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APPLICATION OF ALCALIGENES FAECALIS NO. 4 FOR TREATMENT OF HIGH-STRENGTH AMMONIUM WASTEWATER

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Abstract

Biological ammonium removal from wastewater requires coupling of nitrification and denitrification processes in respective aerobic and anaerobic conditions. This difference in environmental requirements pose a big challenge in the design of systems intended to effect both nitrification and denitrification processes. *Alcaligenes faecalis* strain No. 4, however, has both heterotrophic nitrification and aerobic denitrification abilities in aerobic conditions, eliminating the need to provide anaerobic conditions for denitrification. Batch and continuous experiments in a mixed culture of *A. faecalis* No. 4 and activated sludge were performed to examine ammonium removal and microbial stability of No. 4 in aerobic reactors. At an inflow ammonium load of 500 mg L^{-1} d^{-1} in continuous experiment, the ammonium removal rate (21 mg L^{-1} h^{-1}), under No. 4, was approximately 2 times higher than that of the control (i.e., without No. 4), and the denitrification ratio was approximately 66%. The proportion of intracellular nitrogen converted from removed ammonium in the continuous mixed culture was reduced, compared to control batch and continuous cultures. These results demonstrated stable growth as well as the heterotrophic nitrification-aerobic denitrification abilities of No. 4 in activated sludge systems.

Key words: activated sludge, aerobic denitrification, ammonia removal, heterotrophic nitrification, nitrogen balance

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1. Introduction

Ammonia (either as NH_4^+, NH_3, or a combination of both) is one of the major constituents of most wastewater and can be converted to various nitrogen species depending on the prevailing dissolved oxygen level, carbon to nitrogen ratio, pH, and microbial community. This ammonia can also contribute to air pollution because the NH_3 species is volatile (Koirala et al., 2014; Vaddella et al., 2013). Ammonia released from agricultural source is also of importance to particulate air pollution because it is a precursor to the formation of secondary aerosols, such as ammonium sulfate and ammonium nitrate (Sarawati et al., 2019; Zhang et al., 2010). Therefore, development of effective treatment technologies for high-strength ammonium wastewater such as livestock wastewater are important scientific pursuits.

Activated sludge has been widely applied in many environmental fields for the treatment of organics, nitrogen, phosphate, etc., in wastewater, due to its versatility and verified stability (Chudoba, 1985; Henze, 1992; Mino, 2000; Wang et al., 2016). The conventional activated sludge process is particularly

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ineffective for wastewater with high ammonium and carbon concentrations, such as livestock wastewaters, mainly due to the low efficiency of nitrogen removal (Boonnorat et al., 2017; Eschenhagen et al., 1999; Nsabilmana et al., 2000). The high level of free ammonia in high-strength wastewater inhibits microbial growth (Yang et al., 2004), and, in particular, nitrifying bacteria growth rate is very slow (Wang et al., 2012; Wei et al, 2014). In conventional treatment, nitrification process followed by denitrification process is in two separate units. Carbon, however, is not needed in the nitrification process, but the addition of carbon is necessary in the denitrification process. The nitrification process is the rate-limiting step in ammonium treatment because the growth of autotrophic nitrifiers is extremely slow. In addition, autotrophic nitrifiers and heterotrophic bacteria in activated sludge cannot coexist under aerobic condition because autotrophic nitrifiers are sensitive to organic matter (Eschenhagen et al., 1999). Generally, high strength livestock wastewaters contain both high ammonium and high concentrations of organics (carbon), producing air emissions of ammonia, odor, and greenhouse gases (Qi et al., 2015; Riya et al, 2015), thus the removal of organic compounds should also be considered in any treatment process designed to remove ammonium (Boonnorat et al., 2017; Lai et al., 2011;.Mino, 2000).

As an alternative to the conventional method, several new nitrogen removal processes have been developed, such as anaerobic ammonium oxidation (ANAMMOX) and an aerobic granule process. However, these processes share some limitations with the conventional nitrogen removal system, such as slow growth rate of bacteria and inhibition from high free ammonia concentrations (Neerackal et al., 2016).

Maintaining stability and high-density microbial consortia is important for improving nitrogen and carbon removal efficiencies in the treatment of high-strength ammonium wastewater (Ashok et al., 2014; Dong et al., 2017; Kang et al., 2018; Liang et al., 2015; Seo et al., 2001; Shan and Obbard, 2001; Stuven and Bock, 2001; Uemoto et al., 2000). Some studies have mitigated such limitations using immobilized cell culture to maintain stability and high-density of microbial consortia (Dong et al., 2017; Jittawattanarat et al., 2007), or with a mixed culture of different kinds of microorganisms, such as bacteria and algae for simultaneous treatment of multiple nutrients (Ashok et al., 2014; Kang et al., 2018).

Other research has focused on technologies using genera of bacteria possessing both the ability to nitrify and denitrify, for treatment of high-strength wastewater (Chen et al., 2015; Gupta and Gupta, 2001; Ikeda-Ohtsubo et al., 2013; Li et al., 2017; Nishio et al., 1998; Pai et al., 1999; Shoda and Ishikawa, 2014; Stouthamer et al., 1997; Zhao et al., 2012). *Alcaligenes faecalis* strain No. 4 (No. 4), one such bacteria, was isolated in the laboratory and it was initially studied as an antagonist of various plant pathogens via a nitrified product, hydroxylamine (Honda et al., 1998). This strain has heterotrophic nitrification and aerobic denitrification abilities, and it converts ammonium to nitrogen gas in a single aerobic reactor. The feasibility of No. 4 for treating high-strength ammonium wastewaters in an open system has previously been documented (Joo et al., 2005, 2006; Shoda and Ishikawa, 2014). For more effective treatment compared with previous studies, the combination of No. 4 and activated sludge which has been widely used in field application can be a possible approach, because strain No.4 was originally isolated from activated sludge (Honda et al., 1998).

