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(RESEARCH ARTICLE)

Isolation and characterization of nitrogen removal bacteria from sewage and fecal sludge of dairy cow farms in Cu Chi, Ho Chi Minh City, Vietnam

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Abstract

The bacteria that involved in the removal of nitrogen in wastewater were initially studied. Isolation media were SW-MM (artificial Seawater-Minimal Medium) and 17208 PIA (Pseudomonas Isolation Agar). The pure isolates were streaked on the SW-MM agar plates that added ammonia, nitrate, or nitrite with ascending concentrations to select the isolates had good nitrogen removal potential. The result was that 49 isolates had been collected from 11 samples of sewage and fecal sludge of dairy cow farms, in which including 6 isolates had been isolated by using specialized medium for *Pseudomonas*. All 49 isolates (100%) were able to grow on the SW-MM added 100 mM ammonia/nitrate or more. The ability to withstand ammonia and nitrate of the best isolates reaches 800 mM. Meanwhile, the ability to withstand nitrite had only belonged to 39 isolates (79.6%) with nitrite concentrations of 5 - 15 mM. Nine isolates were investigated the *in vitro* ability to reduce ammonia in the wastewater. Quantitative results of ammonia by phenol-hypochlorite method showed that the ability to reduce NH_4^+ of 9 isolates ranged from 27.9 – 48.9 mg/L on 10 days after inoculation. The six best isolates that had the ammonia- removing efficiency reached 88.5% - 97.5% were identified by using the MALDI Biotyper System (Germany). The identification result showed that XM3, NA6 was *Bacillus megaterium*, M7C1 was *Bacillus pumilus*, NA8 was *Rhodococcus ruber*, MD6 was *Pseudomonas fulva*, and XM5 was *Pseudomonas mendocina*. It is recommended to test the ability of these isolates to reduce nitrate and nitrite in the wastewater.

Keywords: Nitrogen cycle; Ammonia oxidizing bacteria; Nitrite oxidizing bacteria; Nitrate assimilation bacteria; MALDI (Matrix-Assisted Laser Desorption/Ionization) Biotyper System; *Pseudomonas*

1. Introduction

Environmental pollution, especially water pollution due to agricultural activities, is a serious issue facing the world. There were many causes and sources of water pollution, in which soluble nitrogen and phosphorus are two factors contributing to water eutrophication, leading to harms on the environment, ecosystems and health [1]. For soluble nitrogen present in wastewater, there are three most concerned compounds: ammonia, nitrite, and nitrate. The ammonia comes from the decomposition of nitrogen-containing organic compounds. These inorganic nitrogen compounds may also originate from atmospheric deposition, and from agricultural production such as fertilizers, sewage systems and animal manure [2].

Fortunately, many bacteria that have soluble nitrogen transformation activities present in nature have been isolated and successfully used in wastewater treatment. The transformation of ammonia and nitrite can be done by ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) through aerobic pathways in a process called nitrification. Besides that, it is in the nitrogen cycle that a form of coupled nitrification–denitrification called anammox

