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ISOLATION OF LACTIC ACID BACTERIA FROM VANAME SHRIMP (*Litopenaeus vannamei*) AND ITS ANTAGONISM TO PATHOGEN BACTERIA

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ABSTRACT

Vaname shrimp is one of Indonesia's leading export species and is widely cultivated. The main problem in vaname shrimp pond cultivation is the attack of shrimp diseases caused by bacteria or viruses. This study aims to isolate and identify lactic acid bacteria (BAL) from vaname shrimp (*Litopenaeus vannamei*) and test their antagonism against pathogenic bacteria. The research was conducted from February to April 2023. The method used in this study is a survey method. Sampling was conducted at the vaname shrimp pond of the Fisheries Service Task Unit (UPT), Tanjung Punak Village, Rupat Utara District, Bengkalis Regency, Riau. The method used in this research was a survey method. BAL was isolated using MRS Agar + $CaCO_3$ media. The Kirby-Bauer method was used to test bacterial antagonism against pathogenic bacteria. Based on the identification of seven isolates, it was found that only four isolates were positively identified as LAB bacteria, namely 47D, 54T1, 73DB1, and 73BD2. The results of the antagonism test against pathogenic bacteria showed that all BAL isolates could not suppress the growth of *E. coli* bacteria. However, these isolates suppressed the growth of *A. hydrophila* bacteria, with the widest inhibition zone diameter on isolate 54T1 (4.2 mm). On *Vibrio* sp. bacteria, the widest inhibition zone of BAL was on isolate 73DB1 (6 mm). These results indicate that the antibacterial activity produced by BAL is still relatively weak.

Keywords: Lactic acid bacteria, Antagonism, Vannamei shrimp ponds.

1. INTRODUCTION

Vanname Shrimp is one of the leading types of shrimp for Indonesian exports and is widely cultivated. However, shrimp farmers in Indonesia often experience various internal and external problems in shrimp farming[1]. The main problem with the highest percentage of failure in shrimp farming is disease caused by bacteria or viruses. Lactic Acid Bacteria (BAL) produce microbial inhibiting compounds such as hydrogen peroxide, diacetyl, carbon dioxide, reuterin and bacteriocin[2]. Bacteriocins are compounds in the form of proteins produced by bacteria which act as inhibitors of the growth of other microbes. Some bacteriocins have inhibitory activity on some food-pathogenic bacteria. BAL can be classified into 13 genera, namely *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragagenococcus, Vagococcus, Weissela,* and *Bifidobacterium*. Fransiska et al.[3] identified BAL of the genus *Leuconostoc* sp, *Bacillus* sp, and *Lactococcus* sp. found in the digestive tract of pomfret fish. Lactic acid bacteria (BAL) are one group of organisms that can be used as probiotics. One potential effort to overcome disease and increase production in vannamei

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shrimp farming in ponds is using probiotics[4]. Probiotics can maintain water quality and inhibit the growth of pathogenic microorganisms. Pathogenic bacteria are bacteria that can cause disease. The antagonist test is a method used to prove that antagonistic microorganisms can inhibit the activities of nearby microorganisms.

2. RESEARCH METHOD

Time and Place

This research was conducted in February-April 2023. Sampling was carried out at the Vaname Shrimp Ponds Task Implementation Unit (UPT) of the Fisheries Service, Tanjung Punak Village, Rupat Utara District, Bengkalis Regency, Riau and sample analysis was carried out at the Marine Microbiology Laboratory, Department of Marine Science, Faculty of Fisheries and Marine, Universitas Riau.

Method

The method used in this study is a survey method. The stages carried out in this study were determining the sampling location, measuring physical and chemical parameters, taking samples, making media, isolating, characterizing BAL and testing antagonism.

Procedure

Measurement of Physical and Chemical Parameters

Water quality parameters were measured directly in the field on three ponds. Physical parameters were measured, including light intensity using a Secchi disk, a salinity hand refractometer and temperature using a thermometer. In contrast, chemical parameters include pH using universal pH indicator papers, dissolved oxygen (DO) using a DO meter, and ammonia levels using the phenate method.

BAL Isolation

Vaname shrimp samples were washed with sterile distilled water and then mashed and taken as much as one gram then mixed with 9 mL of 0.9% NaCl and homogenized using a vortex to obtain a dilution of 10^{-1} to dilutions 10^{-4} (10^{-1},10^{-2}, 10^{-3},10^{-4}). At a dilution of 10^{-1}, transfer as much as one mL into a dilution of 10^{-2} until the 10^{-4} dilution. From each dilution, 10^{-2}, 10^{-3}, 10^{-4} Taken as much as 0.1 mL and spread in a petri dish containing MRS Agar media plus $CaCO_3$ 1% by Spread plate method. Then, the media was incubated for 24-48 hours at $37^{O}C$[5].

