Aerobic ammonia removal with heterotrophic nitrification and denitrification of *Alcaligenes faecalis* strain No.4 to mitigate nitrogenous pollution caused by piggery wastewater: a feasibility study

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Abstract

The ammonia removal ability of heterotrophic bacteria *Alcaligenes faecalis* strain No.4 isolated from sewage sludge was examined in a batch operation to mitigate ammonia from piggery wastewater, consequently preventing pollution by the inflow of wastewater from piggeries adjacent to rivers. If this process works functionally, it can be effective in controlling nitrous oxide (N_2O) and nitrate (NO_3^-) emissions derived from animal agriculture, the heterotrophic nitrifying and the aerobic denitrifying effect of *A. faecalis* strain No.4 on high-strength ammonium (NH_4^+-N) were evaluated in wastewater collected from a piggery. The removal rate by *A. faecalis* strain No.4 on high-strength ammonium (NH_4^+-N) was 0.97 kg N/m^3/day which was more than 100 fold greater than that achieved using conventional aerobic nitrification and anaerobic denitrification processes. An aerobic one-step denitrification system using *A. faecalis* strain No.4 can be proposed to remove ammonia and phytopathogens from piggery wastewater with high efficiency and prevent water pollution in adjacent rivers.

Ethical Compliance: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Author Contributions: J. Takahashi, M. Shoda, L. Jianhua, and L. Ning contributed to the design and implementation of the research, J. Takahashi and M. Shoda analyzed the results and wrote the manuscript. J. Takahashi conceived the original and supervised the project.

Keywords: *Alcaligenes faecalis* strain No.4, heterotrophic, nitrification, denitrification ammonia, nitrous oxide

Introduction

The world population has been parallel to ammonia synthesis from atmospheric paired nitrogen fixed by the Haber-Bosch process in the early 20th century (Fig.1). According to a quantitative estimation, approximately 50% of the global population seems to depend on synthetic ammonia fertilizers (Erisman, et. al., 2008). Hence, it follows that currently 3.9 billion population depend on the synthetic nitrogen fertilizer. However, the deposition of nitrogen from the atmosphere on terrestrial surfaces was estimated at 125 Tg/year in the early 21st century (Gruber & Galloway, 2008). Eighty% of the deposition is emitted from industrially fixed nitrogen and the remaining 20% is emitted from the combustion of fossil fuels. Once the stable paired nitrogen (N_2) in the atmosphere is chemically transformed to ammonia and urea as nitrogen fertilizers, the reactive nitrogen compounds are no longer under control owing to nitrification and denitrification in the soil, hydrosphere, or atmosphere. Thus, these chemical transformations in reactive nitrogen must be controlled during the ammonia stage. Thus, it is important to reduce the amount of ammonium nitrogen (NH_4^+-N) in the environment, reduce the influx of NH_4^+-N into the environment, and promote the recycling of NH_4^+-N.

Some harmful nitrogenous intermediates are formed and emitted through biochemical reactions, including NO_3^- and nitrite (NO_2^-) in the soil and then the hydrosphere due to leaching (Takahashi, 2006). High concentrations of NO_3^- absorbed as a nutrient by grasses and other plants often cause NO_3^--NO_2^- poisoning and methemoglobinemia caused by nitrate-reducing bacteria in the rumen of ruminants (Takahashi et al., 1989). However, the reduction of nitrate in the rumen inhibits methane production by rumen methanogens, another greenhouse gas, owing to hydrogen uptake (Takahashi et al., 1991). Nitrogen oxides (NOx) are derived from the excess amount of nitrogen fertilizers and manures such as nitric oxide (NO), which contributes to the acidity of rainwater, and nitrous oxide (N_2O), which is an ozone depletion substance in the stratosphere and is a powerful greenhouse gas, although both gases play important roles in medical physiology (Rosselli, et al., 1998; Mennerick, et al., 1998).

