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Screening of amilolytic bacteria in the digestive tract of Red Snapper (*Lutjanus campechanus*) as probiotic candidates

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ABSTRACT

Fish utilize energy from carbohydrate sources in small amounts because of the low secretion of the enzyme amylase for digestion. One alternative is to increase the availability of exogenous digestive enzymes. This study aims to isolate amylolytic bacteria in the digestive tract of red snapper as a probiotic candidate. This study used several methods, namely amylolytic test on starch, acid resistance test (pH 3), observation of bacterial growth for 30 hours, antagonistic test against pathogenic bacteria, stomach attachment test, Gram stain test and pathogenicity test on shrimp. Screening of amylolytic bacteria in the digestive tract of red snapper produced five isolates, namely A1, A2, A3, A4 and A5. From the results of the amylolytic test, only three isolates were further tested, namely A1, A2 and A4. The three isolates were able to survive under acidic conditions of pH 3 and pH 7 for eight hours with density values (OD) in isolates A1 (0.630), A2 (0.597) and A4 (0.601). Isolates A1, A2 and A4 respectively produced antagonistic values with inhibition zones of 8.4 mm, 7.5 mm and 10.3 mm. Next. the ability to attach bacteria to isolates A1 (6.6 x 102 cfu/ml), A2 (11.8 x 102 cfu/ml) and A4 (16.5 x 102 cfu/ml). After that, the bacterial isolates were tested for pathogenicity. Based on the results of the study, there were three bacterial isolates (A1, A2 and A4) in the digestive tract of red snapper that had met the requirements for probiotic candidates in fish/shrimp and the best was A4 isolate.

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1. Introduction

The digestive system in fish is simpler than land animals, so the digestive mechanism or digestibility of feed is limited. According to Castro-Ruiz et al., (2019) that the capacity of digestive enzymes is constantly changing depending on the type of nutrients that can be digested and

absorbed along with its development. Given the limitations on the availability of digestive enzymes, fish utilize energy sources of carbohydrates in small quantities due to the low secretion of amylase enzymes for digestion and insulin for metabolism. According to Kamalam & Panserat (2016), certain differences in digestive mechanisms make fish less able to use digestible carbohydrates to meet energy needs. One alternative solution to this problem is to increase the availability of exogenous digestive enzymes by utilizing bacteria from the digestive tract that have amylolytic activity (digest carbohydrates). Adequate amounts of amylolytic bacteria can provide beneficial benefits for the host by helping the digestive process so that the health of the host is maintained (Valerio et al., 2020).

Amylolytic bacteria are a type of bacteria that can produce amylase enzymes which have the ability to hydrolyze starch or starch into simpler compounds. According to Novitasari (2014) α-amylase hydrolyzes α-1,4-glucoside bonds specifically to produce dextrins. The next process, namely saccharification, requires glucoamylase to break down starch which produces glucose. This process can take place if there is direct contact between the bacterial cell and the substrate. The well-known genera of amylolytic bacteria are Bacillus, Bacteriods, Lactobacillus, Clostridium, Micrococcus, Thermus and Actinomycetes (Myburgh et al., 2019)

Amylolytic bacteria can be taken from the digestive tract of fish, one of which is red snapper which is a carnivorous fish. Based on research by Marlida & Elrifadah (2017) that probiotic candidates isolated from the digestive tract of swamp fish have enzymatic activity such as C3GHDP from cork haruan (carnivores) which has the ability to degrade starch and can be used to substitute fish meal with other starches that contain carbohydrates. in feed ingredients. Screening for amylolytic bacteria in the digestive tract of red snapper has never been done. Based on this, it is necessary to conduct a screening study of amylolytic bacteria from the digestive tract of red snapper which have the ability to digest food as probiotic candidates, have a high amylolytic index value, are resistant to acidic conditions and are not pathogenic. So that the probiotic candidate can meet the criteria to be used as a probiotic.