In this study, ammonium removal in a mixed culture of Strain No. 4 and an activated sludge microbial consortium was initially performed in batch studies. Subsequently, the continuous study was conducted using the mixed culture of No.4 and activated sludge, and compared to a control containing activated sludge system without No.4. These experiments elucidated the role of No. 4 in enhancing ammonium removal.

2. Materials and methods

2.1. Inoculum and pure culture of strain No. 4

A. faecalis No. 4, initially isolated from activated sludge and stored in 25% glycerol solution at -80°C was used in this study (Honda et al., 1998; Joo et al., 2005). The activated sludge (mixed liquor suspended solid (MLSS): 4,000 mg L^{-1}) used in the mixed culture with No. 4 was the return sludge from a local sewage treatment plant (Naruse Sewage Treatment Plant in Tokyo, Japan).

The basal medium used as synthetic wastewater was prepared as follows: 2 g $(NH_4)_2SO_4$, 17 g trisodium citrate dihydrate, 6 g K_2HPO_4, 14 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 mL trace mineral solution in 1 L distilled water. The trace mineral solution contained 57.1 g EDTA·2Na, 3.9 g $ZnSO_4 \cdot 7H_2O$, 7 g $CaCl_2 \cdot 2H_2O$, 5.1 g $MnCl_2 \cdot 4H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.6 g $CuSO_4 \cdot 5H_2O$, and 1.6 g $CoCl_2 \cdot 6H_2O$ (at pH 6.0) in 1 L distilled water. A 1% pre-culture of No. 4 was inoculated into a 150 mL aliquot of membrane-filtered (pore size = 0.2 μm) basal medium and placed in a shaker incubator at 30°C set at 120 cycles min^{-1}. The initial NH_4^+-N concentration was adjusted to approximately 1,200 mg L^{-1} and 2 mL aliquots of the samples were taken periodically for chemical analyses and measurements of pH and colony forming units.

2.2. Batch studies

A total of 38 g of trisodium citrate dehydrate and 6.25 g glucose as carbon sources, and 6 g of ammonium sulfate as a nitrogen source were added to 150 mL of basal medium in a 500-mL shaking flask. The C/N ratio was adjusted to approximately 10, to ensure that the carbon and nitrogen sources were consumed at a balanced rate (Joo et al., 2005). The

initial NH_4^+-N concentration was adjusted to approximately 1,000 mg L^{-1}. Glucose and trisodium citrate dehydrate were added in the basal medium as carbon sources to mixed cultures of activated sludge and No. 4. Aliquots of 10% of the mixture of No. 4 and activated sludge, consisting of 0%, 10%, and 50% pre-culture of No. 4 and 100%, 90%, and 50% activated sludge, respectively, were inoculated into flasks (Table 1). The flasks were cultivated in the shaker incubator at 30°C at 120 strokes per minute for 78 h.

2.3. Continuous studies

A schematic of the reactor-system used in the continuously fed studies is shown in Fig. 1. All operations were conducted under non-sterilized conditions at room temperature and the operational conditions are shown in Table 2. A modified basal medium of synthetic wastewater was prepared by dissolving 1 g Na_2HPO_4, 2 g NaH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 2 mL trace mineral solution in 1 L of distilled water. To complete the basal medium, 12–25 g L^{-1} trisodium citrate dehydrate and 5–7 g L^{-1} glucose were added as carbon sources, and 1–3 g L^{-1} ammonium chloride (200–750 NH_4^+-N mg L^{-1}), and 1.75–2.5 g L^{-1} sodium nitrate (300–400 NO_3^--N mg L^{-1}) as nitrogen sources.

Under these experimental conditions, the C/N ratio of the medium was adjusted to 8–10 (the C/N ratio was adjusted down from 10 to 8 to increase the carbon removal rate at day 36). Influent ammonium concentrations and hydraulic retention times (HRT) were changed and air flow rate was increased with increasing biomass concentration by continuous return of the sludge from the clarifier to the aeration tank. A mixture of activated sludge (1.2 L) and No. 4 culture (1.2 L) was introduced into the aeration tank (2.4 L), and the air flow rate was set at 0.53 L min^{-1}. The solids content of the seeded activated sludge was about 2 times larger than that in No. 4 (Table 1). After performing preliminary batch operation for 3 days, continuous operation was initiated with the synthetic wastewater.

Table 1. Mixed cultures (activated sludge and No. 4) set-up in batch experiments in the shaker incubator.

Name	***Mixed condition***	***Remarks***
AS* 10% 50%	No. 4 (0 mL) + AS (20 mL) No. 4 (2 mL) + AS (18 mL) No. 4 (10 mL) + AS (10 mL)	Activated sludge: TS = 9225 (mg L^{-1}) MLSS = 7841 (mg L^{-1}) No. 4: TS = 3871 (mg L^{-1}) MLSS = 3588 (mg L^{-1})

**AS = activated sludge; No. 4: the culture broth after 70 h cultivation at initial 1,000 mg L^{-1} NH_4^+-N; MLSS: mixed liquor suspended solids.*

