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Research Article
Isolation and Characterization of Anoxygenic Photosynthetic Bacteria for Reducing Ammonia and Probiotics Candidate

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Abstract

Background and Objective: The accumulation of organic matter in an aquaculture pond system might increase the accumulation of ammonia compounds which made toxic in aquatic organisms. Increased ammonia concentrations affected enzymatic mechanisms in animals, increased oxygen consumption and interfered with oxygen transport. This research was aimed to reduce ammonia accumulation by increasing the rate of ammonia reduction by Anoxygenic Photosynthetic Bacteria (APB) which was also a potential candidate for probiotics. **Materials and Methods:** Bacteria were isolated from mangrove forests in the Hanura beach of the Pesawaran district in Lampung province. Strains were grown on SWC media at anoxygenic-photosynthetic condition, morphologically characterized, tested for pH resistance, salinity, antibiotic endurance, ability to compete with *Vibrio* sp. and ability to reduce ammonia. **Results:** A total of 7 isolates grew well at pH 7 and 10 and 3 and 6% of NaCl concentrations. L1 strain reduced ammonia by 62%. L2 strain was the most resistant to nalidixic acid, streptomycin, chloramphenicol, ampicillin and trimethoprim. **Conclusion:** Bacterial strain B has the highest competitive ability against *Vibrio* sp., which was able to survive up to 2.6 log.Σ.cell. Two probiotic candidates were photosynthetic bacteria. They were able to inhibit of *Vibrio* sp. growth, reduce the ammonia content by 22-33% and resistant into antibiotics.

Key words: Vibrio, ammonia, anoxygenic, photosynthetic bacteria, probiotic, vibriosis, aquaculture

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

There were several problems for aquaculture farms, either infectious or non-infectious. *Vibrio* sp. was one of infectious sources, namely vibriosis. Antibiotic utility was applied to treat the diseases. Unfortunately, antibiotics usage was lead to pathogen resistance[1]. Ammonia accumulation was non-infectious problems. Ammonia was accumulated during feed digestion and excreted through their gills and in their feces by fish and shrimp. It was created an anaerobic .condition at the pond[2].

Anaerobic Photosynthetic Bacteria (APB) were bacteria carried out decomposition activities even though oxygen levels in water or sediment were very few or even none at all. This was because they were able to utilize hydrogen donors from compounds that are reduced more strongly (H_2S, ammonia, nitrites and organic compounds) as a substitute for H_2O[3]. According to Canfield *et al.*[4], the presence of anoxygenic photosynthetic bacteria was able to reduce H_2S toxic compounds. Besides, they had good potential as a biocontrol agent[5], as a heavy metal-lowering agent[6] and an effective fishmeal replacement in aquaculture[7]. The effect of Fe ions exerts an increase in the absorption of light in photosynthetic APB at wavelengths[8] of 300-400 nm.

Lampung province, Indonesia, had high production of shrimp hatchery that grew at mangrove habitat. Vibriosis and ammonia accumulation was serious threaten for shrimp aquaculture. Regarding the above reason the objective of the study was to isolate and characterize APB isolates from mangrove forests in the Hanura waters of Lampung as a candidate bioremediation agent in reducing ammonia compounds and candidates probiotic agents in competing against pathogenic bacteria *Vibrio* sp.

MATERIALS AND METHODS

Study area: This research project was conducted at the Department of Biology, Microbiology Lab, University of Lampung, Indonesia from December 2018 to June 2019.