Identification of Bacteria

Bacteria were identified on bacterial colonies growing on MRS Agar+$CaCO_3$ 1% based on observations of colony morphology, cell shape and biochemical tests[5]. Observation of colony morphology includes the colony's shape, edges, color, and size. Observation of cell morphology includes cell shape, Gram properties, and cell motility under a binocular microscope at 10× and 40× magnification. Biochemical tests include catalase tests, Sulfide indole motility (SIM), Simmons Citrate Agar (SCA), Triple Sugar Iron Agar (TSIA), and sugar fermentation tests[6].

Antagonist Test

Bacterial antagonist tests against pathogenic bacteria can be carried out using the Kirby-Bauer method, namely disc diffusion (disk diffusion method), which measures the diameter of the clear zone which indicates a response to the inhibition of bacterial growth by BAL. The test bacteria used are *E.coli*, *Vibrio* sp, and *A. hydrophila*.

3. RESULT AND DISCUSSION

Water Quality Parameters

Water quality parameters were measured for vannamei shrimp ponds at UPT Fisheries Service Rupat Utara, Riau. These parameters included temperature, salinity, pH, dissolved oxygen, and ammonia levels (Table 1). The salinity measurements in vannamei shrimp ponds ranged from 14-15 ppt, the average pH was

7, the average temperature was 30°C, the ammonia levels ranged from 0.96-1.26 mg/L, and the dissolved oxygen levels ranged from 7.5-7.6 mg/L.

Table 1. Water Quality Conditions for Vannamei Shrimp Ponds at the UPT Fisheries Service of Rupat Utara, Riau

Ponds	Temperature (°C)	Salinity (ppt)	pH	Ammonia (mg/L)	DO (mg/L)
Ponds 1 (47 days old)	30	15	7	0,96	7,6
Ponds 2 (54 days old)	30	15	7	1,12	7,6
Ponds 3(73 days old)	30	14	7	1,26	7,5

Table 1 shows that, in general, the water quality parameters tested were not too different between each observed pond. The optimum temperature for the measurement results is that the temperature of the pond waters is 30°C, indicating the optimal temperature for the growth of vannamei shrimp. Temperature is very influential on photosynthetic activity and solubility of the particles in the pond. The growth of vannamei shrimp ranges from 28-31°C and grows well at a temperature of 24-34°C[7]**.** Salinity in ponds ranges from 14-15 ppt. This salinity is still categorized as optimal for the growth of vannamei shrimp. The optimal range of pond water salinity for shrimp farming is 12-20 ppt[8].

In each pond, the optimal pH for the growth of vannamei shrimp was found to be 7. The optimal pH range for shrimp growth was 7-8.5, and it could tolerate pH in the range of 6.5-9[7]. Water pH concentration affects shrimp appetite. Ammonia levels in ponds ranged from 0.96-1.26 mg/L. These results indicate that the ammonia level in pond 3 is slightly high for the growth of vannamei shrimp. Ammonia concentrations of more than 1.0 mg/L can cause death in shrimp[9].

According to the Decree of the Minister of Maritime Affairs and Fisheries no. 28 of 2004[10] concerning general guidelines on pond cultivation, ammonia is expected to be no more than 0.1 mg/L. Ammonia in water comes from the decomposition process of organic matter, which contains many nitrogen compounds (proteins) from leftover feed and fertilization. Other water quality parameters influence ammonia concentration in waters. The results of DO measurements in ponds show relatively high numbers ranging from 7.5-7.6 mg/L. Based on SNI 8037.1: 2014, the optimum dissolved oxygen quality standard for shrimp growth is 4-9 mg/L, which is suitable for survival. Dissolved oxygen conditions in intensive ponds are slightly higher, and this is because these ponds have used a water wheel, which can increase the value of dissolved oxygen.

Lactic Acid Bacteria Isolate

The results of the isolation of lactic acid bacteria (BAL) from vannamei shrimp on MRS Agar media found as many as seven isolates with almost the same size, shape, and color as each bacterial isolate. Adding 1% $CaCO_3$ (calcium carbonate) into the bacterial growth medium aims to neutralize the acid production produced by BAL so that a clear zone is formed around the BAL colony (Figure 1).