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(anaerobic ammonia oxidation) also exists. Anammox is a type of ammonia oxidation under an anoxic condition that was first discovered in 1999. Anammox bacteria oxidize ammonia to nitrogen gas by using nitrite as an electron acceptor but not produce nitric oxide and nitrous oxide, which is considered intermediate products of the denitrification [3-5]. Besides the nitrifying-, denitrifying- and anammox- bacteria have just mentioned, bacteria that are able to assimilate ammonia, nitrite and nitrate have also been reported [6-8]. Nitrate assimilation bacteria (NAB) utilize nitrate as a nitrogen source and synthesize it into organic nitrogen. NAB is widely distributed and quite diverse, including cyanobacteria, proteobacteria such as *Rhodobacter*, *Klebsiella* and *Azotobacter*, and Gram-positive bacteria such as *Bacillus* [9-11]. Similarly, ammonium assimilation in bacteria is known the process of incorporating ammonia-nitrogen incorporation into glutamate and glutamine, which form the biosynthesis donors for all other nitrogen-containing components in cells. Ammonium assimilation bacteria includes streptomycetes, purple and green sulphur bacteria, rumen bacteria such as *Escherichia coli*, *Klebsiella aerogenes*, *Salmonella typhimurium*, and *Bacillus subtilis* [12-14]. Only the nitrite assimilation bacteria is rarely mentioned [8]. Nowadays, there are many research on the possibility of removing nitrogen in wastewater, focusing on each individual mechanism or coordinating many mechanisms [15-19]. In Vietnam, especially in the Mekong Delta, there are many studies on heterotrophic bacteria capable of nitrogen removing. The results showed that the ability to isolate, select and apply these bacteria in reducing ammonium, nitrite, or nitrate of wastewater [20-24]. The diversity of nitrogen-metabolizing bacteria has been discovered. In addition to classic bacteria such as *Nitrosomonas* and *Nitrobacter* [20, 21], Gram-negative bacteria such as *Pseudomonas*, *Klebsiella*, *Acinetobacter*, and Gram-positive bacteria such as *Bacillus*, *Rhodococcus*, *Arthrobacter* have also been reported to be able to remove soluble nitrogen [24-28].

The above is the basis for conducting this study because dairy cow farming is bringing high economic efficiency to farmers in Cu Chi district, but it is also causing the risk of groundwater pollution when animal manure and sewage has not been properly treated. Within the scope of this study, through specialized culture media for heterotrophic bacteria and aerobic culture conditions, the isolated bacteria were able to reduce the soluble forms of nitrogen assumed to be through absorption and assimilation directly or through metabolic reactions in aerobic conditions. We named ammonia-, nitrite-, and nitrate-removal bacteria once they were able to grow in culture media with these compounds as the only source of nitrogen and reduced their concentration. In the current study, the mechanism of these phenomena had not been studied and the experiment evaluating the nitrogen removal ability of bacteria was limited to the ammonia removal of selected bacteria.

2. Material and methods

2.1. Samples collection

Ho Chi Minh City belongs to a transitional region between the Southeastern and Mekong Delta regions of Vietnam. It is located from 10˚10'- 10˚38' North and 106˚22'- 106˚54' East. Ho Chi Minh City has an area of approximately 2,094 square kilometers, in which Cu Chi is a rural district located in the North of the city. Eleven samples of sewage and fecal sludge were collected in two dairy cow farms of Tan Phu Trung and Phuoc Vinh An wards, Cu Chi district (Figure 1). Samples were obtained with sterile tools, transferred into sterile plastic jars, sealed, labeled, refrigerated and transported to the laboratory [24].

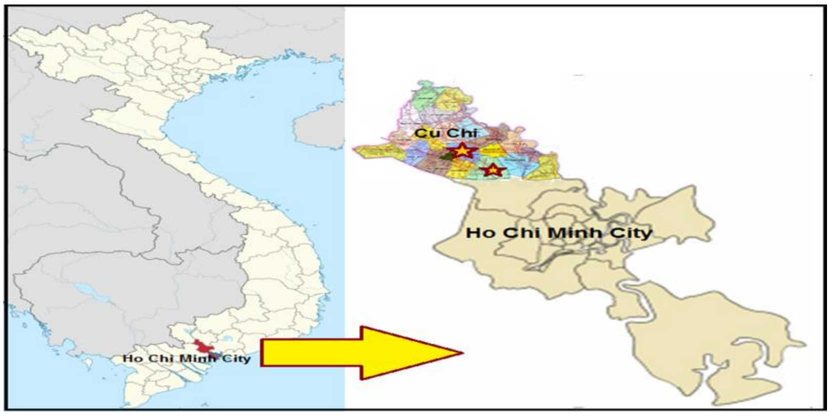

Figure 1 The map of Ho Chi Minh City with Cu Chi district and places of sampling. Star shapes used to illustrate Tan Phu Trung and Phuoc Vinh An wards, Cu Chi district.