Sample preparation: Anoxygenic photosynthetic bacterial isolates were isolated from several sample points in the Hanura Lampung mangrove forest including sea mud, mangrove mud, roots, leaf litter, flowers, snails and crabs. Each bacterial isolate was inoculated in liquid SWC media with a composition (5 g of bacto peptone, 1 g of yeast extract, 3 mL of glycerol, 250 mL of distilled water, 750 mL of sea-water and 15 g of agar) liquid media without agar. It incubated for 6-7 days in front of the lamp tungsten at a distance of 30-40 cm. A single loop of the anoxygenic photosynthetic bacterial isolate was taken and then scratched using the quadrant streak method[9], on SWC agar (SWCA) and re-incubated for 5-6 days in an anaerobic jar in front of a 40-watt accent lamp with a distance of 30-40 cm. Furthermore, the growing bacterial isolates were transferred to the SWCA sloping media to obtain pure isolates[10]. Bacteria characterized through Gram staining, microscopic morphology, colony color and shape of the colony.

pH and salt-tolerant assay: Bacteria were purified at Petri dish containing SWCA media which was given a pH difference treatment of 4, 7 and 10. Then they incubated for 4-5 days by the anoxygenic photosynthetic condition. Bacteria were cultured into modified SWCA media with the addition of salt concentrations of 0, 3 and 6%. It was incubated for 4-5 days by anoxygenic photosynthetic. Their growth parameters observed according to Triyanto *et al.*[11] methods.

Ammonia reducing assay: A single loop of bacteria was grown on a screw tube containing liquid SWC media (5 mL). Each tube was then homogeneous with the vortex. The liquid SWC was added until it was full. The threaded tubes are then incubated at room temperature for 5-6 days in anaerobic photosynthetic conditions. They centrifuged for 10 min at 1000 rpm and 4°C and the supernatant was taken[12]. The amount of 6 ml of bacteria culture supernatant was put into a test tube and add 1 drop of Seignette salt (15 g in 30 mL of distilled water) then homogenate and let stand for 2 min. Nessler (K_2HgI_4) as much as 0.25 mL was added and homogenized again for 15 min until a clear purple solution occurred. Ammonia reduction test was carried out using a UV-Vis Shimadzu spectrophotometer at a wavelength[12] of 420 nm.

Antibiotic assay: Bacteria were cultured at SWC liquid media in photosynthetic conditions for 3×24 hours at room temperature[12]. Antibiotic assay conducted using the diffusion method. Bacteria in 0.1 mL suspension was poured on the surface of the SWCA media and placing antibiotic discs contained Chloramphenicol, Nalidixic Acid, Trimethoprim, Ampicillin and Streptomycin. It was incubated for 3×24 hours by the photosynthetic anaerobic condition. A clear zone around the antibiotic disk paper that indicated the inhibition zone was measured.

Anaerobic photosynthetic-bacteria against *Vibrio* sp. Assay: Bacteria incubated at 14 mL of SWC liquid 3 days in photosynthetic threaded test tubes. A total of 10 mL isolates

of *Vibrio* sp. incubated in Nutrient Broth (NB) media (1×24 hours). They were calculated microscopically together with Gram staining to determine the population density of anoxygenic photosynthetic bacterial and *Vibrio* sp.

Both anoxygenic photosynthetic bacterial and *Vibrio* sp. were taken 1 mL and put into a threaded test tube containing NB liquid media at a density of 10^4 and 10^2 respectively (incubated for 2×24 hours by anoxygenic photosynthetic at room temperature). The population detected on the media used anaerobic photosynthetic pour method, while to detect the growth of *Vibrio* sp. incubated on TCBSA media on Petri dishes at room temperature[13] for 24 hours.

RESULTS

Sample characterization: There were 7 strains of anoxygenic photosynthetic bacteria cultured from 12 location points. They were most Gram-negative isolates in the form of stem colonies while the colonies' color was beige, white, turbid, red, yellow and brown. Thereby they are taken which will be tested further presented in Table 1.

Salt tolerant and pH assay: Anoxygenic photosynthetic bacteria was tested in different pH and NaCl stresses, it was aimed to know the susceptibility of salinity and acidity level. The pH test was carried out using pH 4, 7 and 10 while the NaCl test with concentrations of 0, 3 and 6%. The result was obtained in Table 2 for salt-tolerant, due to all bacteria strains were taken from the mangrove ecosystem it was proven that they were salt tolerant. They were more appropriate to grow well at 3-6% salt concentration or above. Even though some of them kept tolerate the level of freshwater without salt content in the media.