2. Material and methods

This research was carried out in March-May 2021 at the Central Laboratory of the Halal Center at the Islamic University of Malang. The materials used in this study were red snapper fish measuring 15-20 cm taken from Situbondo marine waters, Nutrient Agar (NA) media, Agar, Starch, Tryptic Soy Broth (TSA), Tryptic Soy Agar (TSA), *Vibrio harveyi* bacteria, NaCl, Aquades, Alcohol, Steel Plate, Crystal Violet and Safranin. The tools used are Laminar Air Flow (LAF), Petri Dishes, Test Tubes, Beaker Glass, Erlenmeyer, Hirayama HVE-50 Autoclave, Digital Incubator 30 Liter DSI 300D, Spektophotometer Shimadzu UV-Vis 1900, Orbital Shaker SK-L330-Pro, Vortex BIO-RAD Br-2000, Olympus Microscope and Aquarium for pathogenicity test.

The research stage begins with the sterilization of tools and materials, then proceed with the manufacture of NA media for initial isolation. Isolation was carried out with inoculum sources from the digestive tract of red snapper, namely the intestine and stomach. The isolates obtained were 5 isolates and then incubated at 37 °C for 24 hours followed by morphological and colony observations including color, shape, elevation and optical characteristics (Sousa et al., 2015). Then testing the amylolytic activity with starch agar media after 24 hours of incubation is dripped with iodine solution to observe the clear zone (Saleh et al., 2020). There are 3 isolates that have the highest amylolytic index value and will be further tested. The next test was a resistance test in acidic conditions, namely at pH 3 and pH 7 and then measurements were carried out using a spectrophotometer (Ayyas., et al, 2021). The bacterial growth test was carried out using a spectrophotometer every 2 hours for 14 hours which was modified from research by Meyers et al., (2018). Antagonistic activity test against bacteria using Vibrio harveyi bacteria (Moussaid et al, 2019). Test attachment to the hull using a steel plate substrate with measurements using a spectrophotometer (Janković, 2012). The gram staining test refers to the research of Ako et al., (2020).

In the modified bacterial pathogenicity test from Muliani's study, et al (2017), it was carried out with each isolate with a density of 10^{10} cfu/ml as much as 10 mL poured on 6-7 gram shrimp culture media in an aquarium measuring 60 cm x 30 cm x 30 cm filled with water as high as 20 cm. One isolate per aquarium and a control aquarium is provided. Cultivation was carried out for 12 days by observing clinical symptoms in each aquarium.

3. Results and Discussion

3.1 Isolation and Purification

After isolating the bacteria from the digestive tract of red snapper, morphological observations were made starting from color, colony shape, elevation, edges and optical characteristics. Observation of colony morphology can be seen in Table 1 as follows;

Table 1. Morphology of Bacterial Colonies Isolated from Red Snapper

Isolate	Color	Shape	Elevation	Edge	Optical Characteristic
A 1	Beige	Circle	Flat	Lobate	Not Translucent
A 2	White	Circle	Convex	Undulate	Not Translucent
A 3	Beige	Irreguler	Raised	Entire	Not Translucent
A 4	Beige	Fillament	Convex	Fillamen	Translucent
A 5	White	Ireeguler	Flat	Lobate	Translucent

3.2 Amylolytics Test

Testing for amylolytic activity was carried out by applying each bacterial isolate onto selective starch agar media and incubating it for 24 hours. Amylolytic activity is indicated by the formation of clear zones on the agar medium. The results of the amylolytic activity after treatment can be seen in Figure 1 and the amylolytic index values in Figure 2. Based on the results it is known that the amylolytic index values in isolates A1 (2.14 ± 0.11), A2 (3.60 ± 0.13) , A3 (1.16 ± 0.12), A4 (2.36 ± 0.17) and A5 (1.66 ± 0.20). Isolates with the highest amylolytic index at A1, A2 and A4 will be further tested.

