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ENZYMATIC ACTIVITY OF LACTIC ACID BACTERIA ISOLATES FROM THE GASTROINTESTINAL TRACT OF NILE TILAPIA (*Oreochromis niloticus*) FROM LAKE RANAU, WEST LAMPUNG AS PROBIOTIC CANDIDATES

Aktivitas Enzimatis Isolat Bakteri Asam Laktat dari Gastroinestinal Nila (*Oreochromis niloticus*) Danau Ranau Lampung Barat Sebagai Kandidat Probiotik

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ABSTRACT

Tilapia (*Oreochromis niloticus*) cultivated in Lake Ranau, West Lampung, possesses several superior characteristics, including good growth performance, thick flesh, savory taste, and the absence of muddy odor. These advantages are presumed to be associated with the presence of gut microbiota, particularly lactic acid bacteria (LAB), which have potential as probiotics. This study aimed to obtain lactic acid bacteria isolates from the gastrointestinal tract of tilapia, evaluate their enzymatic activities, and determine potential probiotic candidates. The research employed an exploratory method through bacterial isolation, LAB screening using de Man Rogosa Sharpe Agar (MRSA), enzymatic activity assays, morphological and biochemical identification, and molecular analysis based on 16S rDNA sequencing. The results showed that 12 bacterial isolates were successfully grown on MRSA medium. Ten isolates exhibited amylase activity, while all isolates demonstrated lipase and protease activities, indicated by the formation of clear zones around the bacterial colonies. Isolate UNR.11 showed the highest enzymatic activities with clear zone diameters of 25.5 ± 2.80 mm for amylase, 20.4 ± 1.2 mm for lipase, and 22.7 ± 4.06 mm for protease activity. Based on macroscopic, microscopic, biochemical, and 16S rDNA sequence analyses, isolate UNR.11 was identified as *Bacillus cereus* with 99% similarity. This isolate has potential as a probiotic candidate due to its ability to produce digestive enzymes, namely amylase, lipase, and protease, which may support digestion and nutrient utilization in fish.

Keywords: Enzymatic Activity, Gastrointestinal Tract, Lactic Acid Bacteria, Probiotic Candidate, Tilapia

ABSTRAK

Ikan nila (*Oreochromis niloticus*) yang dibudidayakan di Danau Ranau, Lampung Barat, memiliki karakteristik unggul seperti pertumbuhan yang baik, daging tebal, rasa gurih, dan

tidak berbau lumpur. Keunggulan tersebut diduga berkaitan dengan keberadaan mikrobiota usus, khususnya bakteri asam laktat (BAL), yang berpotensi sebagai probiotik. Penelitian ini bertujuan untuk memperoleh isolat bakteri asam laktat dari gastrointestinal ikan nila, mengevaluasi aktivitas enzimatisnya, serta menentukan kandidat probiotik potensial. Penelitian menggunakan metode eksploratif melalui isolasi bakteri, skrining BAL pada media de Man Rogosa Sharpe Agar (MRSA), pengujian aktivitas enzimatik, identifikasi morfologi dan biokimia, serta analisis molekuler 16S rDNA. Hasil penelitian menunjukkan bahwa diperoleh 12 isolat bakteri yang mampu tumbuh pada media MRSA. Sebanyak 10 isolat menunjukkan aktivitas amilase, sedangkan seluruh isolat menunjukkan aktivitas lipase dan protease yang ditandai dengan terbentuknya zona bening di sekitar koloni bakteri. Isolat UNR.11 memiliki aktivitas enzimatis tertinggi dengan diameter zona bening sebesar 25,5 ± 2,80 mm pada uji amilase, 20,4 ± 1,2 mm pada uji lipase, dan 22,7 ± 4,06 mm pada uji protease. Berdasarkan identifikasi makroskopis, mikroskopis, uji biokimia, dan analisis sekuens 16S rDNA, isolat UNR.11 teridentifikasi sebagai *Bacillus cereus* dengan tingkat kemiripan 99%. Isolat tersebut berpotensi sebagai kandidat probiotik karena memiliki kemampuan menghasilkan enzim pencernaan amilase, lipase, dan protease yang dapat membantu proses pencernaan dan pemanfaatan nutrien pada ikan.

