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Isolation and Identification of Lactic Acid Bacteria as Potential Probiotic from White Snapper (Lates calcarifer)

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Abstract

Lactic acid bacteria are one of the bacteria found in the digestive tract of white snapper (Lates calcarifer) which can potentially be probiotics and have the ability to produce antimicrobial metabolism that is able to suppress pathogenic bacteria. This study aimed to isolate and identify lactic acid bacteria from the intestines of white snapper. This research used qualitative and quantitative methods. This study obtained five solates that showed the characters of lactic acid bacteria. The results of the study showed the characteristics of lactic acid bacteria identified as bacteria of the genus Lactobacillus which are Gram-positive bacteria that have the potential to be probiotics.

1. INTRODUCTION

Bangka Belitung is one of the provinces that is geographically dominated by fisheries potential. In addition many potential capture fisheries resources are suitable for aquaculture. One of the fish that can be used is white snapper (*Lates calcarifer*) which is a potential fishery and leading aquaculture commodity in Indonesia. White snapper has the characteristic of living in groups and has a high tolerance ability to salt content (*euryhaline*). The characteristics of the white snapper cause cultivation to be carried out in the sea or in ponds**[1].** This fish also has a high nutritional value and in the digestive tract of white snapper (*L. calcarifer*), there is a short-sized intestine contained in lactic acid bacteria.

Lactic acid bacteria are bacteria capable of fermenting sugars or carbohydrates to produce large amounts of lactic acid. The classification of lactic acid bacteria is divided into 10 genera, namely Aerococcus, Carnobacterium, Enterococcus, Lactobacillus,

Lactococcus, Leuconostoc Pediococcus, Streptococcus, Tetragenococcus, and Vagococcus. Lactic acid bacteria have common characteristics that are Gram-positive. These bacteria produce products in the form of lactic acid, and antimicrobial compounds [2]. Exploration of lactic acid bacteria can use fish as a source of probiotics potential lactic acid bacteria isolates. It is also closely related to probiotics as preparation of microbial cells and has the ability to produce antimicrobial metabolism that can inhibit pathogenic bacteria. Probiotics are defined as products composed of microbial cultures or microscopic natural feeds that are beneficial to the host[3]. Probiotics can produce antimicrobial metabolisms such as lactic acid, diacetyl, hydrogen peroxide, and bacteriocin compounds. Probiotics are also useful for improving the microbial balance in the digestive tract. The presence of probiotics in the gut of the host and in the lumen, acts as a barrier against the proliferation (growth) of pathogenic effects, including through the mechanism of producing compounds capable of inhibiting the growth of pathogens. Probiotics are intended to help increase the digestive activity in the digestive tract of fish. Therefore, research is needed on the isolation and identification of lactic acid bacteria originating from the digestive tract of white snapper (Lates calcarifer) to increase productivity in white snapper aquaculture in Bangka Belitung province.

2. MATERIALS AND METHODS

Location and time of the research

The research was conducted from September to November 2022. This research was conducted at the UBB Probio_FM Laboratory in Petaling village, Bangka regency, and the Biology Laboratory, Bangka Belitung University.

Instruments and Materials

The instruments used in this study are as follows aluminum foil, autoclave, stirring rod, spreader rod, bunsen, durant bottle, petri dish, funnel, erlenmeyer, beaker glass, magnetic stirrer, measuring cup, object glass, scissors, hot plate, incubator, inoculation loop, preparation glass, convex object glass, measuring flask, micropipette, microscope, analytical balance, tweezers, drip pipette, tube rack, durham tube, test tube, tip, refrigerator, tip box, microwave, laminar airflow and camera digital.

The materials used in this study are as follows agar, 70% alcohol, crystal violet, decolorizer, H2O2, iodine, cotton, sterile gauze, label paper, Sulfide indole motility (SIM), triple sugar iron agar (TSIA), Vogue proskauer (MR-VP), methyl red, MRSBroth, agar nutrients, nutrient broth, MRS agar, NaCl, CaCO3, blood agar, safranins, tissues, and white snapper.

