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Ammonia oxidation capacity of *bacillus* bacteria in swine wastewater after biogas treatment

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Abstract. Nitrogen removal with biological methods plays a crucial role in wastewater treatment technology. The treatment begins with the oxidation of ammonia to nitrite to facilitate the subsequent nitrification and denitrification. Various strains of ammonia-oxidising bacteria have been reported. In this study, we use three *Bacillus* bacteria isolated from swine wastewater to oxidise ammonia. Different initial densities (10^3, 10^4, 10^5, and 10^6 CFU·mL^{-1}) of each strain were examined. The results show that the combination of all the bacteria at a ratio of 1:1:1 and a density of 10^5 CFU·mL^{-1} exhibits the most effect. The findings contribute to the diversity of ammonia-oxidising bacterial species and pose a great potential for applying these strains in wastewater treatment.

Keywords: Bacillus, ammonium-oxidising bacteria, nitrification, denitrification

1 Introduction

Swine wastewater after anaerobic treatment in a biogas tank usually contains a large number of nitrogen compounds, in which total nitrogen ranges from 115–630 mg·L^{-1} [1-3], with an average of about 307 mg·L^{-1} [1]. In total nitrogen value, ammonium (N-NH_4^+) accounts for the most significant proportion, averaging about 289 mg·L^{-1} [1] (94% of total nitrogen). Wastewater containing NH_4^+ poses a severe threat to the safety of water sources [4]. A high level of ammonium discharged into the environment causes eutrophication, toxic algae blooms [5] and is harmful to aquatic animals [6]. For example, the N-NH_4^+ concentration higher than 4.26 mg·L^{-1} is toxic to black tiger shrimp [7, 8]. Therefore, treating ammonium in swine wastewater after biogas is crucial for environmental protection.

There are numerous methods for ammonium treatment published worldwide, such as biological methods [4, 9], the air stripping process [10], precipitation with magnesium ammonium phosphate [11], and electrochemical conversion [12]. Biological methods are often the most studied and applied [4, 9]. These methods consist of two processes: nitrification (oxidation of ammonium to nitrite and then to nitrate) and denitrification (reduction of nitrate to nitrite and then to free nitrogen) [13-15]. Thus, the oxidation of ammonium to nitrite is the trigger process that facilitates the subsequent nitrification and denitrification in biological nitrogen treatment. This process takes place in the presence of

different groups of chemoautotrophic, gram-negative and obligate aerobic bacteria. They use the energy released from these oxidation processes to grow and assimilate CO_2 through the Calvin cycle [16, 17]. *Nitrosomonas* is a group of ammonia-oxidising bacteria (AOB) first described by Winogradsky [18]. They are significant and the most commonly applied bacteria group in ammonium treatment [19-23]. Along with the *Nitrosomonas* group, two other groups of bacteria, namely *Nitrosospira* and *Nitrosoccocus*, are able to metabolise ammonium [24]. However, they have several limitations. They belong to the group of autotrophic bacteria with a low growth rate and development. Their performance is influenced by other microbial groups in wastewaters [25, 26]. They have a low rate of cell division and are highly sensitive to environmental conditions, such as pH, temperature, light, chemical oxygen demand (COD), and dissolved oxygen (DO) [27]. Although they are ammonia-oxidising bacteria, they have poor tolerance to environments with a high ammonium level [25, 28, 29].

Ammonia oxidation with the participation of heterotrophic bacteria groups exhibits superior properties compared with autotrophic ammonium-oxidising bacteria groups [30-32], such as strong growth and development, high cell division rate, good competitiveness against other bacteria groups in wastewaters, and good adaptation to different environmental conditions like pH, temperature, COD, and DO. Notably, numerous groups of heterotrophic bacteria can oxidise ammonium in wastewaters with a very high ammonium level [28, 33, 34]. One of them is the *Bacillus* group, which can metabolise ammonia relatively well [34-37]. Several publications demonstrated that numerous bacteria strains belonging to the *Bacillus* group could oxidise ammonium in the water environment with very high ammonium concentration, above 1 $g \cdot L^{-1}$, and various strains can directly oxidise ammonia to nitrogen [23, 34, 35, 38]. However, very few studies in Vietnam dealt with the ammonium oxidation capacity of heterotrophic bacteria in general and *Bacillus* group in particular. Therefore, this study aims to investigate the ability of *Bacillus* bacteria to oxidise ammonia in swine wastewater after anaerobic treatment and look for a way to apply the technique to wastewater treatment.

