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ISOLATION AND IDENTIFICATION OF BACTERIA FROM SHRIMP POND AS BIOREMEDIATION AGENT CANDIDATE TO REDUCE TOXIC AMMONIA

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ABSTRACT

The accumulation of ammonia in shrimp pond is one major problem that leads to production failure. Ammonia can disturb the metabolic balance of the shrimps and making them more susceptible to disease. An approach to overcome a poor water quality in shrimp culture caused by ammonia is introduced bacteria as a bioremediation agent. The utilization and the development of the local bioremediation agent are expected to be a solution to improve water quality. This study aimed to isolate and identify bacteria from traditional shrimp pond as candidate to reduce ammonia. Bacteria were isolated in liquid enrichment medium. Bacteria identification was done through biochemical tests also molecular analysis of the 16S rRNA gene sequence using primer 27F and 1492R and phylogenetic analysis using MEGA 6.0 program. The results showed that as many as three bacteria were isolated from traditional shrimp pond. These bacteria were NAS_1, NAS_2, and NAS_3. Based on the analysis of 16S rRNA gene sequence, NAS_1 was identified as *Breoghania* sp., NAS_2 identified as *Pseudoalteromonas ruthenica*, and NAS_3 identified as *Halomonas beimenensis*. Ammonia reduction test showed that *Halomonas beimenensis* and *Pseudoalteromonas ruthenica* were able to reduce ammonia with a percentage of 8,4% and 20,3% for five days incubation. Therefore, these bacteria could be potential candidates as a bioremediation agent to improve water quality. Meanwhile, *Breoghania* sp. wasn't show positive result in ammonia reduction test.

Keywords: ammonia; bacteria; bioremediation; shrimp; 16S rRNA

INTRODUCTION

The growing demand for shrimp encourages farmers to improve their farming technology on production targets. Consequently, this will increase organic material from residual shrimp feed and feces. Improper and excessive use of additional feed drive to water degradation in shrimp pond. Suantika *et al*. (2015) stated that excessive feed input that is not balanced with self-purification will generate the accumulation of organic matter in the water together with shrimp excreted metabolism.

The accumulation of organic material from residual metabolism, residual feed, and other waste in the pond will affect the water quality condition as a cultivation medium for shrimp. The decomposition of organic material carried out by bacteria will produce an inorganic ammonia compound. The concentration of ammonia compound will increase in line with shrimp growth due to the amount of additional and uneaten feed given. The high organic matter in the pond system will also lead to an increase in oxygen consumption for bio decomposition process and thus cause a decrease in dissolved oxygen levels that affect shrimp, directly.

High organic compounds either derived from residual feed or shrimp feces will gradually affect ammonia in the shrimp pond system. Ammonia is one of important environmental stress factor in aquaculture. The high level of ammonia may deteriorate water quality (Waikhom and Rosalind, 2018). Long-term ammonia stress also could affect the normal growth in shrimps (Liu, 2020) and can create stress to aquatic species cultured which can increase susceptibility to diseases, eventually mortality (Kathyayani *et al*., 2019). Chang *et al*. (2015) also reported that ammonia interrupted the coagulation and down-regulated transglutaminase gene expression in *Litopenaeus vannamei* thus caused ecotoxicity immune deficiencies and increase susceptibility to infection by pathogens.

Some action needs to be done to overcome this problem. An alternative solution is utilizing bacteria activity through the bioremediation system. According to the previous study by Ardiansyah (2019), shrimp pond water contains bacteria that have the potential to oxidize ammonia compounds. Hence, the utilization and development of the local bioremediation agent are expected to be a solution to improve water quality in the aquaculture system. Karthik *et al*. (2016) stated that the use of probiotic and nitrification consortium in shrimp farms in the laboratory scale experiment had to improve shrimp survival and reduced ammonia toxicity. In connection with that statement, this research urgently needed and this has to be done by exploring finding bacteria for bioremediation agent. This study aimed to isolate and identify bacteria from traditional shrimp pond as candidates for bioremediation agent to reduce ammonia compound.