Instruments and Materials Sterilization

All instruments are washed until clean and dried. Then, materials such as MRSB, MRSA, NaCL, and $CaCO_3$ are heated until dissolved. After that, the instruments and materials were sterilized using an autoclave at a temperature of 121°C and a pressure of 15 psi for 20 minutes.

Preparation Fish Intestine Collection

Fish samples were obtained from catches in the Pagarawan area, Bangka Regency. Then the intestine was taken and put in a plastic sample and the intestine was isolated from a white snapper (Lates calcarifer).

Isolation and Selection of Lactic Acid Bacteria from Intestine of White Snapper

The fish intestines were cut to a size of 1 cm and then the fish intestines were weighed. The intestine was put into a test tube containing 30 ml of MRS Broth selective media and then incubated using an incubator at 37°C for 48 hours. Then make 350 ml of NaCl, with the amount of each test tube containing 9 ml. Then make MRS Agar as much as 350 ml. After that, sterilize all instruments and materials using an autoclave. Then do the dilution and take 1 ml of incubation liquid and make sterile

dilution up to 10-9. The solution resulting from the 10-9 dilution was cultured on MRS Agar using the pour plate method with a continuous streak pattern and then incubated for 48 hours at a temperature of 37°C

Lactic Acid Bacteria Characterization

a. Morphological Observation of Lactic Acid Bacteria Colonies

Identification of bacterial isolates based on morphological characteristics including shape, edge, elevation, margin, color, optical character, and surface[4]. These observations were observed using a colony counter and a bacterial identification book.

b. Gram stain

The object glass was prepared by first rinsing it with 95% alcohol until clean. Drops of distilled water on the object glass. Prepare the bacterial culture, then heat the inoculating loop using a bunsen. After that, take the bacterial culture and apply it to the object glass. Then do the fixation. The bacterial smear is dripped with violet dye for 1 minute, then rinse with distilled water. Then drip iodine, wait for 1 minute, then rinse with distilled water. Then drop the decolorizer, wait for 30 seconds, then rinse with distilled water. Then drop safranin, wait for 30 seconds, then rinse with distilled water. After that, observed under a microscope using an objective lens 40 X. Staining results (purple: Gram-positive, red: Gram-negative).

c. Biochemical Test

The method used in testing biochemical activity is based on the method used by Edy Santoso (2008) such as the catalase test, motility test, triple sugar iron agar (TSIA) test, and methyl red (MR) test [5]

1. Catalase Test

The pure isolate was taken as much as one of ose inoculation loops then placed on top of the object glass then dripped with H_2O_2 3%, then observed for the activity of the gas bubbles produced. Catalase test results (there are air bubbles: positive, no bubbles: negative).

2. Motility Test

The pure isolate was taken as much as one inoculating loops and then put into a test tube containing SIM media, then incubated at a temperature of 37°C for 1x24 hours to determine the motile or non-motile nature of the microbes.

3. Triple Sugar Iron Agar (TSIA) Test

One inoculating loop of the pure isolate was streaked on the surface of the agar upright and the agar slanted, then incubated at temperature 37°C for 3x24 hours.

4. Methyl Red (MR) Test

The pure isolate was taken as much as one inoculating loop, put into a test tube containing MRVP, and homogenized, then incubated for 3x24 hours at a temperature of 37°C. Then drop 3-4 drops of methyl red, then observe the color change.