Improper management of livestock wastewater causes eutrophication in the hydrosphere due to NO_3^- and N_2O emissions in the atmosphere, which is attributed to the excess amount of NH_4^+-N. It is a common issue in Asian and African developing and emerging countries, where abrupt population expansion and urbanization have progressed along with economic development (Nyenje, et al., 2010; Lin, et al., 2021). Thus, excessively fixed reactive NH_4^+-N should eventually return to atmospheric N_2 through complete nitrification and denitrification without the deposition and emission of any pollutant nitrogenous intermediates. To achieve this, it is fundamentally necessary to reduce the excessive input of nitrogen into the environment. NH_4^+-N, which is a pollutant and a burden on the environment, must be removed by efficient nitrification and denitrification. Most biological approaches to ammonia removal from livestock wastewater have conventionally been implemented by aerobic nitrification and anaerobic denitrification using heterotrophs and autotrophs (Carrera et al., 2003). However, autotrophic bacteria are presumably unsuitable for livestock wastewater treatment because of the high concentrations of ammonium and organic matter (Ruiz et al., 2003). Furthermore, the long retention time of autotrophic nitrification has been attributed to the slow proliferation rate of bacteria (Richardson and Watmouth, 1999). In an attempt to determine the biological ammonia removal ability, Joo et al. (2005a, 2005b) isolated heterotrophic bacteria, *A. faecalis* strain No.4, from sewage sludge, which has heterotrophic nitrification and aerobic denitrification abilities. They demonstrated that *A. faecalis* strain No.4 could achieve prompt removal of ammonia from piggery wastewater and efficient denitrification from the removed ammonia under high-strength NH_4^+-N and chemical oxygen demand (COD) (Joo et al., 2006).

The present study deals with a feasibility study in *A. faecalis* strain No.4 removes NH_4^+-N from piggery effluent water according to Joo et al. (2006) and consequently could improve river water quality polluted by flowing piggery effluent into the river.

Materials and Methods

First, the properties of pH, dissolved oxygen (DO), and liquid temperature were surveyed in wastewater containing effluent from a piggery beside a tributary of the Yangtze River located in the suburb of Shanghai, China (Fig. 2). Within a 20 km radius from east to west of this river basin, there is a concentration of 107 piggeries, including the piggery surveyed in this study. There are several factory complexes in the river basin, including textile factories, but industrial wastewater does not flow into the river. Thus, annual changes in water quality (temperature, pH, DO, and EC) in the downstream most reaches of the study area were monitored.

In general, piggeries in this area manage manure without solid-liquid separation. The liquid waste mixed with cleaning water from livestock barns is dumped into the river through a drainage ditch, leading to a tributary stream, and the solid part that settles in the drainage ditch is used as a fertilizer after it dries naturally. Water qualities of pH, DO, liquid temperature, and electrical conductivity (EC) upstream and downstream of the piggery.

Subsequently, NH_4^+-N removal from piggery wastewater contaminated with swine effluent was performed using *A. faecalis* strain 4. (Shoda & Hirai, 2006). Table 1 shows the culture medium used for the growth and cultivation of *A. faecalis* strain 4. Table 2 shows the composition of trace elements added to the medium. The cultured cells of *A. faecalis* strain No.4 were mixed with 50 % glycerol solution and stored at -84°C. The preparation and characteristics of *A. faecalis* strain No.4 were determined according to the procedure described by Joo et al. (2006).

Fig. 3 shows a small-scale (working volume 300 ml) jar fermenter (BMJ-01PI, Able Corp., Tokyo) and the sensors used. To determine the optimal incubation conditions to remove NH_4^+-N derived from piggery effluent, the DO concentration and pH were continuously monitored using a DO sensor (SDOC-12F, Able Corp., Tokyo) and pH sensor (Easyferm Plus 225, Hamilton Bonaduz AG, Bonaduz). NH_4^+-N concentration was monitored using an ammonium sensor (SNH-10, Able Corp., Tokyo). The aeration rate was set at 300 ml/min and the temperature was maintained at 30°C. The agitation speed was set at either 400 rpm (rotation per minute) or 700 rpm. Ammonia removal experiments were carried out by the addition of 45 ml *A. faecalis* strain No. 4 and 2 ml defoaming agent with citric acid (denoted as S in figures) or without citric acid (denoted as C in figures (Control) as a carbon source to 255 ml piggery sample fluid. When vigorous foaming occurred, the defoaming sensor deformed the culture.