2.2. Isolation and collection of bacteria have capable of removing nitrogen

One gram of sludge was suspended in 99 mL sterile water, shaken at 200 rpm for 10 minutes and deposited for 3 hours. After 3 hours, the supernatant was continuously diluted in sterile water up to 10^{-3} by ten-fold serial dilution method. Then 100 µL of the 10^{-5} - 10^{-3} dilutions were collected and spread on Minimal medium [29] agar plates that had prepared with artificial seawater (SW-MM) [30] and 17208 Pseudomonas Isolation Agar (Sigma-Aldrich) plates. This process of dilution and spread method is also applied similarly for the sewage samples. These plates were incubated at 30˚C for 2 – 3 days to observe and select bacterial colonies. Purified isolates was collected by streaking on SW-MM agar plates repeatedly.

Pure isolates were continuously streaked on the SW-MM medium supplemented with 5 mM NH_4^+ (NH_4Cl), or 5 mM NO_2^- (KNO_2), or 5 mM NO_3^- ($NaNO_3$). After that, the concentration of these nitrogen compounds had been increased after each streaking time to select the bacteria that can tolerate high concentration soluble nitrogen. The concentration of NH_4^+ or NO_3^- was increased in a ladder that each scale was 100 mM, and the concentration of NO_2^- was increased in a ladder that each scale was 5 mM. Pure isolates were preserved temporarily in 40% glycerol solution for use in subsequent studies.

2.3. Morphological characterization of bacterial isolates

Colony morphology including form, elevation, margin, surface and size were recorded after 72 hours of growth on SW-MM or PIA media. Cellular size and shape of bacteria were observed by light microscopy. The Gram stain was performed [31]. "KOH String Test" [32] was also used as a complementary method to distinguish between Gram-groups.

2.4. Preliminary survey of ammonia- removing ability of selected isolates

These experiments were limited to the bacterial isolates that are capable of growing on the SW-MM solid medium containing NH4+ and NO3- with concentrations of 500 mM or more.

2.4.1. Bacterial suspension preparation

One colony of each bacterial isolate was inoculated into 5 mL of BM/NO_3^- broth [33], incubated at 30˚C and 120 rpm for 48 hours. Then 1 mL of bacterial suspension was transferred to 50 mL of BM/ NO_3^- broth and continuously incubated at 30˚C and 120 rpm for 3 days to increase cell numbers to 10^9 CFU/mL or more.

The suspension after cultured within 3 days of each isolate had been determined the number of cell by measuring turbidity and subsequently adjusted to the density corresponding to the 4.0 McFarland (1.2x10^9 CFU/mL) [34].

2.4.2. Wastewater sample preparation

The sewage sample was collected from the tank containing wastewater originating from cattle bathing and stables cleaning, including feces and urine of the cattle (Farm No.1 in Phuoc Vinh An ward). The sewage sample was also determined basic parameters including pH, BOD_5 (5 day Biological Oxygen Demand), COD (Chemical Oxygen Demand), concentrations of ammonia and nitrate. After sedimentation of the sewage, the supernatant fluid was undergone sterilizing to eliminate exogenous agents and then diluted by adding distilled water with the ratio 1:1 (v/v).

2.4.3. Survey of ammonia- removing ability of bacteria

An amount of 20 mL of bacterial suspension of each isolate that prepared as described above was added to the flask containing 180 mL of sterilized sewage. The experimental flasks were put on the shaking incubators at 120 rpm and 30˚C to monitor the result 2 days at a time until the ammonia level drop to the allowable limit [35]. The control treatment was conducted in a similar manner but the bacterial suspension was replaced with 200 mL of distilled water. The determination of ammonia in the sewage was conducted by using phenol-hypochlorite method [36]. Every two days, 10 mL of suspension was collected, centrifuged at 12,000 rpm in 5 minutes to obtain the supernatant for the next colorimetric analysis. The ratio of sample and reagent was 5:1 (v/v). The absorbance of the sample was measured by spectrophotometer with 640 nm wavelength after 30 minutes of reaction stability.