2 Material and methods

2.1 Sampling

Three wastewater samples after biogas treatment were collected from three swine farms in Son Kim 1 commune, Huong Son district, Ha Tinh province, Vietnam. Four litres of each sample were stored in a special sterile plastic container, kept cold in an insulated Styrofoam box containing dry ice (5 °C), and brought to the laboratory. The samples were then cultured for up to 36 hours after collection, followed by shaking vigorously and filtering through sterile cotton swabs prior to isolation.

A wastewater sample for testing the ammonia oxidation ability of the isolated bacterial strains was obtained from a private swine farm in Quang Thai commune, Quang Dien district, Thua Thien Hue province, Vietnam. The sample was collected in a 20-litre plastic can wrapped in black bags to avoid direct sunlight during transportation. Before testing, the sample was settled and decanted to remove suspended particles. The studied wastewater samples have the following characteristics (Table 1).

Table 1. Characteristics of wastewater sample

No.	Parameter	Unit	Value
1	pH	–	7.7
2	COD	$mg \cdot L^{-1}$	1.600
3	$N\text{-}NH_4^+$	$mg \cdot L^{-1}$	400

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2.2 Chemicals.

$MgCl_2$, NaCl, K_2PO_4, $CaCO_3$, $FeCl_3$, Na_2COONa, $NaHCO_3$, $(NH_4)_2SO_4$, and Nessler reagents (purity 99–99.9%) were provided by Merck (Germany) and Hanna (Romania); low-melting-temperature agarose was sourced from Lonza (USA).

2.3 Methods

Culture and isolation

Winogradsky I mineral medium was used to culture and isolate the strains of ammonia-oxidising bacteria [39]. Nessler reagent was employed to check ammonia metabolism capability. The bacterial colony was cultured for five days, and ammonia metabolism was checked every 24 hours. The change of reagent colour from yellow to colourless indicates the reaction completion (Fig. 1). The culture tubes showing whatever degree of reagent colour change were considered positive and selected for bacterial isolation. The bacterial colonies with different shapes and colours were divided and transferred to new test tubes. The colonies were considered pure when they had the same shape and colour. In addition, the test tubes containing colonies were tested for ammonium metabolism during culture and isolation to eliminate colonies that were not ammonium-oxidising bacteria.

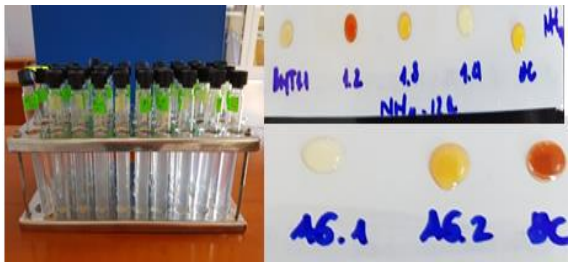

Fig. 1. Qualitative examination of ammonium metabolism with Nessler reagent of cultured bacterial strains

Gram staining

This process is based on the difference between the cell walls of Gram (+) and Gram (–). Gram (+) bacteria have peptidoglycan walls that act as an osmotic barrier preventing the loss of crystal violet. Initially, the bacteria were stained with crystal violet and treated with iodine to increase colour retention. The stain was then decolourised with alcohol, which helped to thicken the pores of the peptidoglycan layer. Therefore, the crystal violet-iodine complex was retained, and the bacteria became violet. Peptidoglycan in Gram (–) bacteria was thin with few crosslinks and had large pores. Alcohol can remove lipids from the Gram (–) wall, enough to increase the pore size. Therefore, in the alcohol-washing step, the crystal violet-iodine complex was removed. Gram (–) bacteria became pink after staining.

Identification and determination of bacteria species

Bacterial strains were identified as pure from their homogeneity on the isolation medium. Species identification was conducted via polymerase chain reaction amplification with 16S rRNA gene sequencing and searched with the BLAST tool. The DNA of the isolated bacteria strains capable of oxidising ammonium was extracted with the Macherey-Nagel kit (Fisher Scientific, USA). The DNA sample was then purified with a Promega kit (USA) before being amplified with a T100 PCR Thermal Cycler (Bio-Rad, USA) by using a 27F forward primer and a 1492R reverse primer. The DNA sample after amplification was checked for purity with an electrophoretic horizontal kit Mini Sub Cell GT (Bio-Rad, USA). The electrophoretic sample was imaged and analysed on the Gel OmniDOC system (Cleaver Scientific, UK). Finally, the DNA was sequenced on an automated

Sanger Sequencing DNA Analyser (Applied Biosystems, USA.

