Heterotrophic Bacteria from the Digestive Tract of Rice Field Eel (\textit{Monopterus albus}) and Its Potency as Probiotic

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Abstract. Normal microbiota in the gastrointestinal tract has a mutual relationship with its host. This research aimed to know the morphological diversity of heterotrophic bacteria that live in the gastrointestinal tract of rice field eel and its potency as probiotic. Bacterial isolation has been done by cultivated of the sample in the nutrient agar medium and then observed colonies and cell morphology. Candidate probiotics of isolates were selected based on the capability of starch, protein and lipid hydrolysis, bacterial resistance to acidity and bacterial resistance to antibiotics. The result of this research showed there are 10 isolates and have various colonies' morphological appearance. All of those isolates consist of 40% cocci, 30% small rod and each 10% long rod, streptococci, and spiral. As many as 8 isolates were gram-positive and 2 isolates was gram-negative. The motility test showed 70% of isolates were motile and 30% non-motile. The results of casein hydrolysis, starch, and fate indicate that 70% of isolates were able to obtain Hydrolyze casein, that 60% of isolates were able to hydrolyze starch and that none of the isolates were able to obtain hydrolyzed fats. All of the probiotic candidates who were tested in isolation are resistant to all antibiotics tested. Thus, it said that not all discoveries meet the criteria as potential candidates.

Keywords: bacteria, diversity, gastrointestinal tract, isolation, rice field eel

1. Introduction

Eel is a freshwater fish that resembles a snake where its body is longer than its tail [1]. There are 2 types of eels that are commonly known in Indonesia, namely rice eel and swamp eel. The body shape of the swamp eel is slimmer with blackish-brown back skin compared to the rice eel [2]. Eels belong to a group of carnivorous animals whose digestion uses hormonal and enzymatic systems, so eels easily digest meat [3]. In the eel digestive tract, microorganisms that are eaten by eels will form colonies called microflora. Microflora is a microorganism consisting of a large number of microbes that generally occupy the digestive tract of living things [4].

In general, fish cannot produce cellulase. Cellulase enzymes commonly produced by microflora that live symbiosis in the digestive tract, as found in carp fish, \textit{Carassius auratus} [5]. In the digestive tract, the microflora that multiplies is not only a cellulase enzyme secretor but also can produce various types of enzymes from groups of protease, lipase and amylase enzymes [6]. Various enzymes produced will then play a role in extracellular digestion in the lumen of the digestive tract [7].

The types of microflora in the digestive tract are very diverse, for example, the microflora of the bacterial group can consist of \textit{lactobacillus sp}, \textit{Vibrio sp}, \textit{Pseudomonas sp}, \textit{Aeromonas sp}, \textit{Bacillus sp}, \textit{Flavobacterium sp}, dan \textit{Citrobacter sp}. [8]. Microflora plays a role in the digestive process (producing various types of enzymes), also acts as an inhibitor of the growth of pathogenic microbes both living
in the digestive tract and the living media of the aquatic biota. The most influential factor in the growth of microorganisms is temperature. Digestion of fish has digestive efficiency 5 to 10 times higher at 28°C compared to 5°C. Thus, in some microbial isolates, the digestive tract of fish used at 28°C [9]. In this study isolation, morphology characterization and bacterial partial selection of digestive tract of rice field eel as a probiotic candidate were carried out.

2. Material and methods

2.1 Sampling
A total of 20 healthy samples of rice field eels with a minimum size of 30 cm were obtained from the catch in the rice fields around the Lingsar area of West Lombok. The samples obtained are stored in sterile plastic. Then all samples are put in the icebox and immediately taken to the laboratory to be cultured.

2.2 Enumeration of total bacteria digestive tract and bacterial isolation
Before surgery, all eels are washed and disinfected with 95% ethanol. The contents of the abdominal cavity (viscera) are surgically dissected on ice, then homogenized in 10 ml of 100 mM potassium phosphate buffer pH 6.9. Then as much as 1 gram of sample added to 10 ml of sterile physiological saline solution. Then serial dilutions were made up to $10^{-7}$, and 100 µl were spread on agar-agar medium and PCA medium, then incubated at 25°C for 24 hours. Then the cup with the amount of 30-300 colonies calculated using a colony counter([10] with modified).

2.3 Morphological characterization
Colony morphological observations (referred to Cappucino & Sherman [11]) include the shape, edge, elevation, and color of colonies that grew apart on the NA media. Observation of cell morphology includes the shape and arrangement of cells, the nature of Gram and motility tests under a binocular microscope at 10 and 40x magnification.