RESEARCH METHODS

Sampling for Bacteria Isolation

Sampling was conducted in October 2019 at the traditional vannamei shrimp pond in Sekuro, Mlonggo Jepara. The traditional shrimp pond was managed conventionally without any probiotic addition. Therefore it's expected that indigenous bacteria can reduce ammonia. The water sample was taken near the bottom of the pond using a sterile glass bottle. Then the water sample was stored in a cool box with ice packs during transportation.

Bacteria Isolation

The medium used for bacteria isolation consisting of 13.5 g $K_2HPO_4.3H_2O$; 0.7 g KH_2PO_4; 0.1 g $MgCl_2.6H_2O$; 0.18 g $CaCl_2.2H_2O$; 0.1 g NH_4Cl; 0.2 g EDTA; 0.5 g $NaHCO_3$; 0.014 g $FeCl_3.6H_2O$; and 15 g agar was added for solid medium (Rodina, 1972). 5 mL water sample put into Erlenmeyer flask containing 25 mL liquid medium and then incubated for 3 days using an incubator shaker at 130 rpm. Bacteria suspension that grew in liquid media was inoculated by streaking in solid medium and incubated for 3 - 4 days. The different bacteria colonies then purified on solid medium.

Colony Identification

The characteristics of bacteria colonies were classified based on the colony color, size, and form. According to Lu *et al*. (2018), some parameters that can be used to identify bacteria are colony characteristics and morphology. Furthermore, Mahon and Lehman (2018) mentioned that a bacteria colony has different morphological properties such as form or margin, elevation, density, consistency, pigment, and odor.

Gram Staining and Biochemical Identification

Gram staining was used as a tool for the differentiation of Gram-negative and Gram-positive bacteria. This method was purposed to identify the characterization of cell wall structure (Bowater, 2017). The biochemical tests include catalase, oxidase, motility, urease, and citrate were used to identify and determine the physiological properties of bacteria.

Bacteria Identification Using Sequences Gene Coding for 16S rRNA

Molecular characterization was carried out for identification process using the gene coding for 16S rRNA. According to Akihary dan Beivy (2020), the 16S rRNA gene contains a various hypervariable region that demonstrates considerable sequence diversity among different bacteria and thus make bacteria identification easier.

Bacteria DNA Extraction

Bacteria isolates were recultured in Zobell solid medium for 2 × 24 hours. DNA extraction was performed using chelex method (Walsh *et al*., 2018). The steps of DNA extraction were: isolated bacteria were put into microtubes containing 100 µL ddH_2O and 500 µL saponin then left for 1 × 24 hours. After left for overnight, the samples were centrifuged at 9000 rpm for 10 minutes. Furthermore, the supernatant was discarded and the pellet was added with 1 mL Phosphate-Buffered Saline, then was mixed using a vortex. The samples were centrifuged again at 9000 rpm for 10 minutes. Then the pellet was taken and add 100 µL ddH_2O and 20% chelex solution. The samples were put on the heating block at 95 ºC for 5 minutes and gently mixed with a vortex then preheated on the heating block at 95 ºC. After that the samples were centrifuged at 9000 rpm for 15 minutes, the supernatant transferred to new microtubes.

Amplification of 16S rRNA Gene

The extracted DNA samples were amplified by Polymerase Chain Reaction (PCR). Amplification was performed with 25 µL mixed solution containing 12.5 µL promega master mix, 1.25 µL primer *reverse* (492F), 1.25 µL primer *forward* (27F), 1 µL DNA *template*, and 9 µL ddH_2O. The amplification process was conditioned in the following stages: pre denaturation at 95 ^{0}C for 3 minutes, followed by 29 cycles at the same temperature for 1 minute, annealing at 52 ^{0}C for 1 minute, extension at 72 ^{0}C for 1 minute, and final extension at 72 ^{0}C for 7 minutes.