Based on Table 3, the pH test results obtained showed that all isolates were unable to grow at pH 4. It was shown that the APB obtained from the isolation results could not grow under acidic conditions. Whereas in alkaline conditions (pH 10), bacterial growth was inhibited in isolates L1.

Table 1: Anoxygenic photosynthetic bacterial that succeeded culture

Sources of strain	Strain codes	Gram staining	Cell shape
Root	AS	Negative	Rods
Root	AM	Negative	Rods
Mud	L1	Negative	Rods
Mud	L2	Negative	Rods
Flower	B	Negative	Rods
Leaves	D	Negative	Rods
Leaves	B2DM	Negative	Rods

AS: Strain was isolated from fibrous root of mangrove, AM: Strain was isolated from root of mangrove, L1: Strain was isolated from mud of mangrove location 1, L2: Strain was isolated from mud of mangrove location 2, B: Strain was isolated from litter of flowers of mangrove, D: Strain was isolated from litter of leave of mangrove, B2DM: Red color-Strain was isolated from litter of flowers of mangrove

Ammonia reducing assay: The results of a decrease in ammonia concentration tests are shown in Fig. 1, that anoxygenic photosynthetic bacterial isolates showed the different abilities in reducing ammonia concentration. Based on the results of the effectiveness test, the greatest effectiveness value is 62% L1 isolate and the smallest is B2DM 12%. Thus the L1 isolate was the best in suppressing the concentration of ammonia compared to other isolates. From this result, we knew that bacteria strain that taken from mud sediment had a high-ability to reduce ammonia. It was due to that they were allegedly doing that ecologically as long as they live at ammonia accumulation inside mud sediment of mangrove.

Antibiotic assay: Antibiotic susceptibility assay was presented in Table 4. The most resistance reactions were streptomycin and ampicillin. There were four isolates namely AS, B, D and

Table 2: Salt tolerant assay toward the anoxygenic photosynthetic bacterial culture

	Concentration NaCl in medium (%)		
APB isolate	0	3	6
AS	+	++	++
AM	+	++	++
L1	++	++	++
L2	++	++	++
B	+	++	++
D	+	++	++
B2DM	-	+	+
K1	++	++	++
B3DK	+	++	++
B4LM	++	+	+

-: No bacterial growth, +: Diameter of bacterial colony less than 0.5 cm, ++: Diameter of bacterial colony between 0.5-1.0 cm

Table 3: pH tolerant assay toward the anoxygenic photosynthetic bacterial culture

	Medium pH		
APB Isolate	4	7	10
AS	-	++	++
AM	-	++	++
L1	-	++	++
L2	-	+	-
B	-	+	+
D	-	+	+
B2DM	-	-	-
K1	-	+	+
B3DK	-	++	+
B4LM	-	++	++

-: No, +: Diameter of bacterial colony less than 0.5 cm, ++: Diameter of bacterial colony between 0.5-1.0 cm

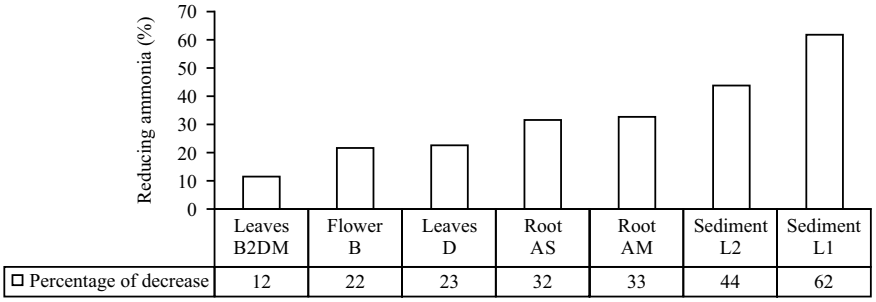

Fig. 1: Percentage of reducing ammonia in anoxygenic photosynthetic bacteria compared to sources of strain isolation habitat