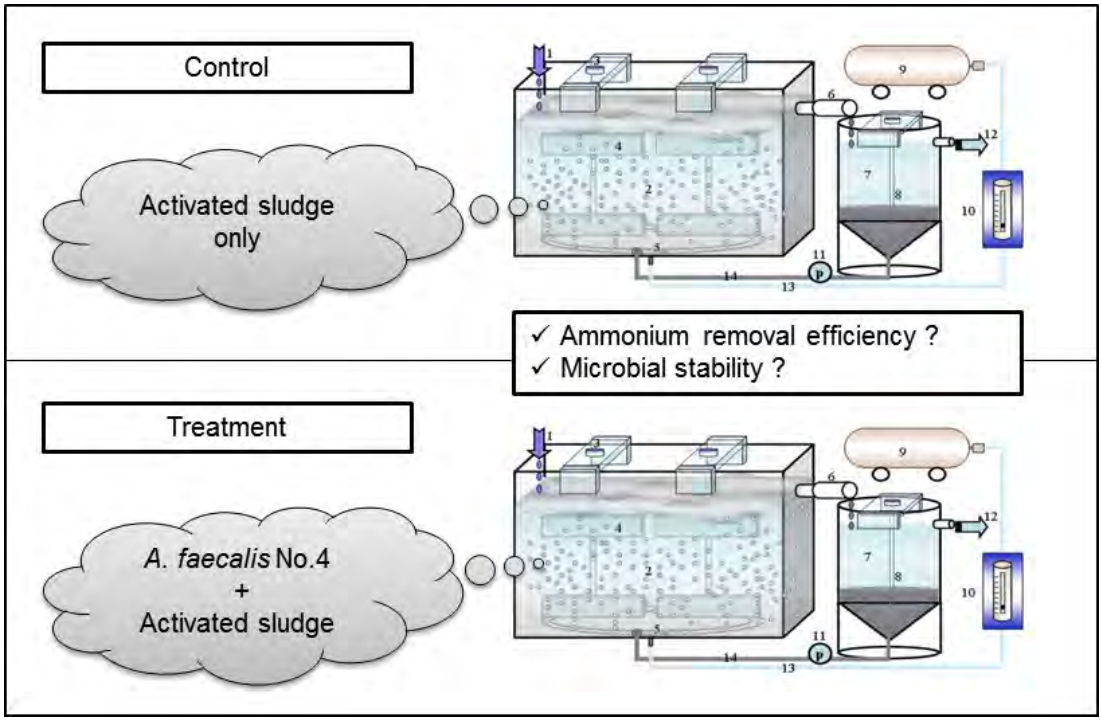

Fig. 1. Experimental approach and schematic of the reactor system (Joo et al., 2006) used in the continuous experiment: 1 = influent; 2 = aeration tank; 3 = agitating motor; 4 = agitating propeller 1; 5 = air dispersing tube; 6 = effluent pipe; 7 = clarifier; 8 = agitating propeller 2; 9 = air compressor; 10 = air flow meter; 11 = peristaltic pump; 12 = effluent; 13 = air and 14 = sludge return

Table 2. Temperature, influent NH_4^+-N concentration, hydraulic retention time (HRT), and air flow rates in the continuous experiments

Name	*Periods (d)*	*Temperature (°C)*	*NH_4^+-N (mg L^{-1})*	*HRT (h)*	*Air flow rate (L min^{-1})*
Activated sludge	1–18	26–29	400	12→18	0.53
	19–38	24–28	400–750	18→24	1.5
	39–52	23–27	200–300	24→14	2.6
	53–60	23–26	200–300	14	3.3
Mixed culture with No. 4 (50%)	1–18	26–29	400	12→18	0.53
	19–38	24–28	400–750	14→24	1.5
	39–52	23–27	200–300	24→14	2.6
	53–60	23–26	200–300	14	3.3

2.4. Aerated fed batch studies

Because of the difficulty in calculating nitrogen balances in continuous culture, modified fed batch culture studies were conducted in a 1-L jar-fermenter using sludge or a mixture of the sludge and No. 4, withdrawn from the aeration tank during the steady-state continuous culture operation. The data obtained in this modified fed batch experiment were used to estimate the nitrogen balance in the continuous culture. A 400 mL sample of the sludge was placed in each 1-L jar-fermenter with 100 mL of fresh basic medium, and cultivated at 27°C at a 0.8 L min^{-1} of air flow rate with an agitation speed of 36 rpm. A 100-mL of culture was removed, and 100 mL of fresh basic medium was added into the jar-fermenter after 5 h of cultivation. Initial concentrations of NH_4^+-N and NO_3^--N as nitrogen sources were set at 90 mg L^{-1} and 100 mg L^{-1}, respectively. Trisodium citrate dehydrate and glucose (at 3:1 ratio) were added as carbon sources. During the 10-hours of operation, concentrations of ammonium, nitrite, nitrate, citrate, and stripped ammonia were measured.

2.5. Chemical and microbial analyses

The samples used for the chemical and microbial analyses were first centrifuged at 10,000 rpm and then filtered using 0.2-μm membrane filter The concentrations of NH_4^+-N, nitrification products (NH_2OH, NO_2^-, NO_3^-), denitrification products (nitrous oxide (NO) and nitrogen dioxide (NO_2)), and citrate were measured as follows: NH_4^+ was analyzed by the standard indophenol method (JIS K 0102, 134–145, 1986); NH_2OH was measured following the method of Frear and Burrell (1995); NO_2^-, NO_3^- and citrate concentrations were measured by ion chromatography (HIC-6A, Shimadzu; Shim-pack IC-A1 column; flow rate 1.5 mL min^{-1}; buffer solution: 2.5 mmol of phthalate and 2.4 mmol trisaminomethane; oven temperature: 40°C). The chemical oxygen demand (COD) was measured by a closed reflux titrimetric method, using a standard ferrous ammonium sulfate (FAS) as titrant after digestion in potassium dichromate and sulfuric acid at 150°C for 2 h. The optical density at 660 nm (OD_{660}) was measured using a spectrophotometer (UV-1200, Shimadzu). L-agar medium (1% peptone, 0.5% NaCl, 0.5% yeast extract, and 1.5% agar at pH = 7.0) was used for the determination of the viable cell number and determinations of colonies. The amount of denitrifying products (denitrification ratio) was calculated by subtracting the amount of total nitrified products (NH_2OH-N, NO_2^--N, and NO_3^--N) and the total amount of nitrogen in dried cells, from the amount of ammonium removed. Intracellular nitrogen content (mg L^{-1}) was calculated from the viable cell number (colony forming unit, cfu) using a spread plate method and nitrogen content of dry cells obtained from the elemental analysis. Dry cells were prepared by centrifuging the culture broth, washing with sterilized water, and drying at 105°C for 24 h. Ammonium removal rate in the batch system was calculated from the graphical-zone indicating the steepest decline. Stripped ammonia in the continuous experiments was estimated using a 350-mL of culture in a sealed 500-mL flask at the same air flow rate (Joo et al., 2006).