PCR products were confirmed by gel electrophoresis and stained with ethidium bromide. Then the reaction product was observed under UV *transilluminator* and the DNA band that forms later compared to markers. DNA amplification products were sequenced to obtain DNA sequences of bacteria isolated from traditional shrimp farming. The purification and sequencing of 16S rRNA gene was conducted by PT Genetika Science Indonesia.

Phylogenetic Analysis

The base sequence of the sequencing results was then compared to sequences in the data bank of the National Center for Biotechnology Information (NCBI) using the program of Basic Local Alignment Search Tool for Nucleotides at http://www.ncbi.nlm.nih.gov/BLAST. Bacterial kinship is presented in the phylogenetic tree image created with MEGA 6.0.

Ammonia Reduction Test

Fresh starter cultures were prepared by inoculated the isolates in liquid medium and incubated on a rotary shaker at 115 rpm for 3 × 24 hours. 5 mL of starter culture put into Erlenmeyer flask containing 25 mL liquid medium and 130 mg/L ammonia then it placed under constant stirring on a rotary shaker for 4 × 24 hours (Widarnani, 2010). Ammonia in the medium was measured with the Nessler method during the treatment. A standard ammonia curve with concentration 0.0, 1.0, 2.0, 3.0, 4.0, 5.0 ppm was used to quantify ammonia in the medium.

Ammonia Reduction Calculation

The percentage of ammonia reduction was calculated using the following formula (Widarnani, 2010):

$$[AO] = \frac{[AK-AP]}{[AK]} \times 100\% \quad \text{..... (1)}$$

Note: AO= the percentage of oxidized ammonia (%); AK= ammonia concentration in control medium (mg/L); AP= ammonia concentration in treatment medium (mg/L)

RESULT AND DISCUSSION

Results

Isolation and Characteristics of Bacteria

The results of morphological and physiological observation can be seen in Table 1 and Table 2.

Table 1. Bacteria Morphology Isolated from Traditional Shrimp Pond

Isolate Code	Colony Form	Cell Shape	Gram
NAS_1	Yellow, medium colonies	Cocci	Gram-negative
NAS_2	Pale yellow, small colonies	Bacilli	Gram-negative
NAS_3	Dark yellow, small colonies	Bacilli	Gram-negative

Table 2. Bacteria Physiology Isolated from Traditional Shrimp Pond

Substrate	NAS_1	NAS_2	NAS_3
Catalase	+	+	+
Oxidase	+	+	+
Motility	+	+	+
Urease	-	-	-
Citrate	-	-	-

Note: + : positive, - : negative

As showed in Table 1, there were three bacteria strains found in the traditional shrimp pond. These bacteria have different colony form, color, and cell shape, whereas gram is negative for all strains.

The biochemical tests (Table 2) were same for all strains. The results showed that NAS_1, NAS_2, and NAS_3 were aerobic, have cytochrome oxidase, motile, weren't produce urease enzyme, and weren't utilize citrate as a carbon source.

Molecular Identification of Bacteria

The PCR product visualization result was showed in Figure 1. While the BLAST result can be seen in Table 3 and phylogenetic analysis was shown in Figure 2.

Gel electrophoresis of the PCR products result (Figure 1) showed a clear amplified band. The size band of strains NAS_1, NAS_2, and NAS_3 were about 1500 bp and have single and quite thick characteristic.