Table 4: Bacterial susceptibility toward antibiotic assay

	Antibiotic assay				
APB isolate	NA	S	C	A	T
AS	8.1	R	21.5	15.3	R
AM	11.7	10	16.4	R	18.1
L1	R	8,8	7.9	R	9.3
L2	17.5	5.2	25.2	6.2	7.1
B	12.4	R	R	R	9
D	9	R	19.1	10	10
B2DM	9.6	R	26.2	14.4	R

NA: Nalidixic acid, S: Streptomycin, C: Chloramphenicol, A: Ampicillin, T: Trimethoprim, R: Resistance

Table 5: Competition test results between APB and *Vibrio* sp.

			Log Σ of initial cell		Log Σ of final cell	
No.	APB versus	*Vibrio* sp.	APB	*Vibrio* sp.	APB	*Vibrio* sp.
1	-	*Vibrio* sp.	-	2.0	-	2.9
2	APB-B2DM	-	4.0	-	2.2	-
3	APB-D	-	4.0	-	2.5	-
4	APB-B	-	4.0	-	2.3	-
5	APB-AS	-	4.0	-	2.3	-
6	APB-AM	-	4.0	-	1.9	-
7	APB-L1	-	4.0	-	2.1	-
8	APB-L2	-	4.0	-	2.2	-
9	APB-B2DM	*Vibrio* sp.	4.0	2.0	2.4	2.2
10	APB-D	*Vibrio* sp.	4.0	2.0	2.2	2.2
11	APB-B	*Vibrio* sp.	4.0	2.0	1.9	0
12	APB-AS	*Vibrio* sp.	4.0	2.0	2.2	1.4
13	APB-AM	*Vibrio* sp.	4.0	2.0	2.6	0
14	APB-L1	*Vibrio* sp.	4.0	2.0	1.6	1.6
15	APB-L2	*Vibrio* sp.	4.0	2.0	2.4	2.4

1: Bacteria of *Vibrio* sp. 2 : APB- B2DM 3 : APB- D
4 : APB- B 5 : APB- AS 6 : APB- AM
7 : APB- L1 8 : APB- L2 9 : BFA B2DM X *Vibrio* sp.
10 : APB- D X *Vibrio* sp. 11 : APB- B X *Vibrio* sp. 12 : APB- AS X *Vibrio* sp.
13 : APB- AM X *Vibrio* sp. 14 : APB- L1 X *Vibrio* sp. 15 : APB- L2 X *Vibrio* sp.

B2DM which are resistant to streptomycin. Whereas AM, L1 and B isolates were resistant to ampicillin antibiotics.

Anaerobic photosynthetic-bacteria against *Vibrio* sp. Assay: The results of the competition test between APB and *Vibrio* sp. shows the competitive ability of each of the different APB isolates. The biggest competition ability of APB isolates was isolate B from mangrove litter samples at 2.6 and AM isolate at 1.9 LogΣcell. The results of the competition test between APB and *Vibrio* sp. can be seen in Table 5.

The results of the competition between APB and *Vibrio* sp. this occurs because isolate B from APB comes from mangrove litter and AM isolates from mangrove roots are deep in the mud, where the aquatic environment is a dirty environment so many decaying bacteria also live in the environment. Therefore, APB B and AM isolates are accustomed to competing with decomposing microorganisms, one of which is Vibrio sp. Also, APB is thought to produce several antibacterial compounds.