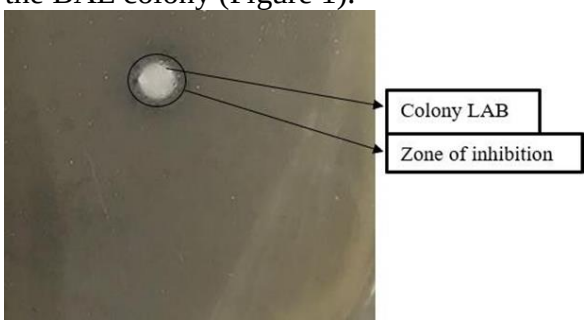

Figure 1. Growth of BAL on MRSA media + 1% $CaCO_3$

The BAL isolates obtained were seven isolates. A total of one isolate came from vannamei shrimp 47 days old, two isolates 54 days old, and four isolates from vannamei shrimp 73 days old. All isolates that have been obtained will then be tested further with physiological tests,

biochemical tests and antagonism tests against pathogenic bacteria, namely *E.coli*, *A. hydrophila*, and *Vibrio* sp.

Morphological and Physiological Characteristics of Lactic Acid Bacteria
Morphology of Bacteria

Colony morphology was identified on all isolates growing on MRS Agar+$CaCO_3$ media. Colony morphology includes color, colony diameter, edges, and elevation. The results of observations of colony morphology on all isolates found that the characteristics of the colonies were almost the same. The results of observations of colony morphology can be seen in Table 2.

Table 1. Morphology of BAL colonies from MRS Agar medium

Code	Diameter (mm)	Colony color	Colony Form	Edge	Elevation	Gram properties	Cell shape
47D	2	White	Coccus	Clear,	Convex	+	Basil
54T1	5	White	Coccus	Clear	Convex	+	Coccus
54T2	5	White	Coccus	Clear	Convex	+	Coccus
73DB1	4	Yellowish	Coccus	Clear	Convex	+	Basil
73DB2	4	White	Coccus	Clear	Convex	+	Basil
73DC	6	White	Irregular	Clear	Convex	+	Coccus
73T	3	White	Coccus	Clear	Convex	+	Coccus

Table 3. Characteristics of BAL isolates

								Sugar Test		
Code	Citrate	Indole	Motility	Catalase	MR	Sulfide	VP	Glucose	Sucrose	Lactose
47D	-	-	+	-	-	-	+	+	-	-
54T1	-	-	-	-	-	-	-	+	+	+
54T2	-	-	-	+	+	-	-	+	+	+
73DB1	+	-	-	-	+	-	+	+	+	+
73DB2	-	-	-	-	+	-	-	+	+	+
73DC	-	-	-	+	+	-	-	+	+	+
73T	-	-	-	+	-	-	-	+	+	+

Table 2 shows from the morphological observations that there was a slight difference between the isolates obtained. The largest BAL colony diameter is 6 mm, and the smallest is 2 mm. BAL isolates are milky white, have smooth edges and raised elevations, and the shape of the bacterial colonies is generally round. The characteristics of BAL growing on MRS Agar media are white to yellowish-white colonies, round in shape (coccus), colony size 0.5–3 mm, transparent edges and not fibrous[11].

All bacterial isolates were Gram-positive, indicated by the formation of purple color in the isolates observed. Observations also showed that bacterial isolates had cocci and bacilli-shaped cells.

Bacterial Physiology

Biochemical tests are carried out to see the physiology of bacteria. The results showed that almost all BAL isolates were citrate negative, catalase-negative, indole negative, did not produce sulfide (-), and sugar test (glucose, lactose and sucrose); all isolates were able to ferment glucose, except isolate 47D, which was only able to ferment sucrose and lactose (Table 3).

In the citrate, test carried out using SCA media, one positive citrate isolate was found, marked by a change in the color of the agar medium from green to blue, and six other isolates showed negative results. Citrate-positive bacteria are bacteria that can utilize citrate as a carbon source. The catalase test results in Table 5 showed that

four isolates were catalase-negative, and three were catalase-positive. This indicated the bacterial isolates could break down H_2O_2 and produce oxygen gas. Based on Nurhamidah et al.[12], positive catalase is indicated by the presence of air bubbles in the isolate, indicating the presence of the catalase enzyme produced by bacteria that can convert hydrogen peroxide into water and oxygen. According to Holt et al.[13] BAL is catalase negative.

Three tests were conducted on SIM media: gas/sulfide (H_2S), indole, and motile. The observations showed that all isolates were negative for sulfide, which was indicated by the absence of a change in the color of the SIM media to black. Based on Holt et al.[13], LAB of the genus *Aerococcus, Enterococcus, Lactococcus, Streptococcus,* and *Vagococcus* produce no gas. From the observations, all bacterial isolates were indole negative. Indole can be seen from the formation of a red ring on the top of the media that has been dripped with kovac reagent. A positive result on the indole test indicates that the bacteria contain the tryptophanase enzyme, which is a catalyst for decomposing the indole group contained in the tryptophan amino acid.