Probiotic Potency Test

a. Temperature Resistance Test

The growth ability of bacterial isolates with various temperature treatments was tested by growing bacterial isolates on MRSB media and incubated at 3 temperature treatments such as room temperature (27°C), incubator temperature (37°C), and freeze temperature (4°C) for 48 hours. Then the growth of bacteria was observed in each tube.

b. Antimicrobial Test

Nutrient agar (NA) was put into a petridish as much as 15 mL, then waited until it was solid. After that, scratch the to stand until it solidified. Then scratch the Staphylococcus aureus bacteria on the nutrient agar (NA) medium. Place the disc paper that has been soaked with lactic acid bacteria on the medium that has been scratched with bacteria. Incubation for 48 hours at 30 °C.

c.Haemolysis test

Measure the weight of the media for blood agar as much as 20 Gram. Then, put it into the Erlenmeyer. Then, add distilled water to a volume of 500 mL then stir until homogeneous. After that, heat the media, then do the sterilization using an autoclave. The sterilized solution will be added as much as 25% blood from 500 mL of media and then homogenized. Then, put the media into the petri dish. Then, place 1 ose of lactic acid bacteria on the blood agar medium and then incubate for 24 hours. After that, observe the zone of hemolysis.

Production of Lactic Acid

The tests carried out were qualitative, lactic acid bacteria were grown on an MRSA medium with CaCO3, then incubated for 48 hours. Observe the growth of bacteria which is indicated by the presence of a clear zone around the lactic acid bacteria colonies.

Molecular Identification with the 16S rRNA Gene

In this study, the process of sequencing the 16S rRNA gene was carried out by sending bacterial isolates to the Department of Biology FMIPA UNIB Bengkulu City for PCR processing. After that, the bacterial samples were sent to Singapore for sequencing.

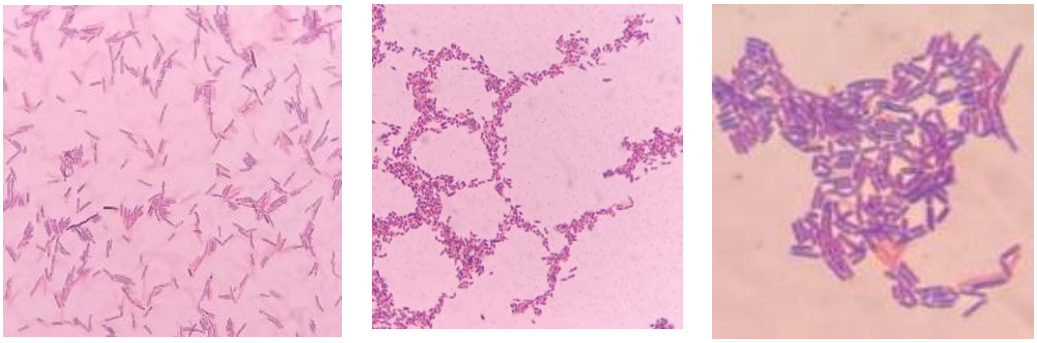
3. RESULTS and DISCUSSION

The results of the research that has been done are 5 bacterial isolates namely SDF1, SDF2, SDF3, SDF4, and SDF5. The characteristics of the three bacterial isolates are shown in table 1.

Table 1. Results Characteristics of Lactic Acid Bacteria

Characteristic	SDF1	SDF2	SDF3	SDF4	SDF5
Colony size	Punctiform	Punctiform	Moderate	Punctiform	Moderate
Colony shape	Circular	Circular	Circular	Circular	Circular
Elevation	Corvex	Corvex	Corvex	Corvex	Corvex
Margin	Entire	Entire	Entire	Entire	Entire
Texture	Shiny	Shiny	Shiny	Shiny	Shiny
Pigmentation	Cream	Cream	Cream	Cream	Cream
Optical property	Opaque	Opaque	Opaque	Opaque	Opaque
LAB Population (CFU/g)	$263x10^3$	$137x10^5$	$210x10^5$	$215x10^3$	$78x10^5$
Gram characteristic	+	+	+	+	+
Cell shape	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Biochemical Test					
Catalase	-	-	-	-	-
Motility (SIM)	-	-	-	-	-
MRVP	+	+	+	+	+
TSIA Test					
Slant/Butt	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y
Gas	-	-	-	-	-
H_2S	-	-	-	-	-
Temperature resistance test					
4° C	-	-	-	-	-
27° C	+	+	+	+	+
37° C	++	++	++	++	++

Information: + : positive, - : negative, Y/Y : Yellow/Yellow
- :Low, +: More, ++:Most