Effects of initial microbial density on ammonium metabolism capacity

Isolated bacterial strains were added separately to the swine wastewater samples after biogas treatment with a density from 10^3 to 10^6 CFU·mL^{-1}. The wastewater was loaded into cylindrical plastic tanks of three litres and a reaction volume of one and a half litres. The aerator was placed at the bottom of the tanks for continuous air supply (DO = 4÷6 mg·L^{-1}). The pH in the tanks fluctuated between 7 and 7.5. The control tank did not contain bacteria. The samples were collected daily for three consecutive days to assess the oxidation ability of each bacteria strain. The experiment was replicated three times. The optimal values achieved from this experiment were used for the following experiments.

Comparison of ammonia metabolising between single and combined strains at optimal microbial density

The isolated bacteria strains were combined in a ratio of 1:1:1 with the optimal density determined in the previous experiment (Previous section) and added to the swine wastewater. The experiment was performed as in Previous section to evaluate the bacteria's ammonium oxidising ability.

Environmental parameter analysis

Environmental parameters, including pH, temperature, dissolved oxygen, ammonium (N-NH_{4+}), chemical oxygen demand, and microbial density, were measured/analysed with the methods summarised in Table 2.

Table 2. Analytical methods for environmental parameters

No.	Measurement/ analytical parameter	Unit	Method Description [a]
1	pH		Measured with a portable pH meter (Toledo, Switzerland), accuracy ± 0.01
2	Temperature	°C	Measured with EXTECH equipment (Chinese), temperature range 0–50 °C, accuracy 0.5 °C
3	DO	mg/L^{-1}	Measured with a HI9146 dissolved oxygen meter (Hanna, Romania), range 0–45 ppm, accuracy ±1.5%
4	N-NH_4^+	mg/L^{-1}	Measured with a Martini equipment (Hungary), range 0–9.99 mg·L^{-1}, accuracy ± 0.01 mg·L^{-1}
5	COD	mg/L^{-1}	SMEWW 5220 D – Standard Methods for the Examination of Water and Wastewater – Determination of COD
6	Microbial density	CFU/mL^{-1}	Dilute the sample and inoculate it on a Petri dish containing a suitable medium. Temperature 28–30 °C for 24 hours. Count the number of colonies formed on the agar plate and calculate the number of microorganisms in 1 mL of the sample.
7	Ammonia removal efficiency	%	N-NH_4^+ (%) = {(C_{in} – C_{out})/C_{in}} × 100, where C_{in} and C_{out} are the N-NH_4^+ concentrations in influent and effluent water in mg·L^{-1}.

Note: [a] For samples with too high concentrations that exceed the measuring scale of the equipment, a sample dilution was performed, and the result was then multiplied with the corresponding dilution factor; CFU: Colony Forming Unit

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The experiments were conducted at the Department of Microbiological Technology of Hue Industrial College and Hue Hard Bee Scientific Research and Technology Transfer Joint Stock Company.

3 Results and discussion

3.1 Isolation and identification of bacteria strains

We isolated nine pure bacteria strains in test tubes in a mineral medium under aerobic conditions from the three wastewater samples collected from three swine farms after biogas treatment. Three of them were capable of oxidising ammonium. Gram staining shows that all three isolated strains were Gram-positive bacteria (Fig. 2). Comparing the 16S rDNA sequence of the isolated bacterial strains with the NCBI database with the BLAST tool, we identified them as Bacillus megaterium, Bacillus licheniformis, and Bacillus subtilis with 100% similarity (Fig. 3). We named them as *Bacillus megaterium* HT1, *Bacillus licheniformis* HT1, *and Bacillus subtilis* HT1.