Results

The wastewater quality in the piggery was indicated such as pH 8.44, DO 0.28 mg/l, fluid temperature 9.5°C, chemical oxygen demand Cr (CODcr) 2160 mg, biochemical oxygen demand (BOD) 1020 mg/l, and NH_4^+-N 1100 mg/l. The total content of the observed acids (oxalic acid, citric acid, lactic acid, formic acid, acetic acid, propionic acid, iso-butyric acid, and butyric acid) was approximately 1000 mg/l. These organic acids were the main carbon sources for *A. faecalis* strain No. 4. For the upstream water quality, pH 6.85, EC 472 µS/cm, DO 5.12 mg/l, and fluid temperature 9.5°C were observed. In downstream water, pH 6.83, EC 470 µS/cm, DO 4.32 mg/l, and fluid temperature 9.5°C were quantified. The EC values in the upstream and downstream areas were slightly above the surface water standard limit (400 μS/cm). The DO concentrations were below the WHO (2011) standard limit for drinking water and below the surface water standard limit (6 mg/l); however, the lower values in the downstream area indicated slightly higher contamination (Mahadevan, 2020).

Table 3 shows annual changes in water quality (temperature, pH, DO, and EC) in the downstream most reaches of the study area. Water temperature in rivers is affected by temperature and has large seasonal variations, but DO also fluctuates widely. A negative correlation (-0.61, $p<0.05$) was found between water temperature and DO. The average river DO throughout the year was 4.82 mg/l, below the lower limit of the guideline. The annual mean value of EC was 527 μS/cm, well above the standard value.

Fig. 4 shows the effect of 20 g/l sodium citrate addition on ammonia removal by *A. faecalis* strain No.4 at an aeration rate of 300 ml/min, temperature of 30°C, agitation speed of 400 rpm, and initial pH of 8.9. There was no significant difference in the change in ammonia concentration between the experimental (S) and control (C) incubations. However, DO in C leveled off in 20h, but in S, the gradual decline in DO indicated continuous consumption of oxygen by *A. faecalis* strain No.4 using citrate as a carbon source, which reflected a constant decrease in ammonia concentration to 0 in 70h. The initial pH value of 8.9 in the sample fluid was relatively high and inhibited the activity of *A. faecalis* strain No.4, and the pH increased to 9.0. This led to decreased activity of *A. faecalis* strain No.4, especially for *A. faecalis* strain No.4 in C. Total organic acid content in the original wastewater of approximately 10 g/l was presumably consumed in 20h.

Fig. 5 shows the effect of the initial pH 8.0 and 10 g citrate addition on ammonia removal by *A. faecalis* strain No.4. The other operational conditions are the same as those shown in Fig. 4. The progressive removal of ammonia by active *A. faecalis* strain No.4 in S medium was confirmed because oxygen deficiency was observed after 10 h of incubation. At 20 h, an increase in DO corresponded to almost complete exhaustion of the carbon sources. Foaming is not a problem. To prevent oxygen deficiency in the bacteria, the agitation speed was set at 700rpm, and the initial pH was adjusted to 8.0 in the next experiment.

Fig. 6 shows the effect of adding 10 g/l citrate on the ammonia removal effect of *A. faecalis* strain No.4 at an aeration rate of 700 ml/min, temperature of 30 °C, agitation speed of 300 rpm, and initial pH of 8.0. There was no significant improvement in the removal rate of ammonia due to heavy foaming.

Discussion

A survey of annual changes in water quality (temperature, pH, DO, and EC) collected in the downstream most reaches of the study area indicated a relatively higher value of EC and lower value of DO than the WHO standard (WHO, 2011). DO was shown to be negatively affected by water temperature, but the variability of these values appears to depend on the amount of piggeries effluent flowing into the river. DO was lower upstream than downstream of the drainage outlets of the piggery surveyed, but there was little difference in EC. This suggests that the entire river area under study is already being contaminated by piggeries effluent. Because the effluent polluted with swine manure from the piggeries adjacent to the river flows into the river, nitrogen compounds such as ammonia derived from livestock manure or its oxidation product, NO_3^--N, are thought to have increased the concentration of dissolved ions, resulting in effluent with high EC. This suggests that ammonia in the effluent is one of the major factors for NO_3^--N contributing to river eutrophication in rivers (Tedengren, 2021). Furthermore, the emission of N_2O into the atmosphere due to the reduction of NO_3^- produced from ammonia derived from livestock manure is considered to contribute to global warming as a powerful greenhouse gas along with ozone layer depletion (Torres, et al., 2016). Therefore, the removal of ammonia from swine effluent is an important issue for environmental health, not only in the hydrosphere but also in the atmosphere. Biological denitrification is an environmentally friendly method for treating wastewater containing livestock manure. Thus, ammonia removal from piggery wastewater using *A. faecalis* strain No.4 was conducted under three conditions. From the results of three different culture tests each removal rate of NH_4^+-N was calculated as follows, 1st culture test (Fig. 3): 0.35 kg N/m^3/day, 2nd culture test (Fig. 4): 0.97 kg N/m^3/day, and 3rd culture test (Fig. 5): 0.70 kg N/m^3/day. These values were similar to those obtained in Japanese piggery wastewater treatment (Joo et al., 2006) despite the different qualities of the wastewater in different places. These values are more than 100 times higher than those of the conventional nitrification and denitrification methods. A. faecalis strain No.4 has the following mechanism (Joo et al., 2005a).