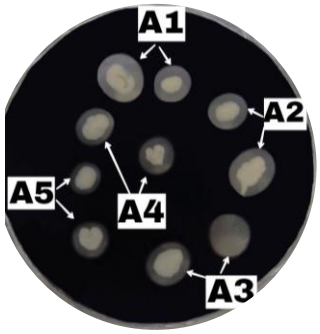


Figure 1. Amiylolytic activity

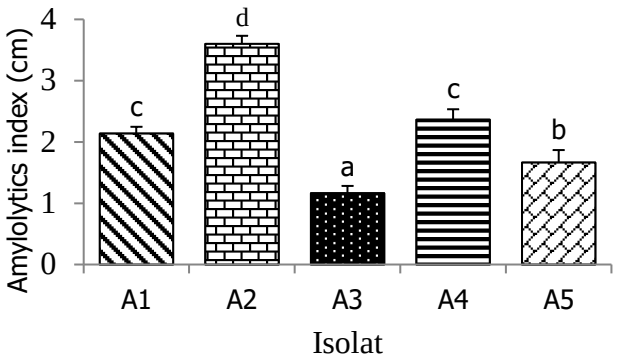


Figure 2. Amiylolytic index

3.3 Resistance Test in Acid Conditions

This test was carried out by inoculating isolates on TSB media with pH 3 and 7. Then measurements were made using a spectrophotometer with an OD value of 620 for 8 hours. Following are the results of the test for measuring the density of bacteria based on the OD values at pH 3 and pH 7 in Figure 3. In isolate A1 after being tested at pH 3 the density of bacteria increased from 0.347 to 0.630 and at pH 7 from 0.293 to 1.299. The pH 3 test on isolate A2 was seen at the 6th hour, which was 0.580, almost the same as the 8th hour, which was 0.597, but the bacterial density continued to increase. Whereas at pH 7 from 0.115 to 1.249. In isolate A4, pH 3 was from 0.336 to 0.601 and at pH 7 from 0.514 to 1.862. Based on these results it can be concluded that the bacterial isolates can survive in acid, namely pH 3 and the density of the bacteria for 8 hours increases.

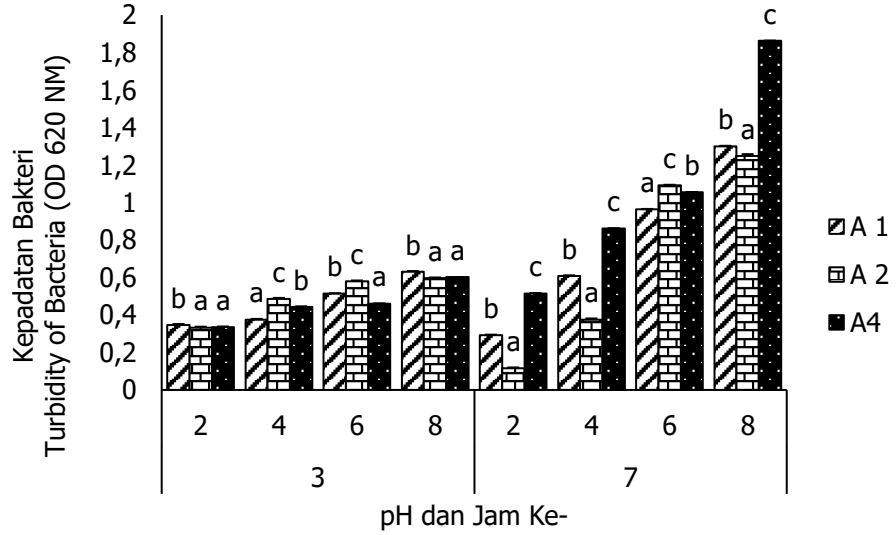


Figure 3. Test results for measuring bacterial density based on OD values at pH 3 and pH 7

3.4 Bacterial growth

In the growth test, 2 methods were carried out, namely measuring the density of bacteria using a spectrophotometer for 30 hours, i.e. every 2 hours, observations were carried out and the TPC (Total Plate Count) method was carried out, namely from the results of the spectrophotometer at crucial hours. The graph of growth in isolates A1, A2 and A4 can be seen in Figure 4. The lag phase occurs at the 2nd hour. The exponential phase of isolates A1 and A2 occurred from 4 to 6 hours, whereas in isolate A4 it occurred from 4 to 8 hours. The stationary phase of isolates A1 and A2 occurred at the 8th hour, whereas for isolate A4 it occurred at the 10th hour. The death phase in isolates A1 and A2 started from the 10th hour, while in isolate A4 the death phase started from the 12th hour.