2.4 Casein hydrolysis test
The agar culture media containing casein was added to the petri dish. Inoculation of isolates to be tested into the agar medium by placing one dose eye culture in the middle of the cup, then spreading an area of 0.5 cm. Then incubated at 28°C for 24-48 hours. If the casein hydrolysis process occurs, the clear area around the colony is seen, on the other hand, if there is no hydrolysis around the microbial colonies it remains cloudy.

2.5 Starch hydrolysis test
The agar culture media containing starch was put into a petri dish. Inoculation of isolates to be tested into the agar medium by placing one dose eye culture in the middle of the cup, then spreading an area of 0.5 cm. Then incubated at 28°C for 24-48 hours. Starch hydrolysis was measured by giving a few drops of Lugol's iodine solution on the surface of the agar medium. Lugols iodine solution used sufficiently to cover the surface of the agar media. If the starch hydrolysis process occurs, clear areas around the microbial colonies are seen.

2.6 Lipid hydrolysis test
Nutrient medium to be thawed and cooled until the temperature is 50°C. Added 0.3-0.4 ml of aseptic sterile plant oil into the tube then stirred until the oil mixed homogeneously. Then the mixture is poured aseptically into the petri dish and allowed to solidify. Then pure culture is inoculated by scratch and incubated in reverse at 28°C for 3-7 days. After the isolates were incubated then the CuSO4 solution was poured into a petri dish and left for 10-15 minutes. The CuSO4 solution which was still present in the petri dish was discarded and observed a shiny greenish color change [12].

2.7 Antibiotic resistance test
Isolates were tested for resistance to 3 types of antibiotics representing the category of antimicrobial agents commonly used in aquaculture. The antibiotics used in this study included Amoxicillin, Chloramphenicol, and Erythromycin in disc form. Antibiotic paper disks are placed in the middle of the media so that the solid contains bacteria in the petri dish container. Antibiotic resistance calculated based on the size of the zone of resistance formed around the disc. Interpretation of resistance zones, intermediates, and vulnerabilities determined based on Cappucino and Sherman [11]. Isolates that have a resistance profile to one of the 3 antibiotics are eliminated as probiotic candidates.
3. Results and discussion

3.1 Total bacteria in the digestive tract

The animal's digestive tract is home to various types of bacteria. Based on the results of the TPC it was found that the total number of bacterial cells in the digestive tract of rice field eel was \(3.5 \times 10^7\) cfu/ml. The below is a comparison of the total number of bacterial cells in the digestive tract of several types of fish.

| Fishes          | Total bacteria (cfu/ml) | Reference                  |
|-----------------|-------------------------|----------------------------|
| A. bicolor      | \(3.0 \times 10^8\)     | Lestari et al., 2016 [13]  |
| Rice field eel  | \(3.5 \times 10^7\)     | This research              |

The existence of microbiota provides benefits to its host through improving nutrient metabolism, providing vitamins and other growth factors, producing ulang extracellular enzymes that help digestion or eliminate toxic residues [14]. On the other hand, the host provides the living space and nutrients needed by microbes to grow and develop. For microbial growth requires the formation of cellular and energy compounds. Microorganisms can process large molecules such as carbohydrates, proteins, and fats by producing extracellular enzymes or hydrolyzing these complex molecules into simpler forms before being transported into cells [15]. Nutritional needs for this process are obtained from the environment (digestive tract) through food processed by the microbes themselves. In addition to the availability of nutrients, bacteria also need environmental conditions that allow them to grow optimally.

3.2 Morphological appearance of isolates

The bacteria that were isolated from the digestive tract of paddy eel were 10 isolates. The colony morphology of these isolates on the NA media is white, orange, yellow, light yellow, and brown with varying edges, which are flat, branched, and wavy. The shape of the elevation is convex, flat, flat and uneven. The results of isolation can be seen in Table 2 below.

| Isolat | Colour         | Edges   | Elevation | Formed     | Gram | Cell Form |
|--------|----------------|---------|------------|------------|------|-----------|
| BS1    | Orange         | Waved   | Convex     | Small circular | Positive | Short Rod |
| BS2    | Light yellow   | Waved   | Convex     | circular   | Negative | Streptococcus |
| BS3    | Pale Yellow    | Regular | Convex     | circular   | Positive | Coccus    |
| BS4    | Milk white     | Regular | Convex     | circular   | Positive | Short Rod |
| BS5    | White          | Rizoid  | Convex     | Agak oval  | Positive | Coccus    |
| BS6    | White          | Berserabut | Plate     | Big Circular | Negative | Spiral    |
| BS7    | Light          | Regular | Convex     | Small circular | Positive | Coccus    |
| BS8    | Brown          | Waved   | Plate      | circular   | Positive | Coccus    |
| BS9    | Light          | Branched | Plate     | Oval       | Positive | Long Rod  |
| BS10   | Light yellow   | Berserabut | unplate   | Big circular | Positive | Short Rod |