$$NH_4^+ \rightarrow \text{Hydroxylamine } (NH_2OH) \rightarrow N_2O \rightarrow N_2$$

The production of N_2O was reported only by less than 1% of used ammonia, and almost no NO_2^- and NO_3^- were produced in the process. Moreover, the growth rate of *A. faecalis* strain No.4 was more than a few hundred times higher than that of nitrification bacteria. Thus, a higher proliferation rate leads to a smaller treatment reactor and a higher treatment rate when *A. faecalis* strain No.4 was used. Furthermore, approximately 40% of NH_4^+-N is used as N_2 gas, and the remaining 60% is used for microbial protein synthesis to form the cell mass of *A. faecalis* strain No.4 (Joo et al., 2005c). This indicates that cell mass production is larger than that in the conventional biological denitrification process.

The conventional biological denitrification method consists of a nitrification process using aerobic nitrifying bacteria and a denitrification process using facultative anaerobic denitrifying bacteria. Therefore, an aeration-capable reaction tank for increasing DO in the nitrification process and an anaerobic tank for the denitrification process are required. In contrast, the biological denitrification system using *A. faecalis* strain No. 4 requires only one aerobic reaction tank that can be aerated. Another property of *A. faecalis* strain No.4 has been reported to effectively inhibit the growth of plant pathogenic fungi (Honda et al., 1998).

In consequence, an aerobic one-step denitrification system using *A. faecalis* strain No.4 can be proposed to remove ammonia and phytopathogens from piggery wastewater with high efficiency and prevent water pollution in adjacent rivers (Fig.7).

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Authorship
J. Takahashi, M. Shoda, L. Jianhua, and L. Ning conceived and designed the study. Shoda, Jian, and Ning gathered the data. J. Takahashi and M. Shoda wrote the manuscript.

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Conflict of Interest
The authors declare there are no conflicts of interest.

Ethical Approval
Not applicable.

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Figure Captions

Fig. 1. Parallel increase in the world population to the global increase in nitrogen input (FAOSTAT, 2021)

Fig. 2. Location of a surveyed piggery and water qualities in the river basin

Fig. 3. Fermenter (Type: BMJ-1L, ABLE Corp. Tokyo Japan) and sensors attached to the reactor

Fig. 4. Changes in ammonia removal by *A. faecalis* strain NO. 4 and DO at 300 ml/min of aeration rate, at 30℃ and 400 rpm of agitation speed(Initial pH8.9) with or without 20 mM citrate

Fig. 5. Changes in ammonia removal and DO at 300 ml/min of aeration rate, at 30℃, and 400 rpm of agitation speed (Initial pH8.0) with or without 10 mM citrate.

Fig. 6. Changes in ammonia removal and DO at 300ml/min of aeration rate, at 30℃, and 700 rpm of agitation speed (Initial pH8.0) with or without 10 mM citrate.