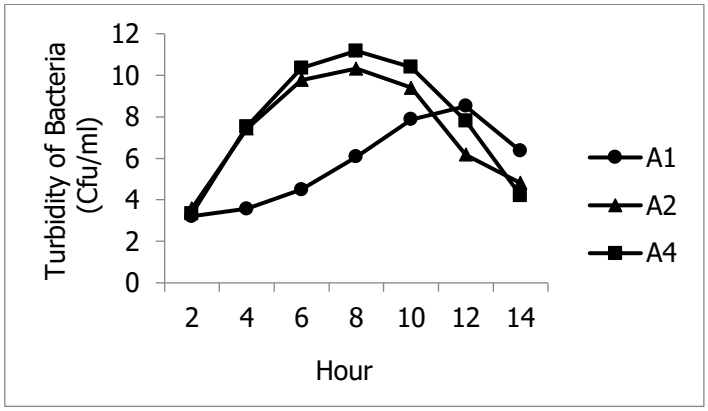


Figure 4. Growth Chart of Probiotic Candidate Bacterial Isolate from Snapper Digestive Tract

3.5 Antagonistic Test Against Pathogenic Bacteria

The pathogenic bacteria used for this test are Vibrio Harveyi bacteria. This bacterium was chosen because it often attacks farmed shrimp and can cause mass death. This test aims to determine the ability of bacterial isolates to inhibit pathogenic bacteria by observing the clear zone. The diameter of the clear zone of each isolate can be seen in Table 2, while the clear zone formed can be seen in Figure 5. Based on the results, isolate A1 can inhibit pathogenic bacteria by up to 8.4 mm, isolate A2 is 7.5 mm and isolate A4 is 10.3mm. Each bacterial isolate was able to fight pathogenic bacteria (Vibrio Harveyi) with the presence of a clear zone formed.

Table 2. Diameter of Inhibition Zone in Antagonistic Test

Isolate	Diameter of inhibition zone (mm)
A1	8,4
A2	7,5
A4	10,3

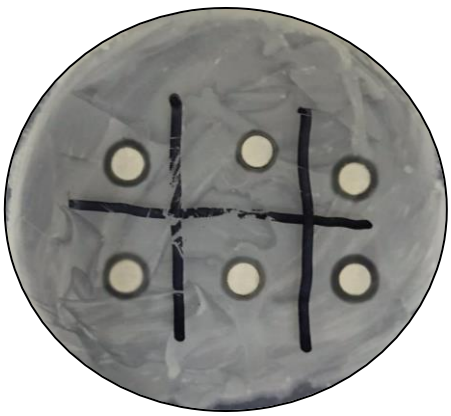


Figure 4. Inhibition zone

3.6 Bacterial Attachment Test

The attachment test aims to find out whether the isolated bacteria can stick to the intestines or stomach of the fish or even be wasted along with the leftovers. The bacterial attachment test used a steel plate substrate which was soaked by the bacterial isolates. Then the steel plate is wiped with oil paper and then tested using a spectrophotometer. The results of measurements with an OD value of 620 nm can be seen in Table 3. Observations using a spectrophotometer isolate A1 yielded a value of 0.034 with an attachment capacity of 6.6 x 102 cfu/ml, in isolate A2 it was 0.062 with an attachment capability of 11.8 x 102 as well as in isolates A4 is 0.088 with the ability to attach 16.5

x 102. Isolates A1, A2 and A4 are able to attach to the substrate with the results of measurements with a spectrophotometer and the ability to attach.