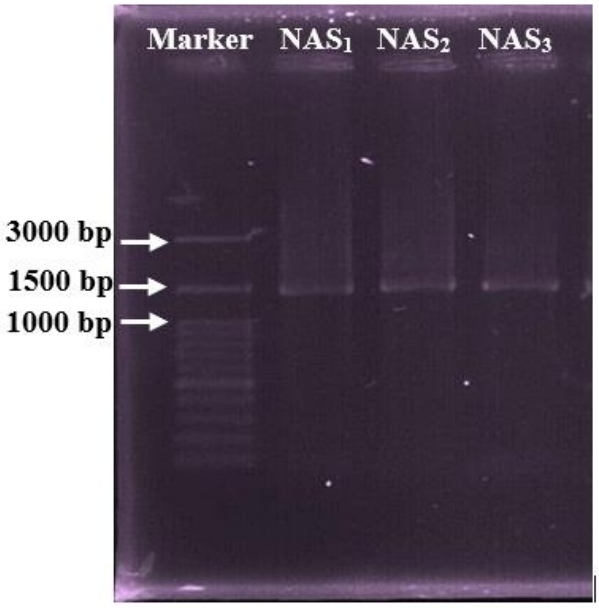

Figure 1. DNA Amplification with 27F and 1492R Primers

Table 3. BLAST-N Result of Bacteria Strains

Isolate Code	Bacteria Species (BLAST)	Accession Number	Ident (%)	Query Cover (%)
NAS_1	*Breoghania* sp.	MG709463.1	99.17%	100%
NAS_2	*Pseudoalteromonas ruthenica*	MH746023.1	99.27%	100%
NAS_3	*Halomonas beimenensis*	MT180859.1	100%	100%

The BLAST-N result of the PCR product sequence indicated that NAS_1 was closely related to *Breoghania* sp (MG709463.1) with 99.17% homology, strain NAS_2 shared 99.27% similarity with *Pseudoalteromonas ruthenica* (MH746023.1), and strain NAS_3 had 100% similarity to *Halomonas beimenensis* (MT180859.1).

The phylogenetic tree (Figure 2) was constructed by neighbor-joining method showed the phylogenetic relationship among 16S rRNA sequences of bacteria isolated from traditional shrimp pond. Bootstrap values were shown at branching point. *Thermococcus onnurineus* and *Thermococcus litoralis* was used as an out-group

Bacteria Ability to Reduce Ammonia

The ability of bacteria to reduce ammonia was quantified based on ammonia reduction in the medium. The results can be seen in Table 4.

Bacteria species that were found in the present study showed different ability to reduce ammonia. *Pseudoalteromonas ruthenica* has high ammonia-oxidizing percentage between two other bacteria, *Halomonas beimenensis* can reduce ammonia with a percentage of 8.4%, whereas *Breoghania* sp. was not able to reduce ammonia.

Table 4. Ammonia Oxidizing Percentage

Bacteria Species	Ammonia Oxidizing Percentage (%)
Breoghania sp.	-
Pseudoalteromonas ruthenica	20.3 %
Halomonas beimenensis	8.4 %