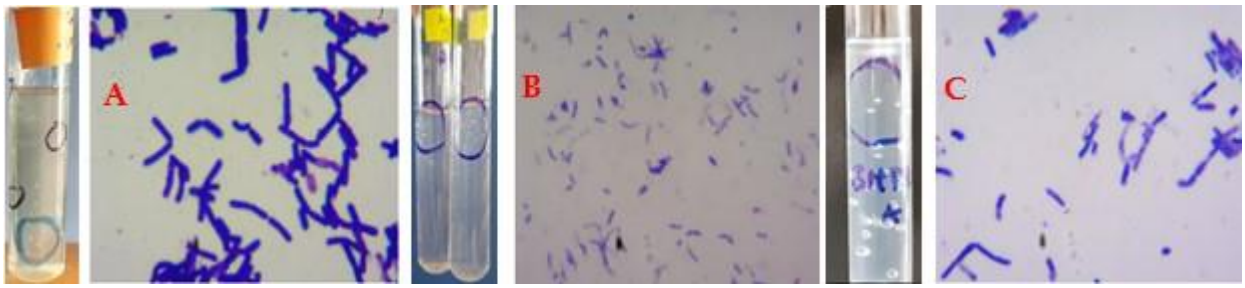

Fig. 2. In vitro colony growth and Gram staining of the bacterial strains: **A:** *Bacillus megaterium* HT1; **B**: *Bacillus licheniformis* HT1**; C:** *Bacillus subtilis* HT1

A

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus megaterium strain FDU301 chromosome, complete genome	Bacillus megaterium	2711	37702	100%	0.0	100.00%	5272433	CP045272.1
Bacillus megaterium strain S188 chromosome, complete genome	Bacillus megaterium	2711	32369	100%	0.0	100.00%	5278689	CP049296.1
Bacillus megaterium strain 5-3 chromosome, complete genome	Bacillus megaterium	2711	35186	100%	0.0	100.00%	5171845	CP047699.1
Bacillus aryabhattai strain IGND-13 16S ribosomal RNA gene, partial sequence	Bacillus aryabhattai	2711	2711	100%	0.0	100.00%	1522	MN133922.1

B

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus licheniformis strain KUBOTAB1 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	2884	2884	100%	0.0	100.00%	1545	MK865401.1
Bacillus licheniformis strain P8_B2 chromosome, complete genome	Bacillus licheniformis	2884	23041	100%	0.0	100.00%	4343379	CP045814.1
Bacillus licheniformis strain KNU11 chromosome, complete genome	Bacillus licheniformis	2884	23006	100%	0.0	100.00%	4201713	CP042252.1
Bacillus licheniformis strain HN-5 16S ribosomal RNA gene, partial sequence	Bacillus licheniformis	2884	2884	100%	0.0	100.00%	1545	MK648261.1

C

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain ZIM3 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2969	2969	100%	0.0	100.00%	1544	MT539995.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	Bacillus subtilis subsp. sub...	2969	29595	100%	0.0	100.00%	4316079	CP053102.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	Bacillus subtilis subsp. sub...	2969	29595	100%	0.0	100.00%	4398844	CP052842.1
Bacillus subtilis subsp. subtilis strain UCMB5021 chromosome, complete genome	Bacillus subtilis subsp. sub...	2969	29625	100%	0.0	100.00%	4060035	CP051466.1

Fig. 3. 16S rDNA sequences of isolated bacterial strains compared with NCBI database: **A:** *Bacillus megaterium* HT1; **B**: *Bacillus licheniformis* HT1**; C:** *Bacillus subtilis* HT1

3.2 Effects of initial microbial density

The wastewater of an initial microbial density level of 10^3, 10^4, 10^5, and 10^6 CFU·mL^{-1} was studied for the ability of *Bacillus megaterium* HT1, *Bacillus licheniformis* HT1, and *Bacillus subtilis* HT1 to convert ammonia. The results are presented in Fig.s 4, 5, and 6.