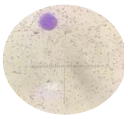
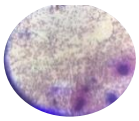
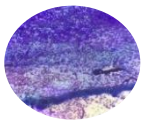
Table 3. *Bacterial Attachment Test*

Isolate	OD 620 nm	Attachment Ability (cfu/ml)
A1	0,034	$6,6 \times 10^2$
A2	0,062	$11,8 \times 10^2$
A4	0,088	$16,5 \times 10^2$

3.7 Gram Stain Test

Based on the results, isolates A1, A2 and A4 are classified as gram-positive bacteria that have thick peptidoglycan so that they can retain the crystal violet color. The results of the gram staining test can be seen in Table 4 as follows;

Table 4. Gram Staining Test of Probiotic Candidate Bacterial Isolates

Isolat	A1	A2	A4
Gram	+	+	+
Gambar			

3.8 Pathogenicity test

Pathogenicity test aims to determine whether the bacterial isolates have a good or bad impact on Vanamei shrimp cultivation. The results of the pathogenicity test can be seen in Table 5. The movement of the shrimp in the treatment of isolates A1 was good and in isolates A2 and A4 it was very good, the appetite of shrimp was very good, there were no body injuries, there were no attacks, there was no disease and in the treatment of isolates A1 , A2 and A4 SR reach 100%.

Table 5. Pathogenicity test

Parameter	Isolate		
	A1	A2	A4
Movement	++	+++	+++
Appetite	+++	+++	+++
Wounds	-	-	-
Disease	-	-	-
Survival rate	100 %	100 %	100 %

Note:
+ + good
+ + + very good

Isolation of bacteria from the digestive tract of red snapper on NA (Nutrient Agar) media which was incubated for 24 hours at 37°C. Then observing the characteristics and morphology of the bacterial colonies including color, colony shape, elevation and optical characteristics can be seen in Table 1. The dominant morphological characteristics of the isolated bacterial colonies are cream color, round and irregular shape, lobate elevation and opaque optical characteristics. According to Bella, et al., (2020), amylolytic bacteria isolated from fish intestines have the characteristics of

colonies that are milky white, round in shape with smooth edges, usually in the form of long, almost round rods, short chain forms, non-motile, and colonies irregular.

Bacteria that have been successfully isolated can hydrolyze starch on starch selective media which is indicated by the presence of a clear zone on the starch agar media, meaning that the bacteria are amylolytic. According to Ardiansyah, et al., (2021), isolates that have amylolytic activity can produce clear zones due to the bond between the starch group and the iodine/lugol compound. Although all bacteria are capable of synthesizing intracellular enzymes, only certain bacteria are capable of producing extracellular enzymes. The ability of all tested isolates to produce extracellular amylase enzymes whose role is to hydrolyze starch into glucose, maltose and dextrin. Based on the amylolytic hydrolysis index value, the highest in isolate A2 was 3.60 ± 0.13 and the lowest in isolate A3 was 1.16 ± 0.12. In the study of Wulandari et al., (2017) the amylolytic index values reached 2.89 ± 0.30 and 2.27 ± 0.19. According to Ginting et al., (2020), differences in amylolytic activity are influenced by several factors including nutrient content, degree of acidity of the media, osmotic pressure, level of aeration and temperature.

Based on the results of the bacterial density based on the OD value at pH 3 it was smaller than at pH 7, but the bacterial isolates could still survive in acidic conditions or pH 3. It can be seen that the bacterial isolate test tube at pH 7 was more turbid than the bacterial isolate test tube at pH 3. Probiotic bacteria are able to survive at low pH but there will be cell damage and decreased viability in bacteria exposed to low pH resulting in a decrease in colonies. According to Uni et al., (2014), in acidic conditions for a long time will cause the death of bacteria. In order for bacteria to be resistant to acidic conditions, isolates must be able to maintain an environment where the internal pH is always higher than the external pH. In the acid resistance test, the isolated bacteria were able to survive in acidic conditions for 8 hours. According to Agestiawan, et al., (2015), the time it takes for food to pass through the stomach is 3 hours.