Fig. 7. Biological denitrification system with an only aerobic one-step process using *A. faecalis* No. 4 strain

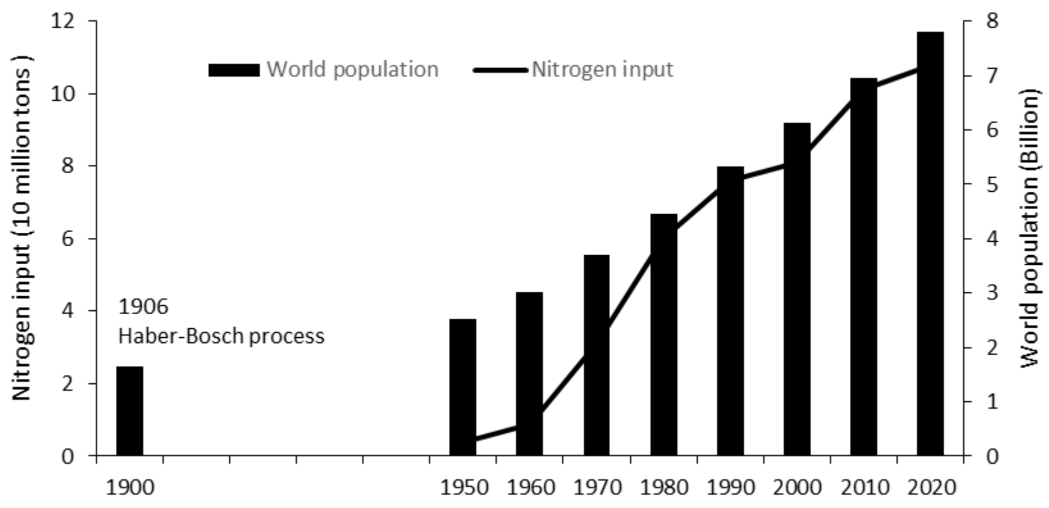
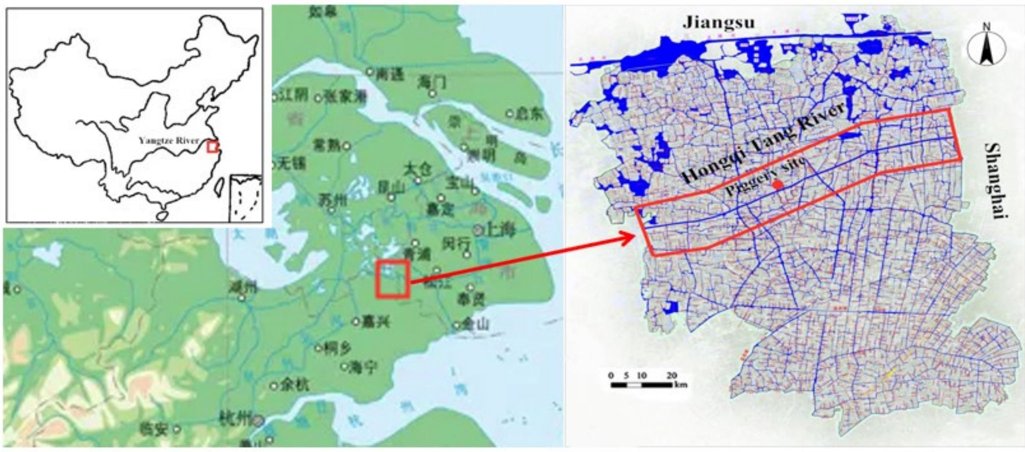

World population
Nitrogen input
Nitrogen input (10 million tons)
World population (Billion)
1906
Haber-Bosch process
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
1900
1950
1960
1970
1980
1990
2000
2010
2020

Fig. 1.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南通
海门
江阴
张家港
启东
常熟
无锡
太仓
昆山
宝山
苏州
嘉定
上海
青浦
闵行
湖州
奉贤
嘉兴
金山
桐乡
海宁
余杭
杭州
临安
Jiangsu
N
Hongqi Tang River
Piggery site
Shanghai
0 5 10 20
km