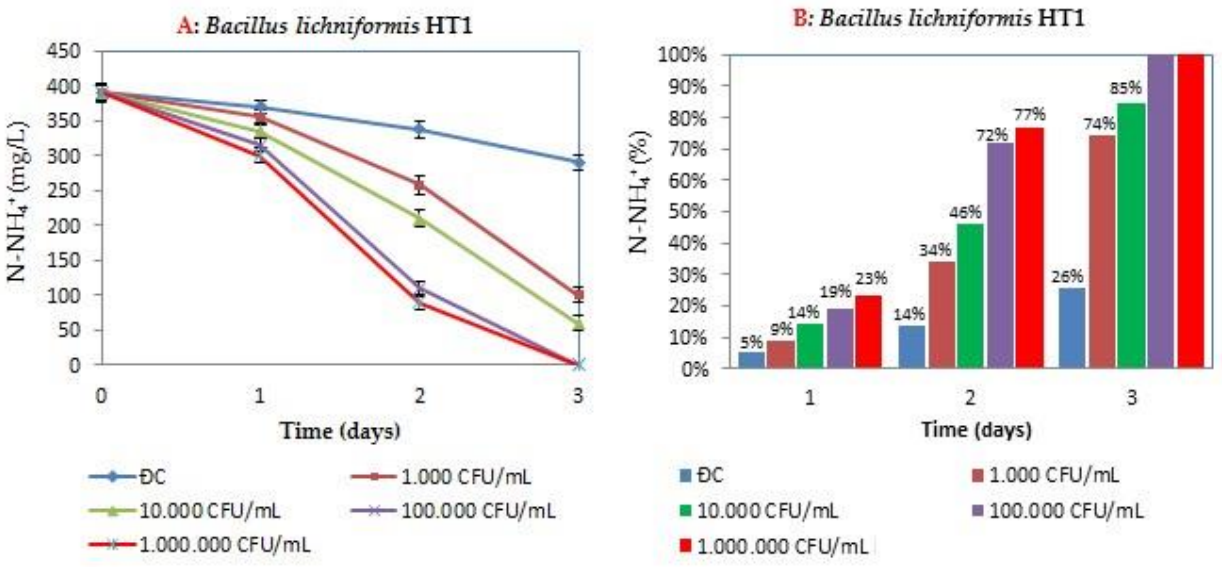

Fig. 4. Effects of microbial density on N-NH_4^+ metabolism capacity (**A**) and N-NH_4^+ treatment efficiency (**B**) in swine wastewater after biogas treatment of *Bacillus megaterium* HT1

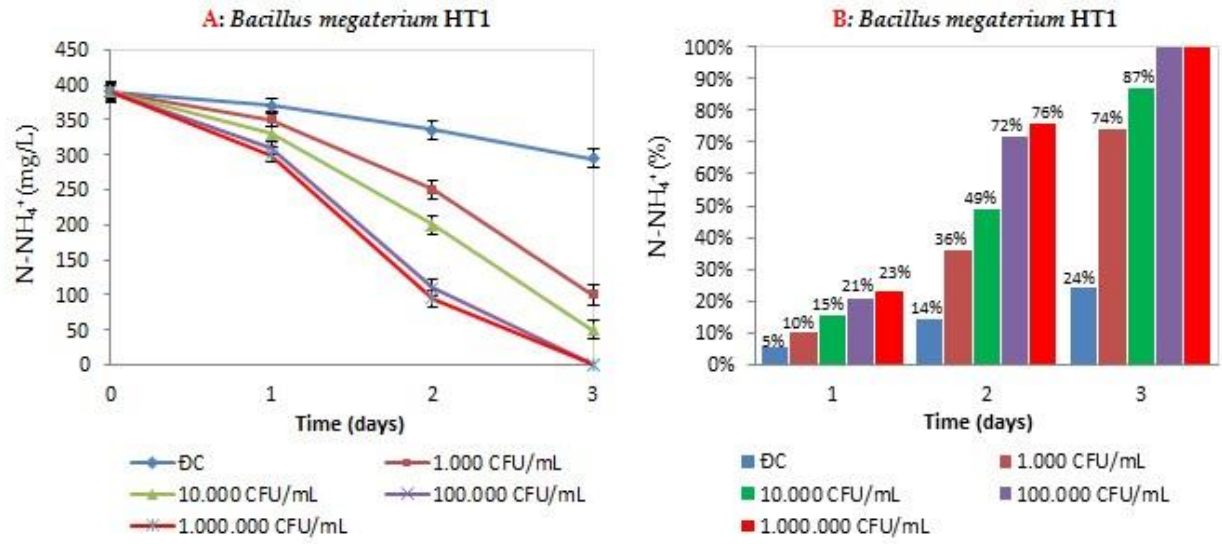

Fig. 5. Effects of microbial density on N-NH_4^+ metabolism capacity (**A**) and N-NH_4^+ treatment efficiency (**B**) in swine wastewater after biogas treatment of *Bacillus Lichniformis* HT1