After observing the growth of bacteria for 30 hours, it was found that after the decrease that occurred at the 14th hour, the growth phase fluctuated. Seen in Figure 4. isolates A1, A2 and A4 experienced a lag phase in the first 2 hours, there were few bacteria that grew. The lag phase does not take a long time because the isolated bacteria are able to adapt in a short time. Then isolates A1 and A2 underwent an exponential phase at 4 to 6 hours, while isolate A4 underwent an exponential phase at 4 to 8 hours. Shown by the increase in the volume of bacterial cells and the number of bacteria that grow on solid media and on liquid media which looks increasingly cloudy. According to Sudin et al., (2020), the exponential phase is the growth of cell mass and volume which increases because the nutrients in the media are sufficient so that the bacteria can grow optimally. The stationary phase in isolates A1 and A2 was at the 8th hour while in isolate A4 it experienced a stationary phase at the 10th hour. The stationary phase or the peak phase and begins to decline, this is caused by cell metabolic activity and media nutrients that are running out and competition for nutrients occurs, so some cells will die and others will continue to grow (Wahyuningsih & Zulaika, 2019). After the stationary phase, there is a declination or decline phase, namely at 14 hours until the death phase. You can see a little live bacteria and the liquid media looks increasingly clear. The decline phase until death occurs due to the depletion of nutrients, then the dead cells will be more than the living cells.

Based on the research results, isolates A1, A2 and A4 were able to inhibit pathogenic bacteria. However, the isolate that had the highest inhibition zone value was isolate A4 with 10.3 mm, which had moderate antagonistic ability. According to Sarfina, et al (2017), the classification of the response diameter of the inhibition zone if ≤10 mm is classified as weak, 10-15 is classified as moderate, 15-20 is classified as strong and if ≥20 is classified as very strong. The existence of metabolic activity carried out by amylolytic bacteria as a form of defense against Vibrio Harveyi bacteria. In addition to the production of secondary metabolites, the size of the inhibition zone by amylolytic bacteria is also influenced by physical and chemical factors, as well as the length of incubation (Batubara, et al., 2017).

One of the requirements as a probiotic candidate is having the ability to stick to the substrate. Based on the test results, all isolates were able to stick to the substrate, although their attachment abilities were different. Based on Table 3, the isolate that has the ability to attach to the substrate is isolate A4 with an OD value of 0.088 and a bacterial density of 16.5 x 102 cfu/ml. According to Priadi, et al., (2020), apart from having high antibacterial activity against pathogenic bacteria, isolated bacteria must have the ability to colonize the intestinal surface. Bacteria that are unable to colonize are released by intestinal contractions.

Based on the test results, isolates A1, A2 and A4 are gram positive which are marked with a purple color because they can form complex bonds with the main dye (crystal violet), namely purple. According to Hamidah et al., (2019), gram-positive bacteria contain a cell wall consisting of two layers, namely a thick peptidoglycan and an inner membrane. This peptidoglycan layer can bind crystal violet dye.

Pathogenicity testing aims to determine whether a given bacterial isolate is pathogenic or not. It can be seen in Table 5 that the bacterial isolates are not pathogenic and there are no significantly bad clinical signs, although the movement of the shrimp in isolate A1 is in the good category because the movement of the shrimp in the aquarium isolate A1 is slightly less active than the aquarium isolates A2 and A4. According to Novitarizky et al., (2018), probiotic bacteria improve fish health by controlling pathogens and improving water quality by regulating and modifying the composition of the microbial community.

4. Conclusion

Isolation of bacteria from the digestive tract of red snapper can produce amylase enzymes, namely isolates A1, A2, A3, A4 and A5. Based on the test results that have been carried out, three isolates (A1, A2 and A4) meet the requirements as probiotic candidates and the best isolate A4.

5. Acknowledgment

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