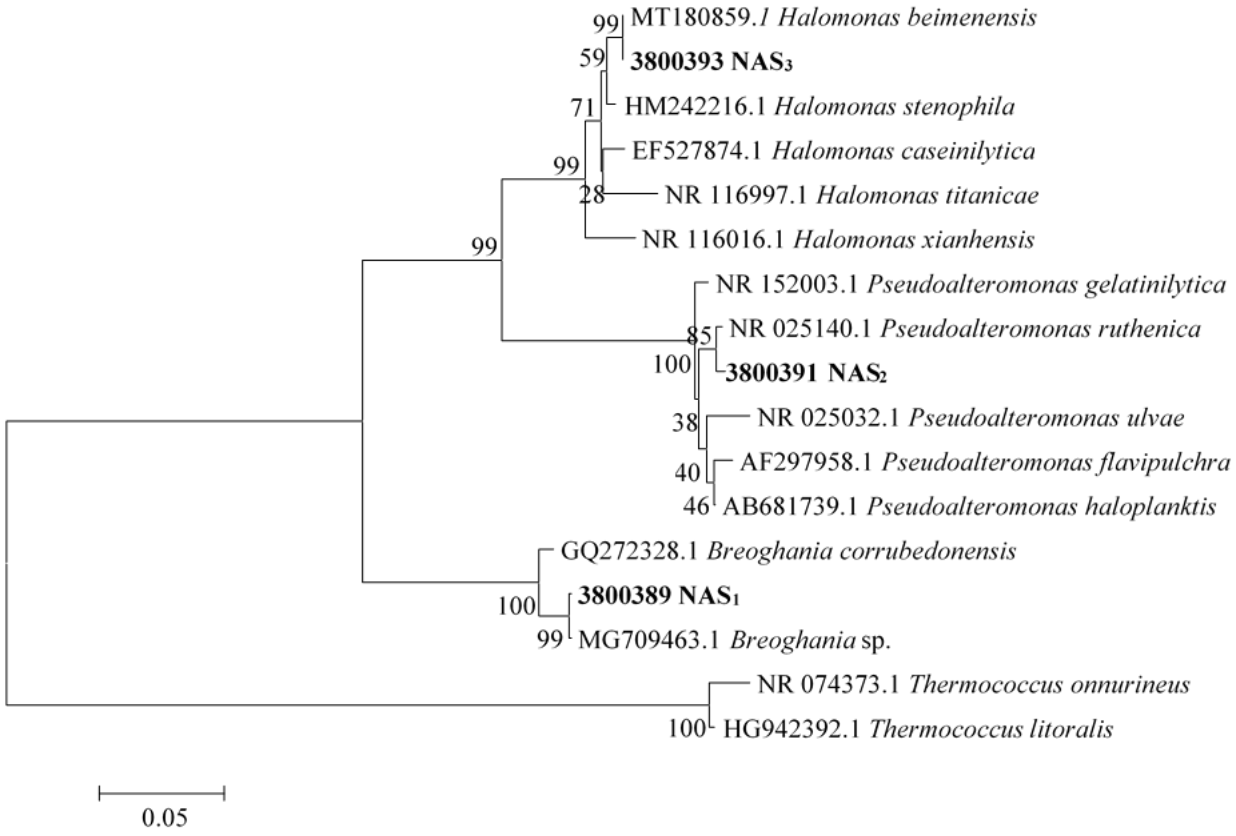

Figure 2. Phylogenetic Tree Isolates Using Neighbor-Joining

Discussion

Isolation and Characteristics of Bacteria

Three bacteria strains were isolated from traditional shrimp pond in Sekuro, Mlonggo Jepara including NAS_1, NAS_2, and NAS_3. Similar results also reported by Widarnani (2010), using the same culture medium bacteria obtained in the traditional pond were as many as two to three strains. The least bacteria that isolated from vannamei shrimp pond are very limited because these bacteria only use ammonia as an energy source, thus making them grow slowly. Fujitani *et al*. (2015) revealed that isolation of nitrifying bacteria was difficult due to the slow growth of these bacteria.

The morphological characteristic features of the isolates were performed in Table 1. Those three bacteria strains had almost similar characteristics. All strains were yellow colonies and Gram-negative. Meanwhile for size colonies and cell shapes were different. Strain NAS_1 showed medium colonies and cocci cell, whereas NAS_2 and NAS_3 showed small colonies and bacilli cell. Morphological characteristics of bacteria in the present study were relatively similar to Ardiansyah *et al*. (2019) the bacteria isolates are mainly Gram-negative cocci bacilli isolates and can remove ammonia. Silhavy *et al*. (2010) mentioned that Gram-negative bacteria have thin peptidoglycan cell wall, which itself is surrounded by a membrane containing lipopolysaccharide.

The results of biochemical tests for all strains were similar as seen in Table 2. Strains NAS_1, NAS_2, and NAS_3 were positive for catalase, oxidase, motility, and negative for urease and citrate. Catalase positive for all strains showed that these bacteria produce catalase enzyme to catalyze the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen (O_2) (Nandi *et al*., 2019). Kaushal *et al*. (2018) mentioned that hydrogen peroxide produced during the aerobic conditions and this enzyme has been used as an important enzyme in many biotechnological areas including bioremediation.