3. Results and discussion

3.1. Ammonium removal in No. 4 pure culture

The changes in the concentrations of ammonium, nitrification products, and citrate in pure culture of No. 4 in basal medium, with incubation time, are shown in Fig. 2. Utilizations of ammonium and citrate were well-balanced when the initial C/N ratio was adjusted to approximately 10. No. 4 produced the maximum amount of hydroxylamine at 60 h, which thereafter decreased. The total amount of nitrite and nitrate at 93 h was only 28 mg L^{-1}, although approximately 800 mg L^{-1} NH_4^+-N was utilized.

Fig. 3 shows the results of the batch study with No. 4 when 120 mg L^{-1} NH_2OH was added to the basal medium instead of $(NH_4)_2SO_4$. About 90 mg L^{-1} NH_2OH-N was consumed in 24 h, and only 0.7 mg L^{-1} NO_2^--N was produced, without the growth of No. 4 (microbial growth usually results in the increase of cell mass). No NO_3^--N was detected. When 170 mg L^{-1} NO_2^--N and NO_3^--N were added instead of ammonium, the utilization and concentration change of NO_2^--N and NO_3^--N were not observed (data not shown). These data indicate that No. 4 converted most hydroxylamine to denitrified products. A complete verification of denitrification was done in a previous ^{15}N tracer experiment using $(^{15}NH_4)_2SO_4$, where 90% of the denitrified product was N_2 gas and 10% of the

denitrified product was N_2O, and NO was detected (Joo et al., 2005).

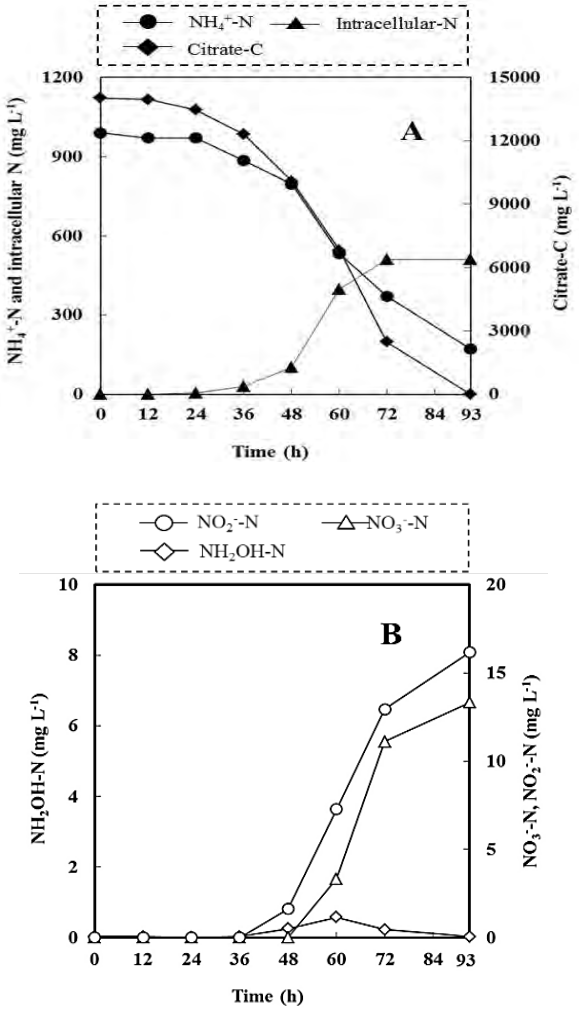

Fig. 2. Profiles of: ammonium (NH_4^+-N), intracellular nitrogen (N), and citrate concentrations (A); and nitrified products - NH_2OH-N, NO_2^--N, and NO_3^--N (B) in basal medium using a pure culture of *A. faecalis* No. 4

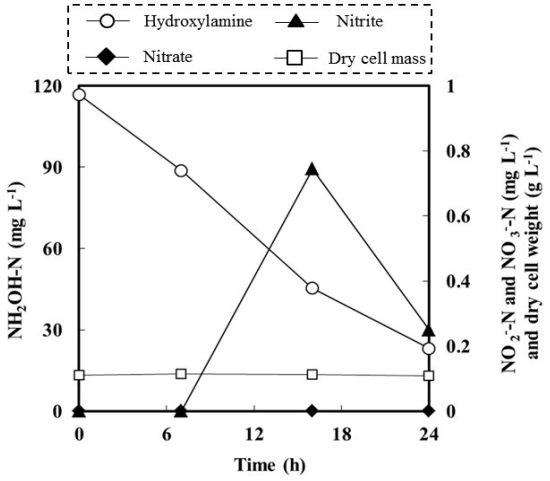

Fig. 3. Profiles of hydroxylamine, nitrite, and nitrate concentrations, and dry cell mass in the basal medium (in pure culture) with hydroxylamine instead of ammonium (i.e. replacing ammonium with hydroxylamine)

3.2. Batch studies

Fig. 4 shows the changes in concentrations of nitrogenous compounds and carbon in batch cultivation in both the control and treatments. Intracellular nitrogen (intracellular N) leveled off after 40 h when citrate was exhausted. The intracellular N in the mixed culture was 25% lower than in the control.

The latter suggested that the MLSS of activated sludge was almost two-folds that of No. 4. A mixture of activated sludge with No. 4 thus resulted in reduced cell mass. However, ammonium removal and citrate consumption were accelerated by mixing No. 4 with activated sludge.