The amino acid tryptophan is an amino acid component commonly found in proteins, so microorganisms can easily use this amino acid. The motility test results showed that one isolate (47D) was motile, and six others were non-motile. The positive motility test was indicated by the growth of bacteria that spread on SIM media. Methyl red is used to determine the ability of bacteria to ferment mixed acids. The results of the test methyl red showed as many as four positive isolates. A positive result is indicated by a change in color on the MRVP media after dropping the solution methyl red. Some bacteria ferment glucose and produce various acidic products that will lower the pH of the growth medium to 5 or lower. Adding a pH indicator methyl red can show a change in pH to acid[12].

Vogesproskauer conducted to determine the ability of LAB to form acetyl methyl carbinol (acetoin) from glucose fermentation. Two positive isolates (47D and 73DB1) were indicated by the change in the media from yellow to red after dropping 5% naphthol indicator and 40% KOH.

The sugar test was carried out using TSIA media. The test results showed that six isolates were able to ferment the three sugars (glucose and lactose, and sucrose) marked by a change in colorslunt and butt turn yellow. One isolate (47D) was only able to ferment glucose-type sugar, marked by a change in the color of the medium to yellow on the slanted medium (slunt), and the medium is red in the vertical medium (butt).

Activity of BAL **Isolates against Pathogenic Bacteria**

An antagonism test was carried out to determine the ability of BAL to inhibit the growth of pathogenic bacteria (*E. coli, A. hydrophila, Vibrio* sp). The results obtained in Table 4 show that no inhibition zones were formed in the antagonism test performed on bacteria *E.coli,* which indicated that the BAL isolates could not suppress the growth of these bacteria. Romadhon et al.[14] found BAL isolates capable of inhibiting the growth of bacteria *E. coli* with the largest inhibition zone of 2.5 mm.

In the antagonism test performed on bacteria *A. hydrophila,* it was found that almost all isolates produced an inhibition zone with inhibition zone values ranging from 1.2 to 4.2 mm, indicating that the antibacterial activity was relatively weak. These results are based on selecting the largest inhibition zone in each isolate. Previous research found that BAL could inhibit the bacterial growth of *A. hydrophila*. Antibacterial activity is classified as weak if the inhibition zone formed is less than 5 mm, moderate if the inhibition zone ranges from 5-10 mm, strong if the inhibition zone ranges from

10-20 mm, and very strong if more than 20 mm[15].

Table 4. Zone of Inhibition of BAL Isolates against Pathogenic Bacteria

Isolate	Zone of Inhibition (mm)		
	E.coli	*A. hydrophila*	*Vibrio* sp
47D	0	0	2
54T1	0	4.2	1.3
73DB1	0	1.2	6
73DB2	0	5	4
NaCl	0	0	0
Cloramfenikol	21	21	33
Ampicilin	8	2	7

In the antagonism test performed with bacteria *Vibrio* sp. It was shown that all bacterial isolates produced zones of inhibition, indicating that all isolates could suppress the growth of bacteria such as *Vibrio* sp. The inhibition zone formed due to the interaction between BAL isolates, which urged the growth of pathogenic bacteria. From the measurement of the inhibition zone formed around the bacterial isolate colonies, it was found that the inhibition zone on isolate 47D was 2 mm, isolate 54T1 was 1.3 mm, 73DB1 was 6 mm, and 73DB2 was 4 mm. Previous research by Nursyirwani et al.[6] obtained 21 bacterial isolates isolated from grouper fish that had antibacterial activity against *V. alginolyticus*. The inhibition zone was formed due to the interaction between BAL isolates, which urged the growth of pathogenic bacteria. BAL is known to produce bioactive compounds in the form of hydrolytic enzymes that can degrade and damage the structural components of the cell walls of pathogenic bacteria[16].

4. CONCLUSION

From 3 samples of vannamei shrimp observed, four bacterial isolates were found which indicated BAL, i.e. in the 47-day shrimp sample, one isolate (47D), 54-day-old shrimp, one isolate (54T1), and 73-day shrimp, two isolates (73DB1 and 73DB2). The results of morphological identification found bacterial colonies with a round shape (coccus), white color, smooth elevation and Gram-positive. Almost all BAL isolates were citrate negative, catalase-negative, indole negative, did not produce sulfide (-), sugar test (glucose, lactose and sucrose); all isolates were able to ferment glucose, lactose and sucrose, except isolate 47D, which was only able to ferment glucose. The results of the antagonism test against pathogenic bacteria showed that all BAL isolates could not suppress bacterial growth in *E. coli*. BAL isolates can suppress the growth of bacteria *A. hydrophila* with the widest inhibition zone diameter indicated by isolate 54T1 (4.2 mm) and on bacteria *Vibrio* sp with the widest BAL inhibition zone by isolate 73DB1 (6 mm).

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