Fig. 2

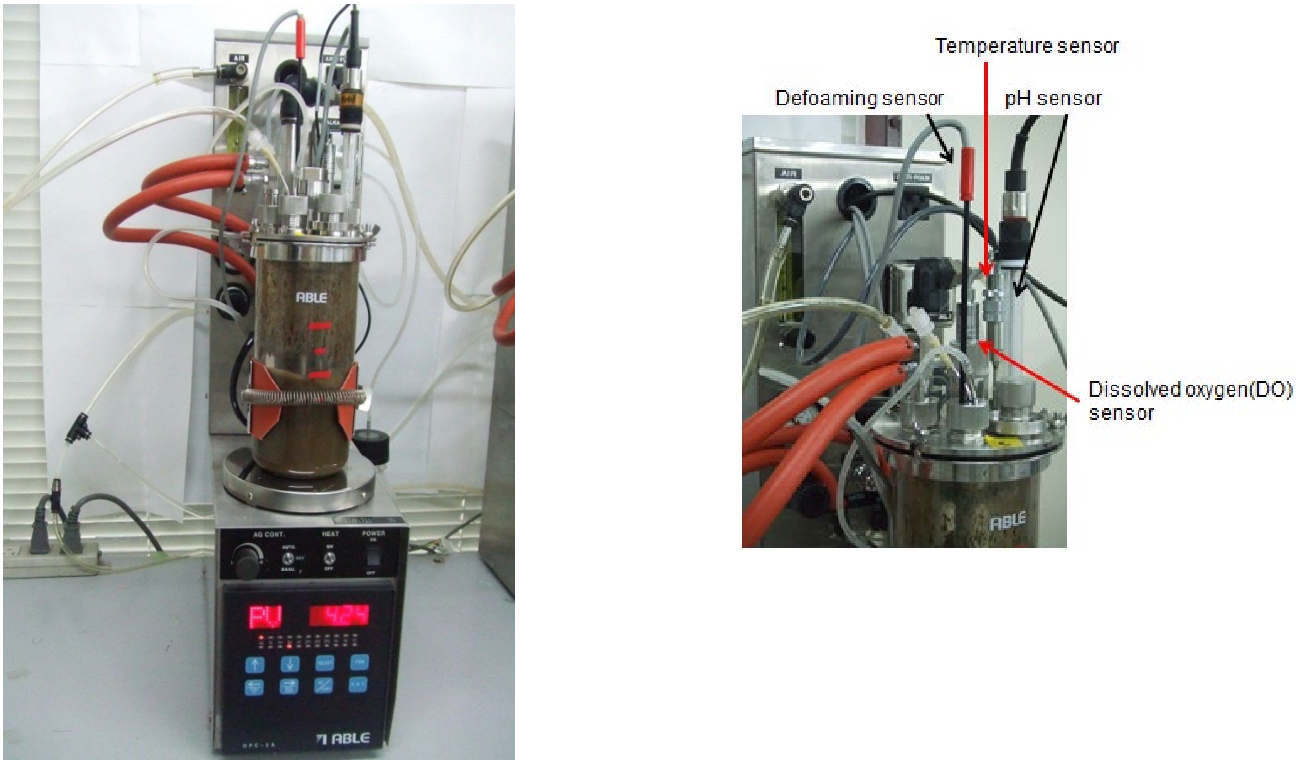

Temperature sensor
Defoaming sensor
pH sensor
Dissolved oxygen(DO) sensor
ABLE

Fig. 3.