Table 3 shows the nitrogen balance in these experiments. About half of the removed ammonium was assimilated by the cells in the mixed culture, while two-thirds of the removed ammonium was detected as intracellular nitrogen in the control (activated sludge only), suggesting that No. 4 in the mixed culture improved the denitrification ratio, instead of cell synthesis.

The mix ratios of 10%, 50% and 100% No. 4 did not significantly affect ammonium removal or its heterotrophic nitrification and aerobic denitrification capabilities (Table 3). Hydroxylamine, a major nitrification product of No. 4 was detected in the mixed culture, but no production of hydroxylamine was detected in the control (Fig. 4B). In the mixed culture, less than 5 ppm nitrite and 12-25 ppm nitrate were produced, but neither nitrate nor nitrite were detected in the control culture. These results confirmed that No. 4 exhibited nitrification ability during the activated sludge process.

Table 3. Nitrogen mass balances in the treatment and control systems in the batch experiments

	100% (No. 4 only)	***10%****	***50%****	***0% (AS only)***
Initial N (mg/l)	1234	1118	1104	1107
Removed N (mg/l)	1072	1118	1104	1086
Intracellular N (mg/l)	528	573	559	772
Nitrification N (mg/l)	28	16	31	0.0
Denitrification ratio (%)	48	47	47	29
Removal rate (mg/l/h)	26	30	30	18

**Percentage mixing volume ratio based on the initially seeded amount of strain No. 4 to the activated sludge (AS).*

2.6. Continuous studies

Fig. 5 shows ammonium removal in an open continuous study in the control (A) and treatment with No. 4 (B), at the operation conditions shown in Table 2. For 20 d from the start of the continuous culture, the

difference between the treatment and the control experiments was not clearly defined.

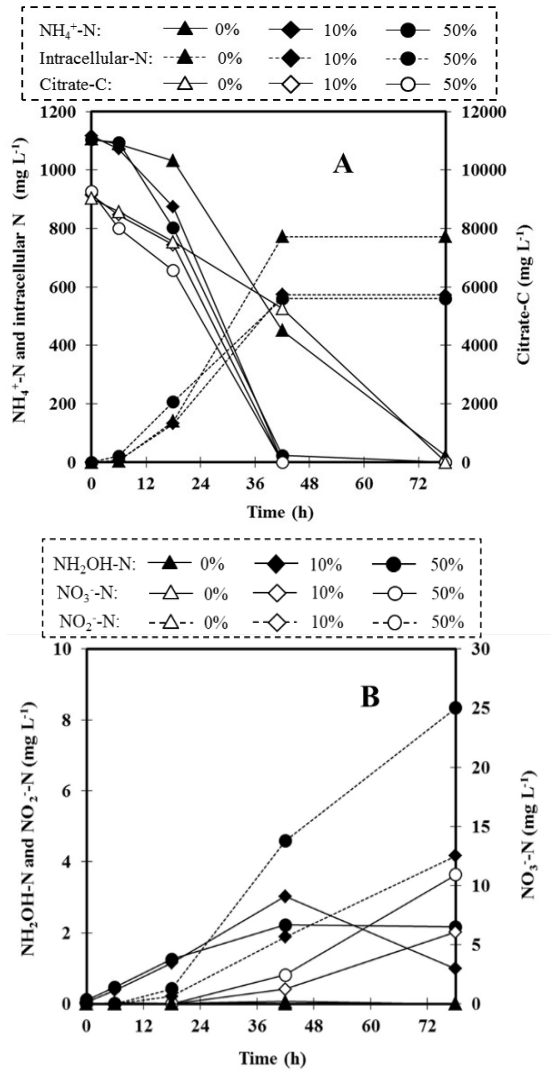

Fig. 4. Changes in nitrogen species in the mixed culture of activated sludge and No. 4 in batch experiments (A) Control; (B) Treatment

However, from the 20th day, distinct differences in the ammonium removal rate and ammonium removal ratio (removed ammonium/inlet ammonium) were observed. The ammonium removal rates in the treatments were significantly greater than those in the control. Ammonium concentration in the effluent of the control system did not reach zero, even at low NH_4^+-N loads of 200–300 mg L^{-1}, and the ammonium removal ratio was in the range of 50–70%. On the other hand, the ammonium removal ratio in the treatment system was almost 100 % after 40 d, while the ammonium removal rate was 60% higher than the control system over the same time periods.

Nitrate removal was almost 100% irrespective of the type of culture, indicating that nitrate initially added was utilized as a nitrogen source or denitrified in the activated sludge, and that a high ammonium load resulted in no adverse effect on the microbial activity with respect to nitrate consumption.

Fig. 6 shows the COD profiles in the treatment and control systems during open continuous experiments. Evidently, the COD was not completely eliminated and about 10–20% of the COD, in the influent, in both systems remained by day 35, mainly because the C/N ratio of 10 was rather high in the continuously operated systems. Therefore, the C/N ratio was lowered to 8 on day 35 by decreasing input COD. Consequently, the COD of the effluent became almost zero and the removal ratios of COD were 97–99% in both cases. This indicated that controlling the C/N ratio is key to enhancing removals of both carbon and ammonium. The pH of the media in the two reactors was maintained in the range of 7–9, which was optimal for the activity of No. 4.