A B C

Figure 2. Gram staining 40x magnification: (SDF1) A; (SDF2) B; (SDF3) C

Characterization of Lactic Acid Bacteria Isolates

The characterization of lactic acid bacteria isolates is done by observing macroscopically and microscopically. Macroscopic observations refer to morphology such as shape, edge, elevation, and colony color. Colonies obtained were round in shape with different sizes and elevations, creamy white and produces a clear zone around it while microscopic observations are made of the structure and shape of the cells, one of which is by Gram staining. Based on the results of Gram staining, showed that the three samples were in the form of bacilli and were Gram-positive bacteria (Figure 1).

Gram-staining cells that cannot release color and will remain colored like crystal violet, namely blue-purple, are called Gram-positive bacteria [7]. Meanwhile, cells that can release crystal violet and bind safranin so that they are red are called Gram-negative bacteria. This is related to the composition of the compounds that make up the cell wall namely Gram-positive bacteria contain more peptidoglycan and less fat than Gram-negative bacteria [8]

Biochemical Test

The results of the catalase test are to identify microbes that are capable of producing the catalase enzyme which is used to break down hydrogen peroxide which is formed from the aerobic respiration process for bacteria. The probiotic bacterial isolates obtained showed negative results for catalase because they did not produce gas bubbles around the bacterial colonies when dripped with H_2O_2 3% solution. Catalase reaction shows a negative result if there are no gas bubbles**[9].** These results are in accordance with studies that have been carried out that obtained negative results on the catalase test of lactic acid bacteria**[10].**

The results of the motility test for all probiotic bacterial isolates were not motile, this can be seen from the absence of movement of probiotic bacteria, the spread of bacterial growth in SIM media only grows on the part of the media that has been given bacteria only. Probiotic bacteria have very limited biosynthetic abilities, so they are non-motile**[11].** Negative results in the motility test indicate that the test bacteria do not have flagella as a means of movement.

Bacteria growing in a test tube containing MRVP media are then added 3-4 drops of methyl red indicator to the tube which shows a positive result when the MRVP medium turns red. The red color formed in the media is due to a decrease in the large amount of acid produced from glucose fermentation**[12].** The methyl red test is used to determine the presence of mixed acid products from glucose fermentation through mixed acid fermentation pathways which are generally lactic acid, acetic acid, formic acid, and succinic acid.

TSIA test results for slant and yellow butt (acid) with or without gas fermentation of glucose, lactose, and sucrose has occurred because lactose and sucrose have higher concentrations so that they can be used for

further fermentation substrates (if glucose runs out) and produces acid marked yellow after 2x24 hours. Lactic acid bacteria isolates of the genus *Lactobacillus* showed an acidic reaction in the TSIA test which was seen from the change in the color of the media to yellow in the slant and butt parts**[13].**

Temperature resistance test

Environmental factors can affect the growth of lactic acid bacteria, namely temperature **[14].** This temperature is controlled to create optimal conditions to encourage the growth of microorganisms in the lactic acid fermentation process. The growth of lactic acid bacteria isolates was tested and the results were obtained as shown in table 6. Based on the results of the temperature resistance test in table 6, showed that at refrigerator temperature the growth rate of bacteria was low (-), medium room temperature (+), and fast at incubator temperature (++).

The optimum temperature for the growth of lactic acid bacteria is grouped into two groups, namely mesophilic (the optimum temperature for growth is 25°C and the maximum temperature is 37°C - 40°C). Then, the thermophilic temperature (the optimum growth temperature is 37°C - 45°C and the maximum temperature is 45°C - 52°C). This research obtained all isolates of probiotic bacteria including mesophilic bacteria because they are able to grow at temperatures of 25°C to 40°C. Therefore, it can be said that at incubator temperature bacteria grow faster than at refrigerator and room temperatures due to refrigerator temperature (-60C) storage for 2x24 hours can inhibit bacterial growth while at an incubator temperature of 37 °C this temperature is the optimal temperature for bacterial growth