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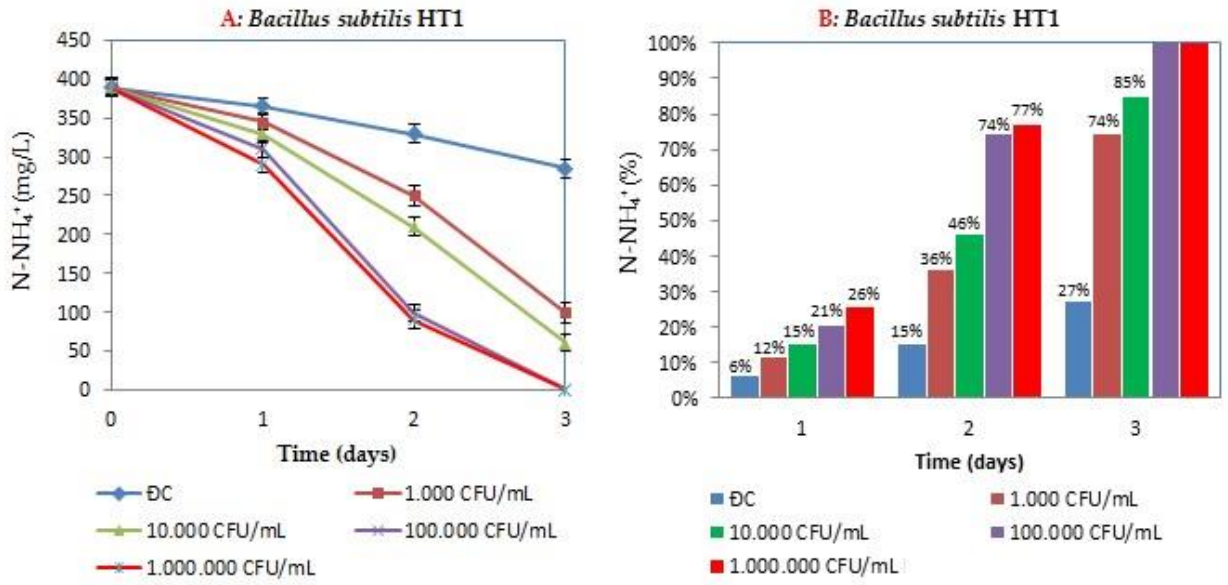

Fig. 6. Effects of microbial density on N-NH_4^+ metabolism capacity (**A**) and N-NH_4^+ treatment efficiency (**B**) in swine wastewater after biogas treatment of *Bacillus subtilis* HT1

It can be seen that, with the initial ammonium concentration at about 400 mg·L^{-1}, the ammonia-oxidising capacity of the isolates increased with the initial microbial density. At the density of 10^3 CFU·mL^{-1}, the ammonia-metabolising efficiency reached 74–77% (ammonium concentrations on day 3 were 90–100 mg·L^{-1}); at the density of 10^4 CFU·mL^{-1}, the value was 85–87% (50–60 mg·L^{-1}). At the density of 10^5 and 10^6 CFU·mL^{-1}, the treatment efficiency was 100%. In the control tank, the efficiency was 24–27% (the remaining ammonium concentration was quantified at 285–295 mg·L^{-1}). At the density of 10^5 and 10^6 CFU·mL^{-1}, there was no significant difference in the ammonium removal efficiency among the three isolated strains. Thus, the initial microbial density at 10^5 or 10^6 CFU·mL^{-1} was suitable for improving the efficiency of ammonia treatment in swine wastewater after biogas treatment for the isolated strains. Concerning treatment costs, at the density of 10^5 CFU·mL^{-1}, one litre of inoculant can treat 10 m^3 of wastewater. However, one litre of inoculant can oxidise 1 m^3 of wastewater at the initial density of 10^6 CFU·mL^{-1}. Therefore, we suggested using wastewater with an initial microbial density supplement of 10^5 CFU·mL^{-1} for ammonia treatment.

3.3 Comparison between single and combined strains

All three strains of *Bacillus megaterium* HT1, *Bacillus lichniformis* HT1, *and Bacillus subtilis* HT1 were added to the swine wastewater samples at a 1:1:1 ratio and microbial density of 10^5 CFU·mL^{-1}. The bacteria's ammonia metabolising efficiency was compared with that of single strains (Fig. 7).

It is obvious that, after two days of treatment, the bacteria can oxidise ammonia with an efficiency of 71–74% when used alone. This Fig. is somewhat higher when used in the 1:1:1 combination (85%). Meanwhile, the control shows only 15% of ammonia removal under the same testing conditions. This proves that adding *Bacillus megaterium* HT1, *Bacillus lichniformis* HT1, and *Bacillus subtilis* HT1 significantly improves the ammonia treatment efficiency in swine wastewater after biogas treatment.