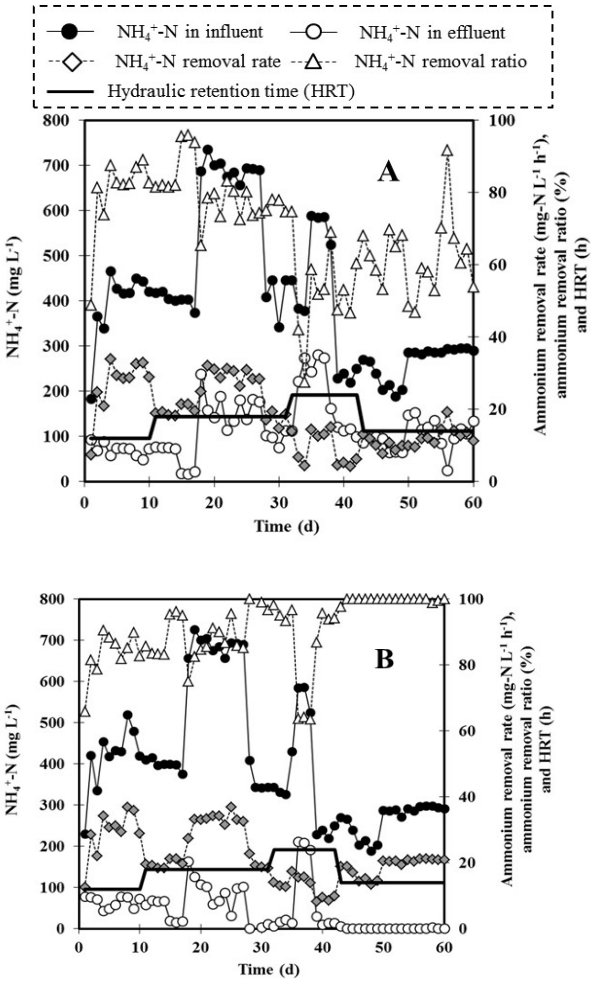

Fig. 5. Changes in ammonium concentrations, ammonium removal (rate and ratio), and HRT in the continuous experiment (A) Control; (B) Treatment

Fig. 7 shows the relationships between the ammonium removal rate (and ammonium removal ratio) with ammonium load in the treatment and the control experiments. In the control system (Fig. 7A), the ammonium removal rates ranged from 5 to 30 mg L^{-1} h^{-1}, while the removal ratios ranged between 40 and 95% (average 65%) in the NH_4^+-N load range of 200–1000 mg L^{-1} h^{-1}. In the treatment culture (Fig.

7B), the removal ratio was mostly above 90% and the removal rates were in the range of 10–25 mg L^{-1} h^{-1} at loads below 550 mg L^{-1} h^{-1}. In the NH_4^+-N load range of 550–1100 mg-N L^{-1} h^{-1}, the removal ratio was 85% and removal rate averaged 30 mg L^{-1} h^{-1}. This demonstrated that the removal ratio in the treatment culture was significantly higher than that in the control culture, in the NH_4^+-N load ranges tested in this experiment (Gupta and Gupta, 2001; Ikeda-Ohtsubo et al., 2013; Nishio et al 1998; Zhao et al., 2012; Li et al., 2019).

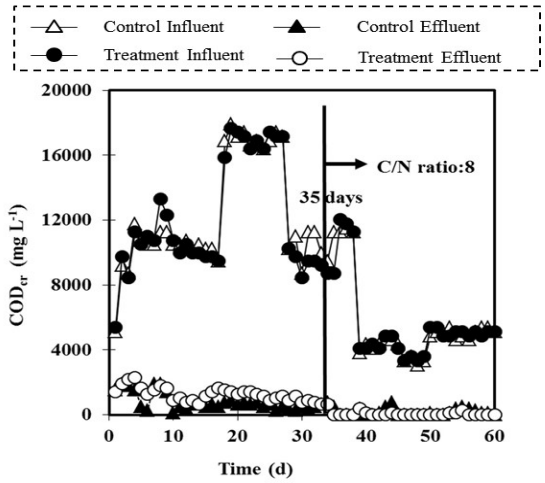

Fig. 6. The changes in the chemical oxygen demand using chromate (CODcr) in the continuous experiments.

The total cell number (cfu mL^{-1}) in the control system averaged 1.3×10^{10} for the entire experimental period and reached 2.5×10^{10} after 40 d. In the treatment (mixed culture experiment), the mean total cell number was estimated at 2.1×10^{10} and reached 4.2×10^{10} after 40 d. The final cell number of No. 4 was estimated to be 60% of the total cell number (based on clear differences in the appearance of No. 4 colonies from those of other bacteria, and the faster growth of No. 4 from other bacteria on the plates).

The higher ammonium removal ratio in the treatment culture compared to the control culture was thus partly due to higher viable cell numbers of No. 4 in the treatment culture. The main factor for the survival of No. 4 and the higher total cell number in the open mixed sludge system was presumably that the acetate supplied as a carbon source was not the primary carbon source for many bacteria in the sludge and was preferentially utilized by No. 4. These results further demonstrated the potential for field application of No. 4, especially from a viewpoint of maintaining stability and high-density of microbial consortia in open systems (Ashok, et al 2014; Dong et al., 2017; Uemoto et al., 2000).

In the control experiment, approximately 44% aerobic denitrification was observed (Table 4). This implied that some strains in the activated sludge had a similar ability as No. 4 to perform ammonium removal through aerobic denitrification. To confirm the ammonium removal mechanism in the control experiment, the isolation of dominant microorganisms from the sludge were conducted.