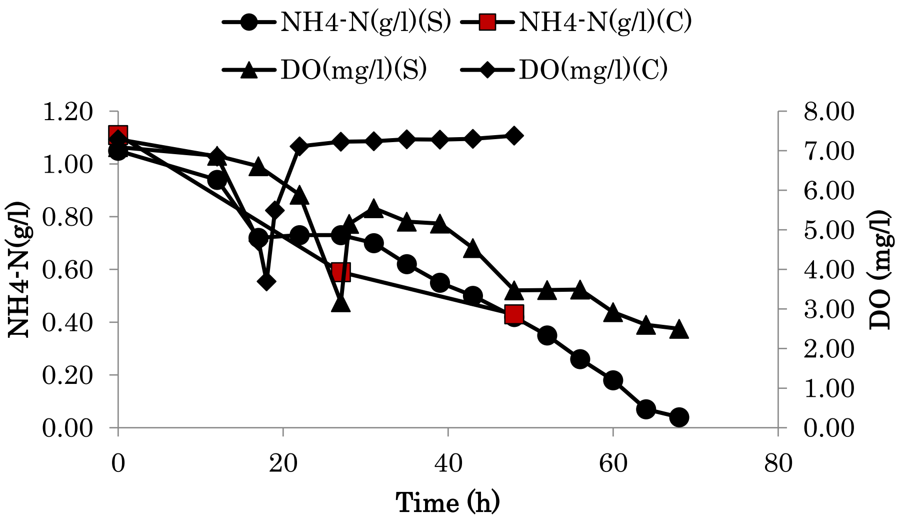
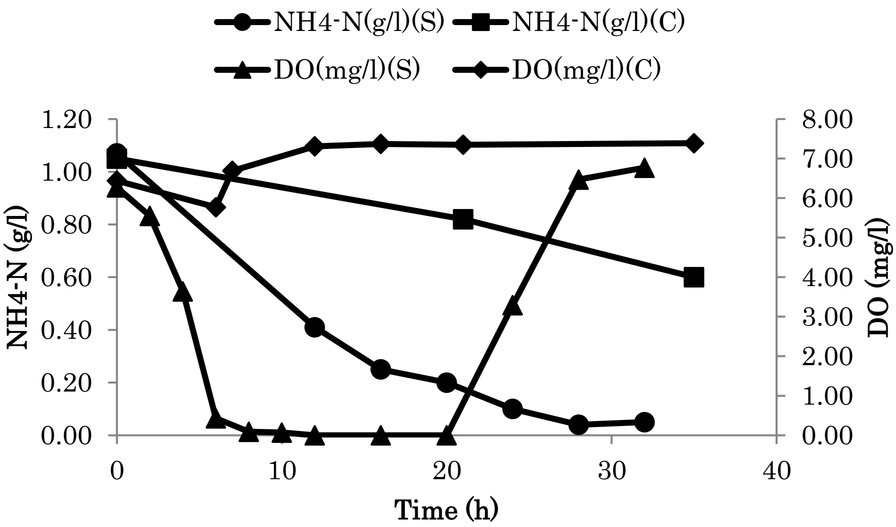

NH4-N(g/l)(S)
NH4-N(g/l)(C)
DO(mg/l)(S)
DO(mg/l)(C)
NH4-N(g/l)
DO (mg/l)
Time (h)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
20
40
60
80

Fig. 4.


NH4-N(g/l)(S)
NH4-N(g/l)(C)
DO(mg/l)(S)
DO(mg/l)(C)
NH4-N (g/l)
DO (mg/l)
Time (h)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
8.00
7.00
6.00
5.00
4.00
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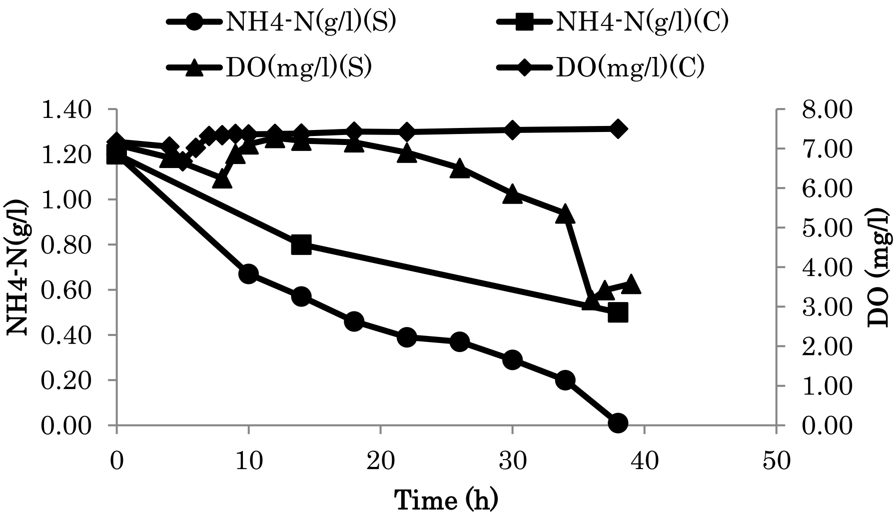
2.00
1.00
0.00
0
10
20
30
40

Fig. 5.


NH4-N(g/l)(S)
NH4-N(g/l)(C)
DO(mg/l)(S)
DO(mg/l)(C)
NH4-N(g/l)
DO (mg/l)
Time (h)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
8.00
7.00
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3.00
2.00
1.00
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0
10
20
30
40
50

Fig. 6.