2.5. Identifying selected isolates by their molecular fingerprinting

This experiment was limited to the selected bacterial isolates with the best results after going through all the survey. These selected isolates were cultured on LB for 24 hours, and identified by using MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) method. The characteristic spectrum pattern of this proteomic fingerprint is used to reliably and accurately identify a particular microorganism by matching thousands of reference spectra from microorganism isolates. According to the specifications of the manufacturer, a highly probable species identification has a score value lying between 3.000 and 2.300; a secure genus and probable species identification has a score value lying between 2.299 and 2.000; a probable gnus identification has a score value lying between 1.999 and 1.700. A low score of <1.700 was considered unreliable for identification [36].

2.6. Experimental design and Data analyses

Quantitative experiments were random assignment (Completely Randomized Design) with three replicates. Negative controls were conducted similarly to treatments, did not use bacterial suspension but put on sterilized corresponding medium instead.

Statistics methods were ANOVA (Analysis of Variance) one factor and Duncan test at α=0.05 by using IBM SPSS Statistics 20.0

3. Results and discussion

3.1. Bacterial isolation

Forty bacterial isolates were collected from spreading on the SW-MM medium and 9 isolates were collected from spreading on the PIA medium specialized for *Pseudomonas* bacteria. After transferred to the SW-MM medium supplemented with 5 mM NH_4^+ or NO_3^- as the only nitrogen source, all of these 49 isolates could grow even with increased concentration of these substances. Meanwhile, there were up to 10 isolates that were unable to grow on SW-MM medium added 5 mM ammonia. The origin and the ability of growing on the specific media of 49 isolates were described in Table 1.

Table 1 Origin and ability to grow on the media of the isolates

Origin and number of samples		**Number (name or ratio) of isolates grew on types of media**				
		PIA	**(SW-MM) +ammonia**	**(SW-MM) +nitrate**	**(SW-MM) +nitrite**	**Total**
Farm No.1	Sewage sample No.1	1 (NA8)	5 (100%)	5 (100%)	5 (100%)	5
	Sewage sample No.2	1 (NXM5)	4 (100%)	4 (100%)	0 (0.0%)	4
	Sewage sample No.3	0	4 (100%)	4 (100%)	4 (100%)	4
	Sludge sample No.1	1 (MD6)	4 (100%)	4 (100%)	3 (75.0%)	4
	Sludge sample No.2	2 (XM5, XM6)	4 (100%)	4 (100%)	3 (75.0%)	4
	Fecal sludge sample No.1	1 (M3P1)	3 (100%)	3 (100%)	2 (33.3%)	3
Farm No.2	Sewage sample No.3	1 (M7C2)	8 (100%)	8 (100%)	6 (75.0%)	8
	Sewage sample No.4	0	5 (100%)	5 (100%)	5 (100%)	5
	Sewage sample No.5	0	5 (100%)	5 (100%)	5 (100%)	5
	Sludge sample No.3	1 (M2P1)	3 (100%)	3 (100%)	3 (100%)	4
	Fecal sludge sample No.2	1 (M1P2)	4 (100%)	4 (100%)	4 (100%)	3
Total:	12	9 (18.4%)	49 (100%)	49 (100%)	39 (79.6%)	49

This study showed that the rate of bacteria growing on the SW-MM supplemented with ammonia was equal to the rate of bacteria growing on the SW-MM supplemented with nitrate (100%). Meanwhile, the number of bacteria growing on the SW-MM supplemented with nitrite was only 79.6% compared to the number of bacteria growing on the SW-MM supplemented with nitrate. Research on nitrogen-removing bacteria in the Mekong Delta, Vietnam showed that the number of bacteria that could grow on the medium containing nitrite as the only nitrogen source accounted for 96.7%

to 98.6% compared to the number of bacteria growing on the medium containing nitrate as the nitrogen source [23, 24].

3.2. Morphological characterization

On the isolation media**,** the color of the colonies was quite varied (Figure 1). Opaque white and translucent white made up the majority, accounted for 59.2% and 14.3% respectively. The remaining were yellow (18.4%), and other colors such as orange, pink, beige (8.2%). The other morphological characteristics of colonies included circular and entire (69.4%), irregular and lobate (30.6%); convex or pulvinate (57.1%), flat or raised (42.9%); smooth (95.9%) or wrinkled surface (4.1%). The majority of colonies have a diameter of ≤ 1 mm (69.39%). Colonies of 2 - 7 mm in diameter accounted for 30.61%.