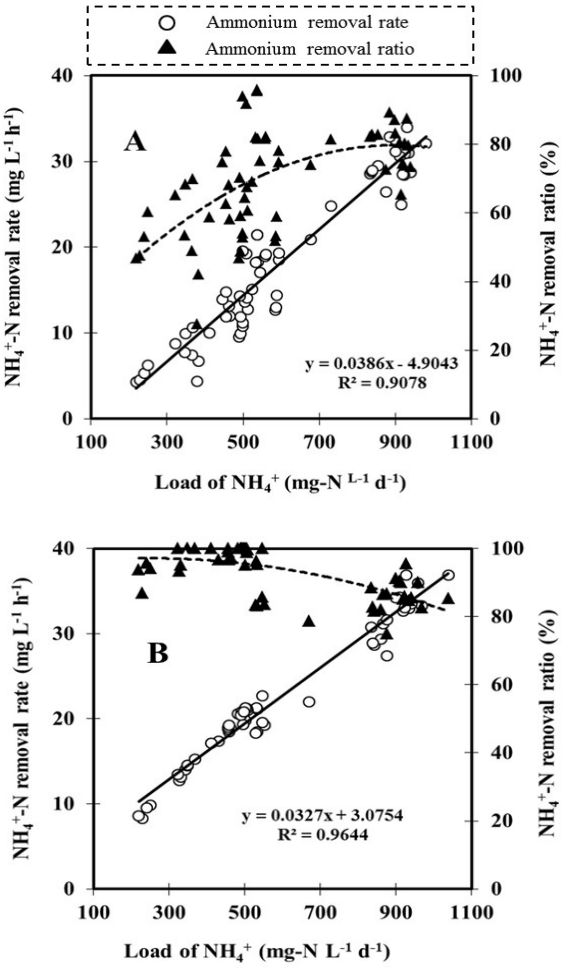

Fig. 7. Correlations between ammonium removal rate (and ammonium removal ratio) with ammonium load in the continuous experiments (A) Control; (B) Treatment

Table 4. Nitrogen balances in the treatment and control systems in the open continuous experiments

	Control	***Mixed sludge with No. 4***
Period (days); HRT (h)	50–60; 14	50–60; 14
Removal rate (mg-NH_4^+-N L^{-1} h^{-1})	13	21
Removal rate (mg-NH_4^+-N L^{-1} h^{-1}) at fed-batch	9.6	16
Removal load (mg-NH_4^+-N L^{-1} h^{-1})	310	497
Removal load (mg-NH_4^+-N L^{-1} h^{-1}) at fed-batch	230	382
Influent NH_4^+-N (mg L^{-1})	289±4.6	290±7.5
Effluent NH_4^+-N (mg L^{-1})	111±35.4	0.4±0.9
Removed N (mg L^{-1}); ratio (%)	178±2.7; 62±12.5%	289±0.5; 100±0.3%
Intracellular N (mg L^{-1}); ratio (%)	83; 47±2.7%	88; 31±4.6%
Stripped N (mg L^{-1}); ratio (%)	16; 9±1.3%	10; 3.5±1.4%
Denitrified N (mg L^{-1}); ratio (%)	79; 44±2.8	191; 66±4.5%

Five dominant strains were tested in pure cultures using basal medium, containing citrate, nitrate, and nitrate. Fig. 8 shows the change of nitrogenous compounds and citrate, as substrates. Four strains consumed ammonium and nitrate, and produced nitrite (conventional autotrophic bacteria). Only one (AS1) of the fives strains consumed ammonium and citrate. Therefore, although this strain exhibited heterotrophic characteristics, the aerobic denitrification of this strain was not clear.

2.7. Nitrogen balances

In open continuous experiments, nitrogen balance could not be accurately determined. Therefore, we performed an aerated fed batch experiment using sludge from the aeration tank to estimate nitrogen balance by calculating intracellular, stripped, and denitrified nitrogen components. Fig. 9 shows changes in the concentrations of nitrogenous compounds, citrate, and stripped ammonia in an aerated fed batch experiment.

Ammonium removal in the treatment system (Fig. 9B) was two times faster than in the control system (Fig. 9A). The ammonium removal rates calculated between 5 h to 10 h were 9.6 mg L^{-1} h^{-1} and 16 mg L^{-1} h^{-1} for the control and treatment system, respectively. Nitrate removals in both cases were not different, implying nitrate removal was accomplished mainly by the activated sludge. The ratios of stripped ammonia to removed ammonium were 3.5% in the treatment culture and 9% in the control culture. Using these data, ammonium removal and nitrogen balance within the 50 to 60-d period are shown in Table 4. Ammonium removal rates in the continuous experiment were 21 mg L^{-1} h^{-1} in the treatment culture and 13 mg L^{-1} h^{-1} in the control culture, indicating almost a 2-fold difference in the removal rates. A similar observation was made in the batch experiments, as shown in Table 3.