The bacterial isolates obtained showed different morphological appearances both in terms of color, shape, and elevation. This color difference was caused by the presence of pigments produced by bacteria. Pigments found in bacteria include carotenoid pigments, anthocyanins, melanin, tripirilmethenes, and phenazine. The carotenoid pigment will give orange, and yellow. while the melanin pigment will give a brown color. These pigments are the result of the decomposition of the amino acid tyrosine by the enzyme tyrosinase [16]. According to Waluyo [17], bacteria and fungi are distributed in almost all types of waters and have different amounts and types. Also, the composition, size, and presence of a microorganism depend on the environmental conditions. In general, water sources in rice fields are more varied and contribute to the diversity of gastrointestinal bacteria. Each water source contains a variety of bacteria, the diversity
of bacteria from several water sources is thought to be the cause of the diversity of bacteria found in the digestive tract of rice eel.

Bacteria have various forms, namely, round, stem and spiral. Rod-shaped bacteria are known as bacilli. Stem-like cell forms: short stems, long stems, single cells or strands. Round-shaped bacteria known as coccus, these bacteria can also be distinguished: monococci, a single round bacteria, streptococci, which are round, grouped bacteria extending to form chains. Spiral forms are divided into 3 forms, namely a spiral group of bacteria that looks like a spiral, vibrio is considered an imperfect spiral form, spiroseta is a group of spiral-shaped bacteria that are flexible. Most of the bacterial isolates that have been isolated are in the form of coccus, which is as much as 50%, short bacillus 30%, streptococcus, long and spiral bacilli, each 1%.

The 10 isolates of the bacteria that were isolated were composed of gram-positive and gram-negative bacteria. 80% of isolates are gram-positive and the remaining 20% are Gram-negative. The bacterial isolate BS2 and BS6 are gram-negative because bacterial cells appear red when microscopic observation. This red color is caused by the loss of the violet crystal dye at the time of decolorization with alcohol, then the bacterial cell absorbs the red dye, safranin. Gram-negative bacteria contain lower lipid concentrations so that bacterial cell walls are more easily dehydrated due to treatment with alcohol. The dehydrated cell wall causes its permeability to decrease so that the crystalline purple substance exits the cell then absorbs safranin [18].

3.3 Hydrolysis of casein, starch, and lipid

The test results of casein hydrolysis activity were characterized by the presence of a clear zone around the isolate colonies, casein hydrolysis characterized by a clear yellow zone around the colony, and fat hydrolysis activity indicated by the presence of green around the colonies of isolates grown. The test results of casein, starch and fat activity are presented in Figure 1.

**Figure 1.** Hydrolysis of the macromolecules of casein, starch, and lipid by the bacterial gastrointestinal tract of rice field eel. The occurrence of hydrolysis is relaxed by the presence of a clear zone around the colony

This shows that the macromolecules which are the source of carbon have been used as an energy source by bacteria. Based on the hydrolysis test, it was found that 5 isolates of bacteria that were isolated could hydrolyze casein and starch at the same time, but 3 isolates could not hydrolyze casein. starch and fat.

**Table 3.** The results of the macromolecular hydrolysis test by bacterial isolates from the rice field eel digestive tract.

| Bacterial Isolates | Hydrolysis casein | Hydrolysis starch | Hydrolysis lipid |
|--------------------|------------------|------------------|-----------------|
| BS1                | +                | +                | -               |
| BS2                | +                | -                | -               |
| BS3                | +                | +                | -               |
| BS4                | -                | -                | -               |
| BS5                | +                | -                | -               |
| BS6                | -                | +                | -               |
| BS7                | +                | +                | -               |
| BS8                | -                | -                | -               |
| BS9                | +                | +                | -               |
| BS10               | +                | +                | -               |
| Percentage         | 70%              | 60%              | 0%              |
Based on Table 3 it can be seen that as much as 70% of bacteria in the eel digestive tract can hydrolyze casein. This is indicated by the presence of clear zones that appear after 24 hours incubation in bacterial isolates BS1, BS2, BS3, BS5, BS7, BS9, and BS10. The results of the starch hydrolysis test in Table 3 show that bacterial isolates BS1, BS3, BS6, BS7, BS9, BS10 showed the activity of amylase enzymes so that these bacteria hydrolyze starch, this can be seen from the presence of clear zones formed around these isolates. For fat hydrolysis tests almost all bacterial colonies isolates do not have lipase enzyme activity. Thus, these bacteria are not able to decompose fat into fatty acids and glycerol in the digestive tract of rice field eels. Based on Table 3 it is known that the percentage of casein, starch, and fat hydrolyzing bacteria are 70%, 60%, and 0% respectively. Thus it can be said that most of the bacteria found in the eel digestive tract are protein hydrolyzing bacteria.