Positive oxidase for all strains showed that these bacteria produce oxidase enzyme. Cytochrome oxidase catalyzes the reduction of molecular oxygen. Khademian and James (2017) reported that cytochrome oxidase enzyme in bacteria plays a fundamental role in aerobic respiration.

Strains NAS_1, NAS_2, and NAS_3 were indicated positive for motility. According to Constant (2017), many bacteria are motile through the action of large complex protein assemblages called flagellum. Ismail (2019) and Yang (2016) also mentioned that bacilli and some cocci have flagella that causes bacteria to move.

Urease test was signified a negative result for all strains. This indicated that these bacteria were not produced urease enzyme to catalyze the hydrolysis of urea into carbon dioxide and ammonia. According to Ainayah *et al*. (2016), urease enzyme was produced by marine bacteria and plays an essential role as non-toxic catalyst.

Citrate test showed a negative result. The result indicated that citrate wasn't utilize as a carbon source. Aditi *et al*. (2017) mentioned that citrate utilization test was done to differentiate among bacteria species based on their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease.

Bacteria Identification Base on 16S rRNA Gene Sequence

As seen in Figure 1 the result of molecular test with 16S rRNA analysis showed that band characteristics of strains NAS_1, NAS_2, and NAS_3 were single and quite thick. The bands have a size about 1500 bp which indicated that the DNA concentration of PCR product was sufficient to be processed into the sequencing stage. Untu *et al*. (2015) mentioned that DNA target must have size about 1400 bp to obtain good sequencing results.

The data results of BLAST Nucleotide analysis showed that strain NAS_1 has 99.17 % similar identity with *Breoghania* sp., strain NAS_2 showed species relatedness to *Pseudoalteromonas ruthenica* with sequence similarity of 99.27%, whereas strain NAS_3 showed a sequence similarity of 100 % to *Halomonas beimenensis* (Table 3). According to Greenwood *et al*. (2012), the species greater than 97 % similarity to the same species but similarity less than 97 % represent different species.

The sequence similarity result was relevant to their phylogenetic tree analysis. As can be seen in Figure 2, phylogenetic tree analysis demonstrated 3 clusters of the isolates. The numbers contained in each tree branch show the bootstrap value. Based on phylogenetic tree analysis, it showed that NAS_1 strain was located in *Breoghania* sp. branch, NAS_2 strain aligned most closely with *Pseudoalteromonas ruthenica*, whereas NAS_3 was in the same branch with *Halomonas beimenensis.*

Breoghania sp. is a Gram-negative moderately halophilic bacterium belong to the family Cohaesibacteracea. These bacteria was proposed by Gallego *et al*. (2010) in the previous study obtained bacteria strain that isolated from coastal water of northwest Spain. The bacteria are Gram-negative, aerobic rods, motile, catalase and oxidase positive. Based on genotypic, phenotypic and chemotaxonomic characteristics supported that the strain was a novel genus and species. He *et al*. (2010) also have successfully isolated *Breoghania* sp. strain L-A4 from the rhizosphere of *Phragmites australis* in the Qinhaungdao coastal wetland in China with characteristics a long rod-shaped aerobic bacterium, approximately 0.2 to 0.4 µm wide.

Pseudoalteromonas ruthenica is a Gram-negative halophilic bacterium with characteristics motile, catalase, and oxidase positive. Previous study by Ivanova *et al*. (2007) reported that these bacteria is Gram-negative, strictly aerobic, oxidase and catalase positive, rod shaped, 0.7–0.9 µm in diameter and 1.0–1.2 µm long with a single polar flagellum. Khalifa dan Munira (2019) also reported that bacteria strain UQAD-3 was isolated from a marine ecosystem in the Al Ahsaa region Saudi Arabia identified as *P. ruthenica.* This bacteria was observed at 15% NaCl indicating the moderate halophilic nature of the strain.