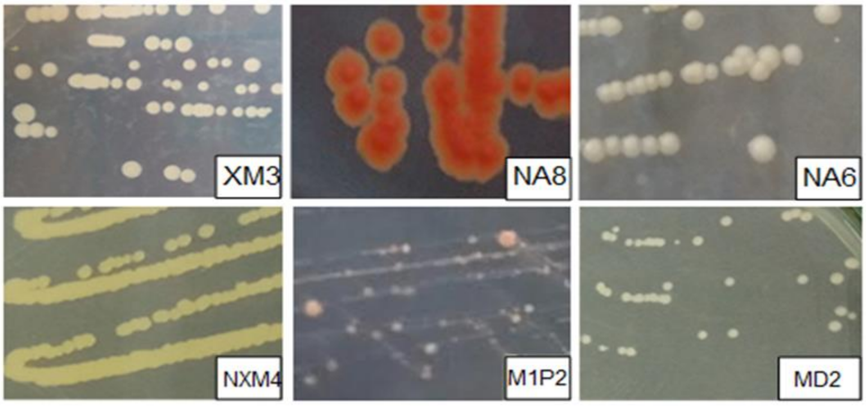

Figure 2 Colony morphology of nitrogen-removing bacteria on SW-MM medium

Through microscopic observation, there were 77.6% of the total bacterial isolates that exhibited their motility and 22.4% of them were non-motile. Rod-shaped (bacillus and coccobacillus) bacteria accounted for 61.2%, while the sphere-shaped (coccus) accounted for 38.8%. The results of Gram-staining and “KOH string test” showed that up to 85.7% of the total isolates were Gram-positive and 14.3% of them were Gram-negative (Figure 2).

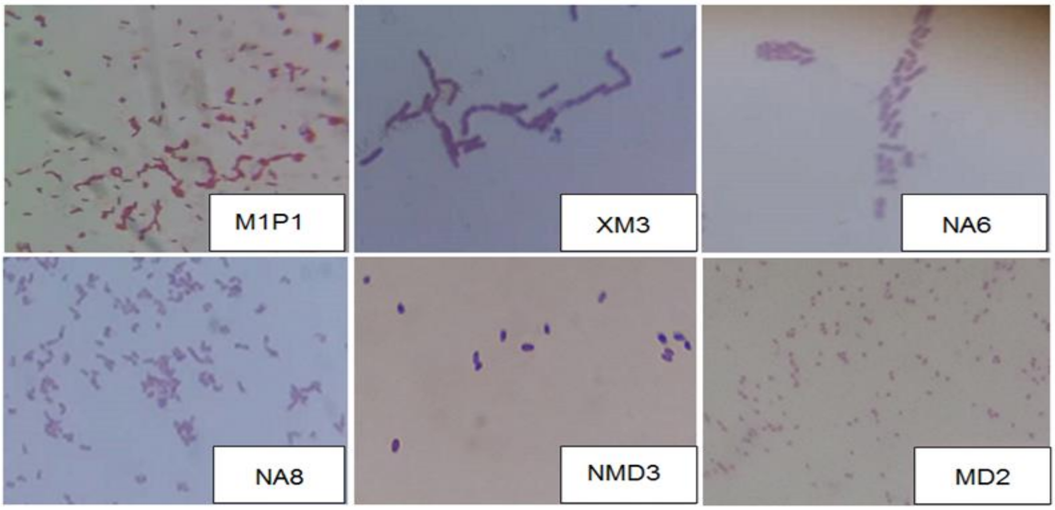

Figure 3 Cell morphology and Gram-staining results of some isolates

The records of morphological characteristics of each isolate were essential to support the subsequent identification. The results of research [23, 24] also showed that rod shape, mobile and Gram-positive were the major characteristics of heterotrophic bacteria which had the ability of soluble nitrogen-removing**.**

3.3. Ammonia-removing ability of selected isolates

Due to castle manure commonly contain large amounts of organic matter and ammonia with high concentration, the anaerobic resolution models for example using AFBR (Anaerobic Fixed Bed Reactors) had been recommended and had partly success [24]. However, the ability of ammonia- and nitrate- removing of bacteria that had been isolated from some kinds of wastewater in the surveys of [22, 37] showed the potential of aerobic resolution by heterotrophic bacteria.