DISCUSSIONS

Bacteria have genes for resistance that are obtained from the environment for a very long time or even after exposure to antibiotic selection. Bacteria can reproduce to propagate resistance genes both vertically and horizontally and have a long-term impact. Some bacteria have a natural ability to be resistant to antibiotics. This can occur because bacteria have enzymes that can damage drugs[14]. Also, streptomycin and ampicillin antibiotics are many types of antibiotics that are often used in aquatic environments. This is supported by research Kusmarwati *et al.*[15] that 100% of *Vibrio parahemolyticus* bacteria resistant to streptomycin and 90% of ampicillin in the cultivation of Vaname shrimp on the north coast of Java.

Furthermore, as shown in Table 5, the types of antibiotics of nalidixic acid, chloramphenicol and trimethoprim have sensitive reactions to APB isolates. This is presumably because all three types of antibacterials are still rarely used in an antibiotic environment. According to research Kusmarwati *et al.*[15] informed that sensitive bacterium *Vibrio parahemolyticus* on chloramphenicol antibiotics by 18%.

Makori *et al.*[16] and Agustiyani *et al.*[17] were described that the optimum aquaculture environment (ponds) requires the pH of soil conditions ranging from 6.34-8.10. The degree of acidity (pH) is one of the conditions for growing a microorganism. The relationship between the pH value and bacterial growth greatly affects the work system of bacterial metabolism itself to produce a substance called an enzyme. Enzyme activity will be maximum if the pH conditions are also optimum. Rice *et al.*[18], was explained that the acidic pH of the bacterial cell membrane will become saturated by hydrogen ions thereby limiting membrane transport. This causes the acid substance that does not decompose to seep into the cell so that the ionization of the cell's pH changes. This change causes the process of sending amino acids from RNA to be inhibited so that it inhibits growth and can even kill the bacteria itself.

Then, the results of the APB isolate growth test with media containing different concentrations of NaCl showed that 8 isolates could grow at concentrations of 3% and 6% NaCl except for isolates K and B3DK. According to Suprayogi *et al.*[19] that if the water contains 0.5-3% salt content, then the water can be called brackish water whereas if >3% can be said to be seawater.

Sodium chloride (NaCl) stress inhibited the growth of bacteria because it increased the osmotic pressure of the substrate which can cause water withdrawal from the microorganism cells so that the cell will lose water and shrink and cause slowing of microorganism activity[18]. This is supported by Membre research[20] where NaCl can cause denatured microbial protein besides NaCl also has high toxicity to microbes, salt ionization will produce Cl-ions that are toxic to microorganisms and can block the respiration system of the microorganism itself. Rubiano *et al.*[21] salt were capable of reducing the solubility of O_2 in water, cause cells to be more sensitive to CO_2 and interfere with the work of proteolytic enzymes in microbial cells that function to break down proteins into amino acids.

In the study of antibiotic tests on APB isolates, the largest clear zone was found in B2DM isolates tested on chloramphenicol antibiotics by 26.2 mm and the smallest clear zone was L2 isolates on ampicillin antibiotics by 6.2 mm, Table 4. According to Li *et al.*[22], the size of the clear zone contained in the antibiotic test is influenced by several factors including 1) the type and age of bacteria, 2) the concentration of antibiotic substances and the amount of inoculum or bacterial density, 3) levels of active substances or functional groups of antibiotic substances. Photosynthetic bacteria are known to have a central role in the nitrogen element cycle. The addition of photosynthetic bacteria can also eliminate organic matter or other harmful substances in improving water quality and is included in the type of probiotics that provide nutrients and enzymatic contributions to fight pathogenic bacteria[23-25].

In anaerobic sediments, some microbes break down organic matter with fermentation reactions that are capable of producing alcohol, ketones and other compounds. According to Madigan *et al.*[26] microorganisms can also use oxygen from nitrates, nitrites, sulfates and CO_2 to decompose organic material, but these microorganisms also emit nitrogen gas, ammonia, ferrous, manganous manganese, H_2S and methane as a result of metabolism.