Halomonas beimenensis is one of halophilic bacterium that dominating shrimp pond (Alfiansah *et al*., 2018). This bacterium is Gram-negative, catalase and oxidase positive, motile. According to Joulak *et al*. (2019), *Halomonas* spp. is comprised of halophilic whose species are widely distributed throughout different hypersaline environments. Dzewit (2013) also mentioned that Halomonas belonging to family Halomonadaceae a chemoorganoheterotrophic, aerobic or facultatively anaerobic bacteria and most of which are halophilic or halotolerant.

The Ability of Bacteria to Reduce Ammonia

The ammonia removal test was used to screen bacteria based on their ammonia removal efficiency. As can be seen in Table 3 the present study showed that *Halomonas beimenensis* and *Pseudoalteromonas ruthenica* were able to reduce ammonia with percentage decrease of 8.4 % and 20.3 %. Meanwhile *Breoghania* sp. was not showed a positive result to reduce ammonia.

The present study showed that *Halomonas beimenensis* and *Pseudoalteromonas ruthenica* are heterotrophic bacteria that can reduce ammonia. According to Stein (2011), several genera of chemoorganotrophic bacteria are capable of oxidizing ammonia, hydroxylamine, various organics, and nitrite in a process termed heterotrophic nitrification. Furthermore, Dauda and Akinwole (2015) mentioned that heterotrophic bacteria will convert toxic ammonia to nitrogen gas instead of nitrates. Wang *et al*. (2018) also stated that heterotrophic nitrifying microorganisms gain several advantages such as higher buffering capacity to organic loads and less oxygen demand.

Several species of heterotrophic bacteria have been shown to affect nitrogen's biological oxidation. These bacteria have been reported to oxidize ammonia into nitrite and nitrate, such as *Halomonas beimenensis*. This also been substantiated by Wang *et al*. (2017) and Sangnoi (2017) who reported that ammonium removal efficiency of all Halomonas strains showed a range of 23-71%. The previous study has higher percentage decrease compared to the present study which is only 8.4%. This can occurred because the different species of *Halomonas* sp. and also differences in environmental factors such temperature, salinity, and ammonia concentration that affect bacteria. The previous and present study provides evidence that Halomonas is halophilic heterotrophic nitrifying bacteria. According to Alfiansah *et al*. (2018), Halomonas bacteria also utilized as probiotic that contributed to the increase of shrimp survival rate.

Pseudoalteromonas is a genus belong to phylum Proteobacteria, a diverse group of heterotrophic bacteria. Pseudoalteromonas widely distributed in the marine environment and associated with a variety of marine organisms (Atencio *et al*., 2018). In the present study *Pseudoalteromonas ruthenica* was able to reduce ammonia with a percentage decrease of 20.3 %. Previously there have been no studies related to the ability of *Pseudoalteromonas ruthenica* in oxidizing ammonia. However, Jiang *et al*. (2019) revealed that Pseudoalteromonas showed a positive role in nitrogen removal in saline wastewater treatment. Several bacteria of this genus also have been used as probiotics in shrimp and fish cultivation to inhibit pathogen bacteria. Wang *et al*. (2018) has suggested that *Pseudoalteromonas* spp. was anti-Vp_{AHPND} probiotic candidates to against acute hepatopancreatic necrosis disease (AHPND) in shrimp farms.

CONCLUSION

According to the results of this study, there were three bacteria isolated from traditional shrimp pond. These bacteria are *Breoghania* sp., *Pseudoalteromonas ruthenica*, and *Halomonas beimenensis*. Among these bacteria, *Pseudoalteromonas ruthenica*, and *Halomonas beimenensis* showed the ability to reduce ammonia with percentage of

20.3% and 8.4 % respectively. Further research is needed to confirm the bacteria before application, including the ability to grow in shrimp ponds and the ability to antagonize against pathogenic bacteria in shrimp ponds.

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