The ability of each isolate to hydrolyze is because each bacterium has a hydrolysis enzyme in its cell. The ability of microbes to hydrolyze nutrients is very important to help the formation of cellular compounds and energy for microbial growth. Microorganisms can utilize large molecules such as casein, starch, and fat by hydrolyzing this complex molecule into a simpler form before being transported into the cell [15].

Even so, most of the bacteria found in the eel digestive tract are protein hydrolyzing bacteria. This is thought to be related to eel eating habits belonging to carnivorous animals, where the need for protein in their feed is 65-70% [2]. Mohanta, et al. [19] suggested that some mechanisms for the absorption of fats, carbohydrates, and proteins can be influenced by the presence of intestinal microflora, thus the presence of bacteria helps in the process of protein synthesis in the eel digestive tract.

### 3.4 Resistance isolates of probiotic candidates to antibiotics

One of the important criteria in selecting probiotic candidates that will be used for humans and animals is the resistance to antibiotics commonly used in humans. Based on the tests carried out, data on the results of testing of isolates resistant to antibiotics amoxicillin, chlorambucil, and erythromycin can be seen in Table 4.

**Table 4.** Data on isolate resistance to 3 types of antibiotics based on the diameter of the inhibitory zone (mm) formed.

| Isolat | Amoxicillin (mm) | Chloramphenicol (mm) | Erythromycin (mm) |
|--------|------------------|----------------------|-------------------|
|        | Ø Status         | Ø Status             | Ø Status          |
| BS.1   | 7 R              | 14 I                 | 0 R               |
| BS.2   | 0 R              | 13 I                 | 0 R               |
| BS.3   | 2 R              | 11 R                 | 10 R              |
| BS.5   | 9 R              | 20 S                 | 13 R              |
| BS.6   | 0 R              | 15 I                 | 0 R               |
| BS.7   | 0 R              | 9 R                  | 3 R               |
| BS.9   | 18 I             | 19 S                 | 18 S              |
| BS.10  | 10 R             | 15 I                 | 13 R              |

The research data (Table 4) shows that only 1 isolate is sensitive to antibiotics, and 10 other isolates are classified as intermediates or resistant to antibiotics. Sensitivity to antibiotics was only shown by isolates BS.9 with inhibition zone diameters reaching 19 mm in chloramphenicol and 18 mm in erythromycin, while 9 other isolates namely BS.1, BS.2, BS.3, BS.6, BS.5, and BS.10 is intermediate and resistant to the three antibiotics used. Bacterial resistance can be seen in BS.6 isolates where no clear zone is formed against the antibiotic activity of amoxicillin and erythromycin.

Resistance to bacteria is caused by several factors including the presence of resistance agents on chromosomes or plasmids and can also be due to changes in genetic material due to methylation, insertion, or recombination, modification of compounds targeted by antibiotics, and bacteria
developing other metabolic pathways that bypass the inhibited reaction by antibiotics [20]. Resistance to antibiotics is a change in the ability of bacteria to become resistant to antibiotics. Antibiotic resistance occurs due to changes like bacteria so that it can no longer be turned off or killed by antibiotics. Bacteria that are resistant to antibiotics will not be killed by antibiotics. Inappropriate use of antibiotics such as continuous use can cause microbes to be resistant to antibiotics. Resistant genes from pathogenic bacteria can be transferred to other bacteria that have not been in contact with antibiotics [4]. This is one of the causes of antibiotic-resistant bacteria.

4. Conclusion
The 10 isolates were obtained from bacterial isolates from the swamp eel digestive tract. These bacteria are BS1, BS2, BS3, BS4, BS5, BS6, BS7, BS8, BS9, and BS10. The bacterial isolates display colony morphology that varies in shape, size, color, edges and elevation of the colonies. Eight bacterial isolates are gram-positive and only BS2 and BS6 are gram-negative. Most of the isolates were able to hydrolyze starch and protein. However, all isolates are resistant to antibiotics, so all of these bacterial isolates cannot be used as probiotics.

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