Table 2. Antimicrobial Test Results

No.	Isolate	Inhibition zone (inch/mm)
		Staphylococcus aureus
1.	SDF1	1.90
2.	SDF2	3.64
3.	SDF3	3.45
4.	SDF4	7.17
5.	SDF5	6.58

Antimicrobial Test Results

Antibacterial test of 3 lactic acid bacteria isolates (S1, S2, and S3) against the pathogenic bacteria Staphylococcus *aureus* (Gram-positive) that causes disorders of the digestive tract, showed that all lactic acid bacteria isolates were able to inhibit pathogenic bacteria. The inhibition zones formed varied in size between 1.90-7.17 mm. The inhibition zone of the probiotic isolates showed antibacterial activity against *Staphylococcus aureus*. Based on the average antimicrobial test results, the antimicrobial activity of lactic acid bacteria was in the moderate category. The diameter of the inhibition zone against pathogenic bacteria was categorized into 3, namely, an inhibition zone of 0-3 mm indicating low antimicrobial activity, 3-6 mm moderate antimicrobial activity, and an inhibition zone diameter of >6 indicated high antimicrobial activity**[15].**

Based on the table 2 of antimicrobial test results, it can be seen that among the isolates, isolate SDF1 has the lowest inhibition zone size of 1.90 inches/mm which indicates low antimicrobial activity while isolate SDF4 has a zone size of 7.17 inches/mm which explains the highest antimicrobial activity. The inhibitory effect caused by lactic acid isolates can be caused by the presence of acids or substances such as bacteriocins which are able to inhibit the growth of pathogenic bacteria [16].

Lactic Acid Production

The parameters of lactic acid production, by using qualitative tests, which was indicated by the presence of a clear zone around the lactic acid bacteria colonies grown on MRSA media supplemented with 1% CaCO3 (Figure 2). The test result explained that all isolates

formed a clear zone, so that it could presumed that these isolates were lactid acid bacteria. Further testing using a quantitative approach are needed to carried out to determine the potential of lactic acid bacteria isolate as probotic agent

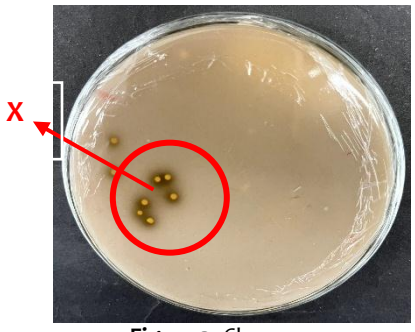


Figure 2. Clear zone

Molecular dentification with the 16S Rrna gene

The 16S RNA gene of all selected isolates has been successfully isolated, as seen by the formation of the corresponding band on the electrophoresis results. Amplification of lactic acid bacteria isolates genom using primers 63f and 1387r resulted DNA fragments sized approximately 1300 bp (Figure 3).

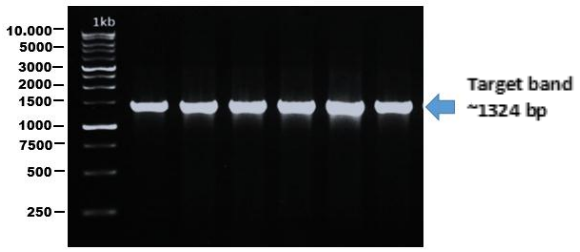


Figure 3. PCR amplification of 16 S rRNA gene from of lactic acid bacteria isolated from white snapper; M = marker 1 Kb ladder (Fermentation); lane 1-6 = PCR product of LAB.

4. CONCLUSION

Based on the research that has been done, it can be concluded that the analysis regarding the identification of lactic acid bacteria from the intestines of White Snapper (*Lates calcarifer*) can be classified that the isolated lactic acid bacteria is Lactobacillus. From the results of microscopic and macroscopic observations, as well as the tests carried out, it can be seen that lactic acid bacteria isolate from the intestine of White Snapper has the potential as a probiotic.

5. ACKNOWLEDGMENT

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