Sea Water Complete (SWC) media used in the study contained organic compounds in the form of nitrogen from peptones which when incubated under anaerobic conditions for approximately 5 days will form ammonia compounds. According to Jeong *et al.*[27] an ammonia test can be carried out using a basic solution of mercury (II) iodide in Potassium Tetraiodomercurate (K_2HgI_4) or it can also be called a Nessler reagent to measure ammonia compounds calorimetric. In the working principle of the Nessler method, Nessler reagents when reacted with ammonia in the form of an alkaline solution will form a brownish-yellow colloidal dispersion.

As reported by Tsang *et al.*[28], ammonia can be reduced with the help of indigenous bacteria through metabolic processes. Ammonia degradation mechanism is found in aerobic conditions by nitrifying bacteria groups, namely Ammonia-Oxidizing Bacteria (AOB) such as Nitrosomonas and Nitrobacter. But apart from that also the anaerobic ammonia condition can be degraded by anaerobic ammonia-oxidizing (Anammox). According to Feng *et al.*[29], that the anoxygenic photosynthetic bacteria Rhodobacter encapsulants can be used as a bioremediation agent because it can reduce ammonia compounds by 56%.

Competition is a negative interaction that occurs between two or many microorganisms when the supply of food sources, oxygen, light and space needed is limited to survive and reproduce[30]. APB which is anaerobic bacteria can decompose organic matter with fermentation reactions that can produce alcohol, ketones and other compounds. This is supported by research by Feng *et al.*[29], which states that anaerobic microorganisms can use oxygen from nitrates, nitrites, sulfates and CO_2 to decompose organic material, but emit nitrogen, ammonia, ferrous, manganous manganese, H_2S and methane as a result of metabolism. It suspected to be toxic to *Vibrio* sp.

According to Yang *et al.*[31] the antimicrobial effect produced by bacteria is caused by several factors, namely the production of antibiotics, bacteriocin, siderophore, lysozyme, proteases, or hydrogen peroxidase and changes in pH values through the production of organic acids. The antimicrobial in question is bacteriocin, which is an extracellular component in the form of an antimicrobial protein compound that shows the opposite response to the target bacteria[32]. The bacteriocin compound that is currently widely used in fishery products is Nisin produced from *Lactococcus lactis*[33]. Antimicrobial compounds which are bacteriocin will cause the target bacterial cells to become lysis. Besides, there was a struggle for nutrition and space between APB and *Vibrio* sp. as stated by Sumardi *et al.*[34] when a challenge test was conducted between Bacillus and *Vibrio* sp. Also, the difference in oxygen demand of each bacterium in which APB is a facultative anaerobic is that it can live in conditions of very little oxygen content until there is no oxygen at all while *Vibrio* sp. is a type of facultative aerobic bacteria wherein.

CONCLUSION

The result informed that bacteria were successfully cultured anaerobic photosynthetic-bacteria. L1 strain reduced ammonia by 62%. L2 strain was the most resistant to nalidixic acid, streptomycin, chloramphenicol, ampicillin and trimethoprim. Bacterial strain B has the highest competitive ability against *Vibrio* sp., which was able to survive up to 2.6 log.Σ.cell. L1 was potentially developed to bioremediation agent but has a low ability to pH and salinity stress it also happened to strain L2. Regarding the result of reducing ammonia, antibiotic susceptibility, pH and salinity stress, anaerobic photosynthetic-bacteria strain B was more appropriate selected as a candidate for bioremediation and prob

SIGNIFICANCE STATEMENT

This study found 2 probiotic candidates, APB-B and APB-AM strains, that were unique photosynthetic-bacteria. They were able to inhibit the growth of *Vibrio* sp. (*in vitro* conditions), able to reduce the ammonia content by 22-33% and resistant into antibiotics. This study will help researchers to uncover resistance antibiotics in BFA bacteria that occur in mangroves habitat. The resistance of these bacterial antibiotics has not been explored by many researchers. It was potentially developed as a probiotic candidate to overcome the shrimp disease.

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