3.3.1. The ability to grow on solid media containing soluble nitrogen compounds

Experimental evaluation of the ability to grow on solid media containing soluble nitrogen compounds showed that all 49 bacterial isolates were able to grow on the minimal medium added of 100 - 800 mM ammonia or nitrate. The diagram in Figure 4 below shows the number of isolates capable of growing on the SW-MM medium with ammonia or nitrate supplemented with the highest concentration. Thereby showing that most bacteria were capable of developing at maximum concentration of 500 mM of ammonia and nitrate: 13 isolates (26.5%) and 10 isolates (20.4%), respectively. There was only one isolate capable of growing on medium containing 800 mM ammonia or nitrate (isolate M7C1).

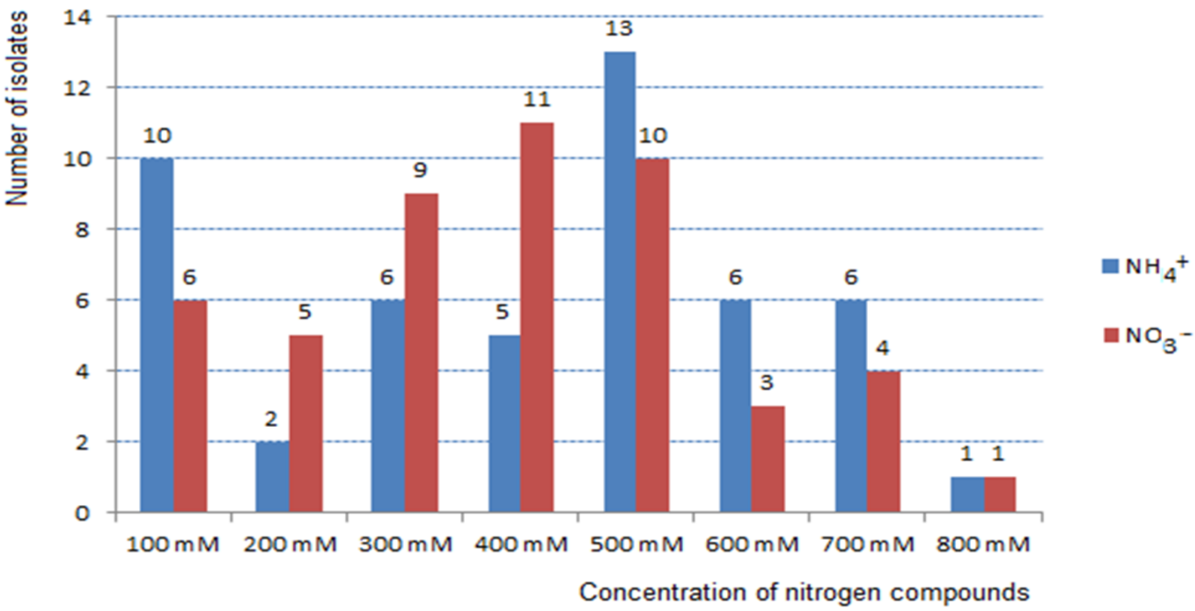

Figure 4 The number of isolates had grown on the SW-MM medium with the maximum concentration of ammonia/nitrate added

Meanwhile, there were only 39 isolates that had capability of growing on the SW-MM medium with 5 - 15 mM of ammonia added. Most of isolates had grown on the medium added 5 mM of nitrite (28 isolates, 71.8%). Ten isolates had grown on the medium added of 10 mM of nitrite (25.6%) and the only one (isolate M1P1) has grown on the medium added of 15 mM of nitrite (2.6%).

