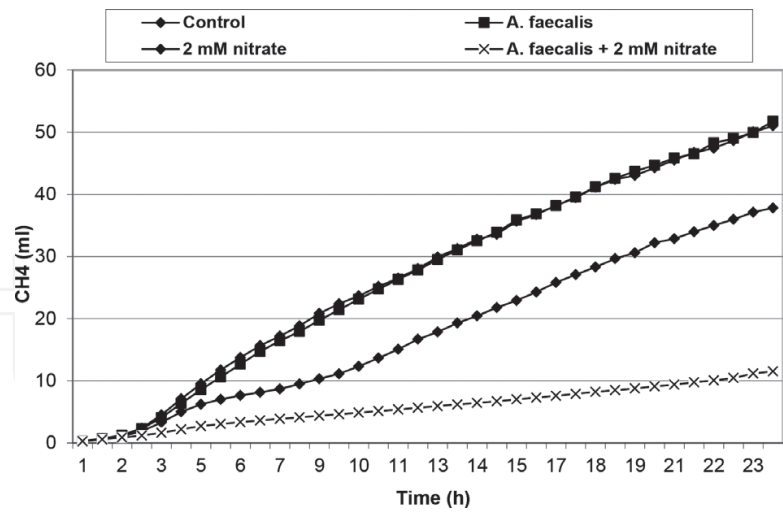


Figure 14. Methane production from rumens when No. 4 was introduced [28].

5. Conclusions

Table 8 shows comparison among conventional ammonium treatment method, anammox method and No. 4 method. No. 4 has many advantages over other methods. No. 4 was effective to remove high-strength of ammonium in several wastewaters when organic acids are supplied. The excess cells of No. 4 are produced during treatment of ammonium because the cell growth of No. 4 is associated with ammonium removal. Possibility of the re-use of the excess cells in agricultural areas was presented. How to collect organic acids as carbon source for No. 4 is a problem to be solved. Production of high concentration of organic acids in anaerobic fermentation and the use of the produced organic acid solution to wastewaters were shown as one possible method.

Acknowledgements

These researches were supported by many organizations of Japan; Able Cooperation (Tokyo), Kanagawa Prefectural Livestock Industry Research Institute, Yokohama Municipal Sewage Treatment Center, Several Chemical Companies, Obihiro University of Agriculture and Veterinary Medicine and Kanagawa Prefectural Agricultural Research Institute.

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