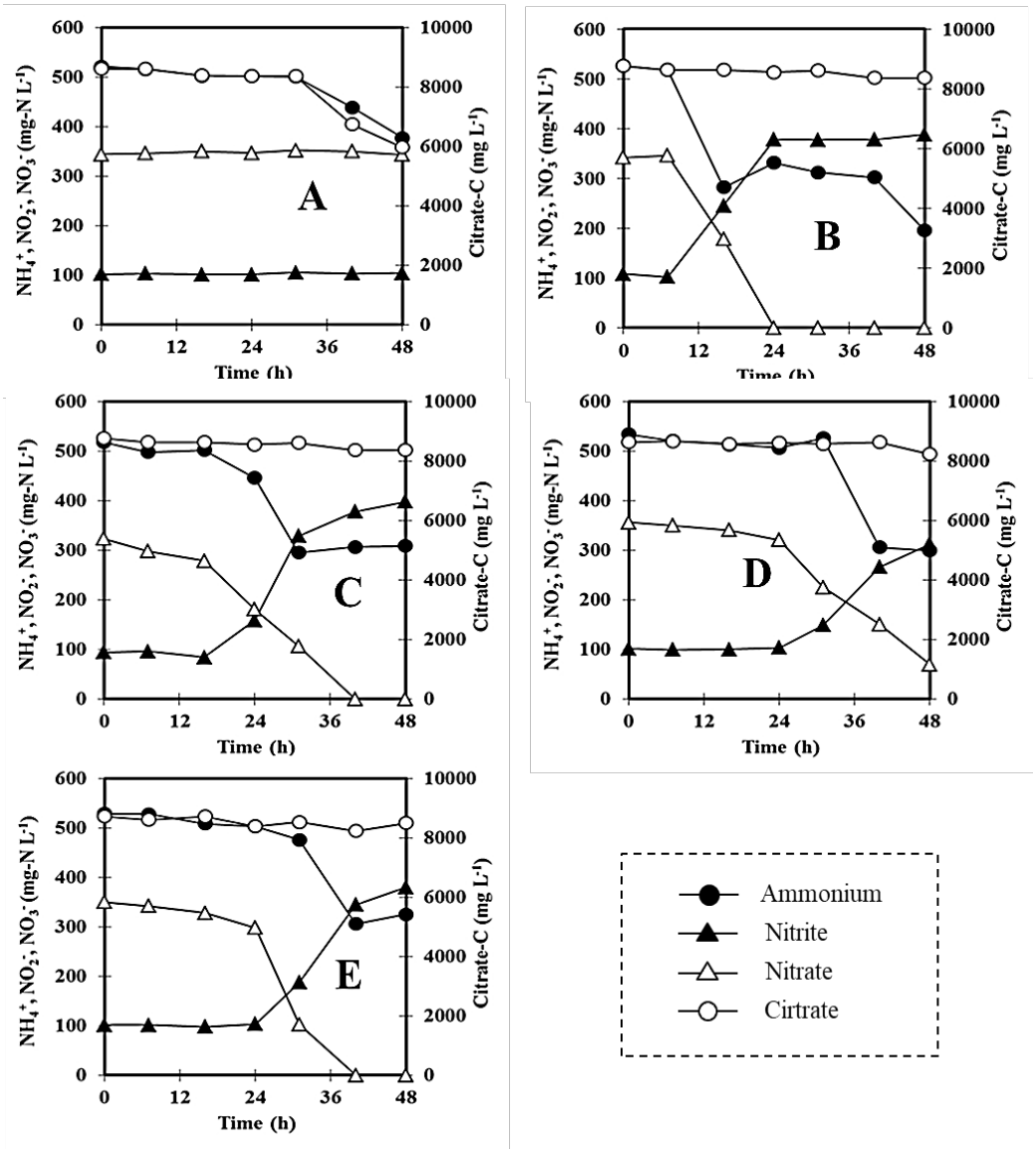

Fig. 8. Changes in ammonium, nitrite, nitrate, and citrate in pure cultures of five dominant strains isolated from the activated sludge: (A) Strain AS1; (B) Strain AS2; (C) Strain AS3; (D) Strain AS4; (E) Strain AS5

The intracellular nitrogen in the treatment culture represented 31% of the removed ammonium, which was lower than that in the control culture, and this value was also lower than that in batch experiment with pure cultures of No. 4 (48%, Table 3). In the control, the amount of ammonium removed was less than half of that in the treatment, and the denitrification ratio of removed ammonium was about 44%. In the treatment system with No. 4, the ratio increased to 66% and this ratio was higher than the 48% seen in the batch experiments with No. 4. These data indicated reduced sludge production in the continuous treatment culture but increased conversion of ammonium to nitrogen gas.

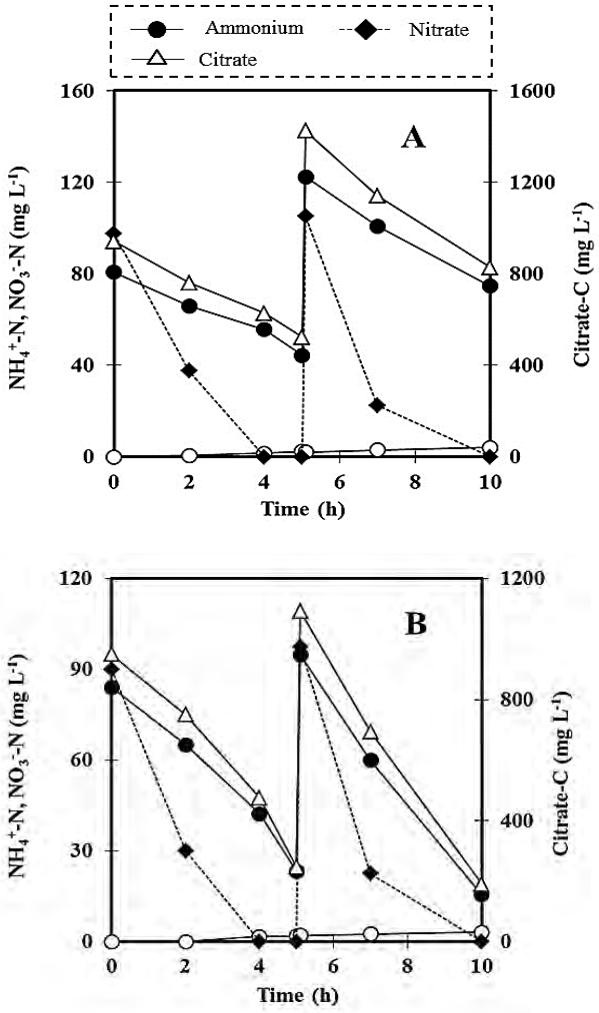

Fig. 9. Change in ammonium, stripped ammonium, nitrate, and citrate concentrations in aerated fed batch experiments in the 1-L jar-fermenter in the: (A) control, (B) treatment

4. Conclusions

The current studies confirmed that No. 4 grew at a stable rate and exhibited its characteristics in activated sludge systems. In the activated sludge treatment with No. 4, ammonium removal rate, removal efficiency, and denitrification ratio were better than those in a control system, with an ammonium removal rate about 2 times greater than that in the control system. The ammonium removal rate was significantly higher in the mixed culture with No. 4, compared to previous results in which similar bacteria were used. In the mixed culture, the denitrification ratio was higher (66%) than that in a control system (44%). This indicated that some of strains in the activated sludge had similar capabilities as No. 4 to perform ammonium removal through aerobic denitrification.

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