3.3.2. The ability of ammonia-removing in the sewage

Nine isolates were selected for the experiment to assess the ability to remove ammonia in the sewage with a volume of 200 mL. The origin and ability to grow on the solid nitrogen-containing medium of selected isolates was described in Table 2 below.

Table 2 *In vitro* Plant Growth Promoting functional characterization of nine selected isolates

Isolates	Origin	Maximum concentration of nitrogen compounds that could withstands		
		Ammonia (mM)	Nitrate (mM)	Nitrite (mM)
MD2	Sludge sample No.1	500	500	5
MD6	Sludge sample No.1	500	500	0
XM3	Sludge sample No.2	700	700	10
XM5	Sludge sample No.2	500	400	5
NA1	Sewage sample No.1	500	500	5
NA6	Sewage sample No.1	700	700	10
NA8	Sewage sample No.1	700	600	10
M7C1	Sewage sample No.3	800	800	10
M7C7	Sewage sample No.3	700	600	10

The sewage sample collected from Farm No.1 in Phuoc Vinh An ward has been determined some quality parameters. The pH was 8.2; the BOD_5 was 490 mg/L; the COD was 4805 mg/L; and the concentrations of ammonia and nitrate were 111.3 mg/L and 3.0 mg/L, respectively. After being diluted 2 times to lower the ammonia concentration to about 50 mg /L, the sewage sample was supplemented with bacteria at concentration of 1.2x10^8 CFU/mL. The results showed that except for the control treatment, all 9 treatments using bacteria showed a gradual decrease in ammonia concentration in wastewater from 2 days after inoculation. The linear regression of 6 isolates including M7C1, MD6, XM5, XM3, NA6, NA8 had the R^2 equal from 0.8604 to 0.9568. The linear regression equation of isolate NA8 was shown in Figure 5 below.

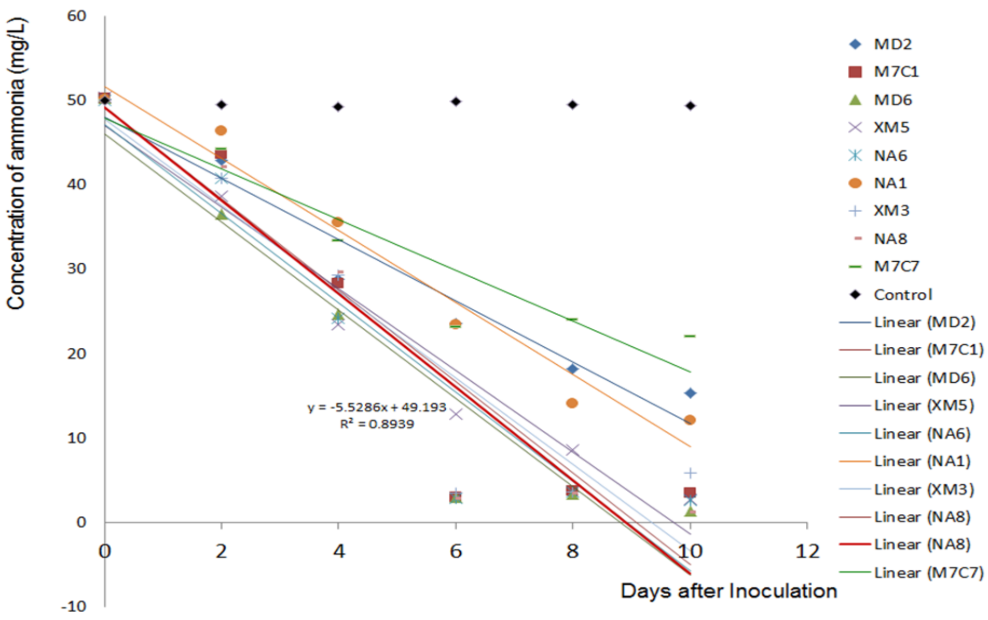

Figure 5 The reduction of ammonia concentration in wastewater under the influence of bacteria

After 10 days of treatment, 6 isolates including M7C1, MD6, XM5, XM3, NA6, NA8 were able to reduce ammonia to the allowable threshold. The amount of ammonia in the sewage decreased from 50 mg/L to 1.5 - 5.8 mg/L. The processing efficiency reached 88.5% to 97.5% (Table 3). Under non-aeration conditions and initial concentration of ammonia in wastewater was 50 mg/L, ammonia-removing efficiency in wastewater of selected isolates reached 74% to 90% within 3 - 4 days. Lowering the concentration of ammonia or aeration condition could increase the treatment efficiency [22, 38].