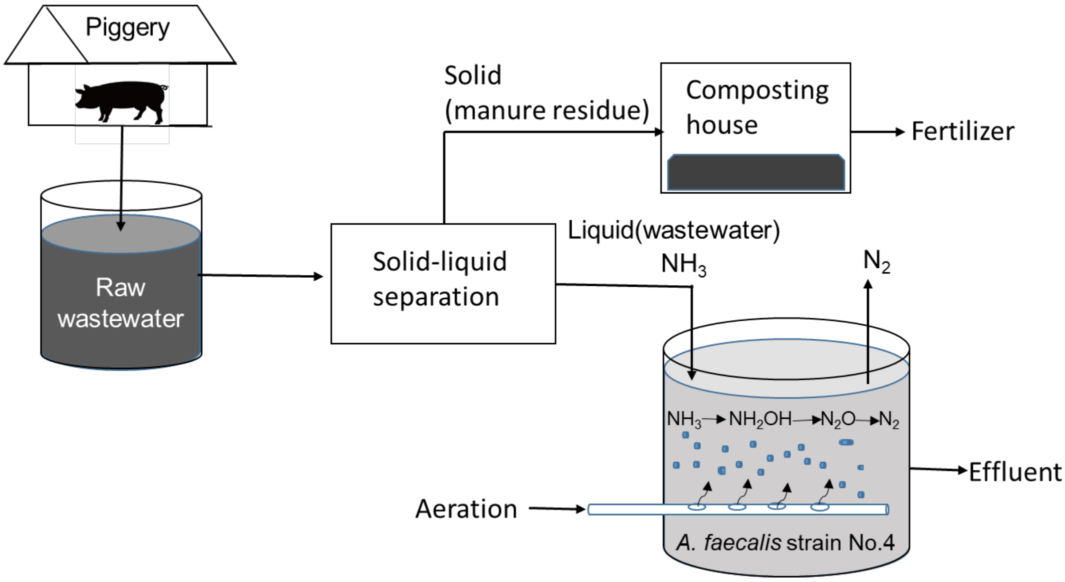
Piggery
Solid
(manure residue)
Composting
house
Fertilizer
Raw
wastewater
Solid-liquid
separation
Liquid(wastewater)
NH3
N2
NH3 → NH2OH → N2O → N2
Effluent
Aeration
A. faecalis strain No.4

Fig. 7

Table 1. Culture medium for *Alcaligenes faecalis* strain No.4

Dipotassium hydrogen phosphate (K_2HPO_4)	14 g /l
Potassium dihydrogen phosphate (KH_2PO_4)	6 g /l
Trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)	15 g /l
Ammonium sulfate ($(NH_4)_2SO_4$)	2 g /l
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.2 g/l
Trace element solution	2 ml

Table 2. Culture medium for *Alcaligenes faecalis* strain No.4

K_2HPO_4	14 g /l
KH_2PO_4	6 g /l
$C_6H_5Na_3O_7 \cdot 2H_2O$	15 g /l
$(NH_4)_2SO_4$	2 g /l
$MgSO_4 \cdot 7H_2O$	0.2 g /l
Trace element solution	2 ml

Table 3. Annual changes in water quality (temperature, pH, DO, and EC) in the downstream most reaches of the study area.

	River water			
	Temp (°C)	pH	DO (mg/l)	EC (mS/cm)
Jan	4.7	7.31	6.21	608
Feb	8.2	7.27	5.43	411
Mar	9.5	7.68	5.69	569
Apr	14.6	7.48	5.82	510
May	16.5	7.50	4.88	525
Jun	23.5	7.41	2.88	629
Jul	28.2	7.31	3.54	541
Aug	31.9	7.16	4.52	467
Sep	28.3	7.15	4.56	553
Oct	21.0	7.16	6.12	518
Nov	20.0	7.18	4.11	532
Dec	14.4	7.48	4.13	460
Mean±SD	18.4±8.6	7.34 ±0.17	4.82±1.06	527±61

Appendix 1. Correlation coefficients and their *p* values among the parameters in river water qualities

	Peason's r			
	Temp	pH	DO	EC
Temp	1			
pH	-0.51	1		
DO	-0.61	0.08	1	
EC	0.02	0.19	-0.16	1
	p value			
	Temp	pH	DO	EC
Temp	-			
pH	0.09	-		
DO	0.04	0.80	-	
EC	0.95	0.55	0.62	-