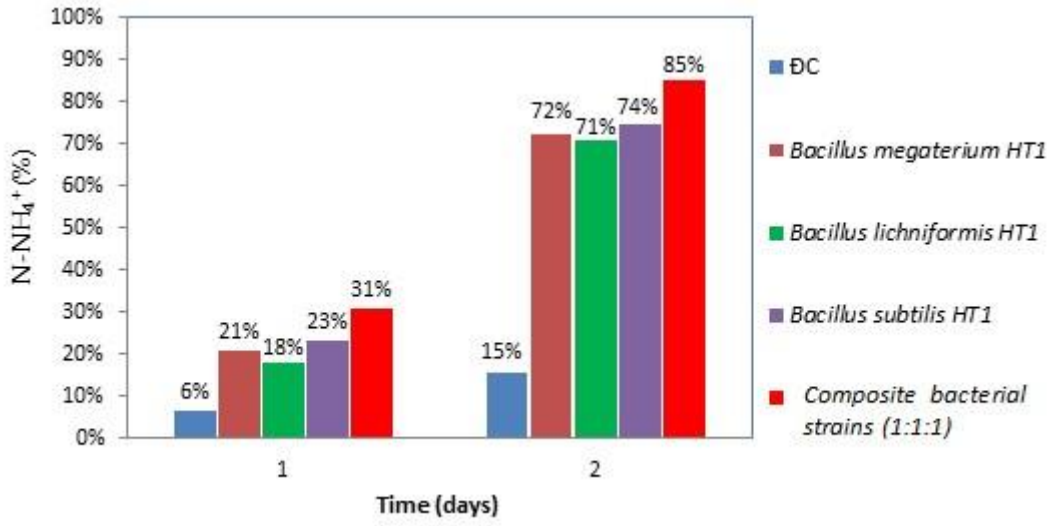

Fig. 7. Comparison of $N\text{-}NH_4^+$ metabolism capacity in swine wastewater after biogas between single strains and combination of isolated strains at initial microbial density of 10^5 CFU·mL^{-1}

4 Discussion

The bacterial strains isolated in our study belong to the genus Bacillus. This genus is widely distributed in nature, especially in soil. They are commonly used in water treatment because they can survive for a long time in the form of spores, easily proliferate, and have a high antibacterial activity [40]. According to previous studies [41-46], the bacteria belonging to the genus Bacillus are often investigated and applied to aquaculture water treatment, in which *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, and *Bacillus pumilus* were evaluated for their water treatment capacity. Some strains were reported to have impressive nitrogen removal capacity [46, 47]. Studies on applying single and mixed strains of bacteria belonging to the Bacillus group in livestock wastewater treatment were also published. Liu et al. [48] used a mixture of *Pseudomonas geniculata* ATCC 19374 and *Bacillus cereus* EC3 to remove ammonia in livestock wastewater, in which the treatment efficiency within 72 hours was 70.06% higher than that of single bacteria treatment. Guo et al. [49] immobilised Bacillus subtilis in a chitosan-sodium alginate composite carrier to remove ammonia from swine wastewater after anaerobic treatment. The findings revealed that both adsorption and microbial activities contributed to the removal of ammonia with a 54.3 and 42.2% efficiency. Huynh Van Tien et al. [50] applied *Bacillus aryabhattai* KG12S, capable of synthesising bio-flocculants, to swine wastewater after biogas treatment, with a 77.8% ammonium treatment efficiency. These publications reinforced the scientific basis and practical application of *Bacillus megaterium* HT1, *Bacillus lichniformis* HT1, *and Bacillus subtilis* HT1 to swine wastewater. Note that the mixture of these three strains exhibited an 85% efficiency after 48 hours, which is an outstanding advantage of this system. The findings pose a great potential for applying these strains to wastewater treatment.

5 Conclusion

In this study, we successfully isolated and applied three *Bacillus megaterium* HT1, *Bacillus licheniformis* HT1 *and Bacillus subtilis* HT1 bacteria to oxidising ammonium in swine wastewater after biogas

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treatment with an 85% removal efficiency. These strains promise great application to improving ammonia oxidation in livestock wastewater.

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Conflict of interest

The authors have no conflicts of interest regarding the publication of this article.

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