Table 3 The ratio of ammonia-removing of 6 selected isolates through the measurement periods

Treatments	The ratio of ammonia-removing of				
	2 DAI	**4 DAI**	**6 DAI**	**8 DAI**	**10 DAI**
MD6	27.3%	50.8%	94.1%	93.4%	97.5%
XM3	18.6%	41.4%	93.0%	92.8%	88.5%
XM5	23.3%	53.5%	74.6%	83.0%	94.7%
NA6	18.6%	51.8%	94.5%	92.8%	94.9%
NA8	16.0%	40.9%	94.4%	93.1%	97.5%
M7C1	13.1%	43.6%	94.1%	92.8%	93.0%
Control	1.0%	1.6%	0.4%	1.0%	1.4%

3.4. Identification of selected isolates

Based on the above results, there were 6 selected isolates had been identified by using MALDI-TOF MS. Profiles of morphology of these isolates had been also checked (Table 4).

Table 4 Morphological characteristics and classification results of 6 selected isolates

Isolates	Colony Morphology				Cell Characteristics			MALDI classification
	Color	Shape	Margin	Elevation	Shape	Motility	Gram	Best match
MD6	Opaque white	Circular	Entire	Convex	Cocco-bacillus	Motile	Negative	*Pseudomonas fulva*
XM3	Opaque white	Circular	Entire	Convex	Rod	Motile	Positive	*Bacillus megaterium*
XM5	Opaque white	Irregular	Lobate	Raised	Cocco-bacillus	Motile	Negative	*Pseudomonas mendocina*
NA6	Opaque white	Circular	Entire	Flat	Rod	Motile	Positive	*Bacillus megaterium*
NA8	Orange	Irregular	Lobate	Raised	Rod	Non	Positive	*Rhodococcus ruber*
M7C1	Opaque white	Irregular	Lobate	Flat	Rod	Motile	Positive	*Bacillus pumilus*

Table 4 showed that 3 isolates XM3, NA6, M7C1 belong to the genus *Bacillus*, 2 isolates MD6, XM5 belong to *Pseudomonas*, and the isolates NA8 belongs to *Rhodococcus*. These genera have also been reported to be able to remove soluble nitrogen [24-28]. *Bacillus pumilus*, *Bacillus megaterium*, and *Pseudomonas mendocina* were found in effluents of GAC (Granular Activated Carbon) beds. They had been identified as nonpathogenic bacteria in nature and had the ability of removal of organics and inorganics including ammonia [39]. *Pseudomonas* spp. and *Bacillus* sp. were reported as nitrgen removal bacteria by heterotrophic nitrification and aerobic denitrification [40]-[42]. *Rhodococcus* sp. CPZ24 was isolated from swine breeding wastewater, had capable of oxidizing ammonia 50 mg/L within 20 hours. The study also showed that 13% of absorbed ammonia was transformed to nitrite and nitrate, 24% of this was converted into intracellular nitrogen, and 48% of this was released in forms of gaseous denitrification products [18].

4. Conclusion

The six isolates had been selected from 49 bacterial isolates that collected from sewage and fecal sludge samples of dairy cow farms. The effectiveness of the removal of ammonia from wastewater of the 6 isolates ranged from 88.5% to 97.5%. They had also been identified as nonpathogenic bacteria present in nature including *Bacillus pumilus*, *B. megaterium, Pseudomonas mendocina, P. fulva,* and Rhodococcus ruber. The recommendation was studying the ability of nitrite and nitrate removal of the selected isolates.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest.

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