Kata Kunci: Aktivitas Enzimatis, Bakteri Asam Laktat, Gastrointestinal, Ikan Nila, Kandidat Probiotik

INTRODUCTION

Aquaculture is a crucial sector in supporting food security and economic growth in Indonesia (Sambuaga et al., 2016). Tilapia (*Oreochromis niloticus*) is a leading commodity widely cultivated due to its rapid growth, high nutritional value, and increasing market demand. According to data from the Ministry of Maritime Affairs and Fisheries, tilapia production in the fourth quarter of 2022 reached 482,250 tons, representing a growth rate of 43.71% (KKP, 2022). Tilapia can be cultivated in various waters, including Lake Ranau in West Lampung, which boasts favorable environmental conditions for fish growth (Wibowo et al., 2021). Tilapia from Lake Ranau are known to have larger bodies, thicker flesh, a savorier flavor, and a less muddy odor than typical tilapia. These advantages are thought to be influenced not only by water quality but also by the presence of bacteria in the fish's digestive tract, which play a role in nutrient absorption and maintaining fish health.

Fish digestive tract bacteria have the potential to be used as probiotics because they can improve the balance of intestinal microflora, feed digestibility, and fish growth (Nayak, 2010). Several probiotic candidate bacteria, such as *Bacillus* sp. and *Lactobacillus* sp., have been successfully isolated from the digestive tract of tilapia (Sarbaini et al., 2016; Pangaribuan & Sembiring, 2022). In addition, probiotic bacteria are known to produce extracellular enzymes such as amylase, protease, and lipase, which aid the digestion of carbohydrates, proteins, and fats (Wang et al., 2018). However, studies on bacteria from the gastrointestinal tract of tilapia in Lake Ranau and their enzymatic activities are still limited. Therefore, this study was conducted to isolate bacteria from the gastrointestinal tract of tilapia in Lake Ranau and test their enzymatic activities as potential probiotic candidates for fish farming.

RESEARCH METHODS

Research Time and Location

This research was conducted from March to November 2024 in floating net cages (KJA) in Lake Ranau, West Lampung, and in the Productivity Laboratory, Department of Fisheries and Marine Sciences, Faculty of Agriculture, University of Lampung.

Research Design

The study used an exploratory method to isolate, test, and identify candidate probiotic bacteria from the gastrointestinal tract of tilapia (*Oreochromis niloticus*) in Lake Ranau.

Materials and Equipment

The materials used included de Man Rogosa Sharpe Agar (MRSA), Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), skim milk agar, starch agar, olive oil, phosphate buffer saline (PBS), Lugol's solution, hydrogen peroxide (H_2O_2), crystal violet, and safranin. The equipment used included surgical instruments, petri dishes, an incubator, an autoclave, a microscope, a laminar air flow tube, a vortex tube, a micropipette, and an eyepiece.

Research Procedures

Sampling

The research samples were obtained from tilapia (*Oreochromis niloticus*) cultivated in floating net cages (KJA) in Lake Ranau, Lumbok Seminung District, West Lampung. Three fish were used as research samples. Fish samples were collected using a net, then anesthetized with clove oil before being aseptically dissected using sterile surgical instruments. The digestive tract organs, including the stomach and intestines, were isolated and then crushed using a sterile porcelain mortar. The digestive tract samples were then suspended in sterile Phosphate Buffer Saline (PBS) and homogenized using a vortex until a homogeneous suspension was obtained.

Isolation, Purification, and Screening of Lactic Acid Bacteria (LAB)

The gastrointestinal samples were crushed and homogenized using sterile PBS. Bacterial isolation was performed by inoculating 100 µL of the sample suspension into Tryptic Soy Agar (TSA) media using the spread plate method, then incubating at 37°C for 24–48 hours to observe bacterial colony growth. The colonies that grew were then purified based on differences in morphological characteristics, including color, shape, and size. Each colony with distinct characteristics was inoculated using a sterile loop needle onto a TSA slant and incubated again at 37°C for 24–48 hours until a pure isolate was obtained. Lactic acid bacteria (LAB) screening was performed by growing the pure isolate on de Man Rogosa Sharpe Agar (MRSA) selective media using the streak plate method. The isolate was then incubated at 37°C for 24–48 hours. Colony growth on MRSA media indicated the isolate's ability to grow as a lactic acid bacteria.

Enzymatic Activity Testing

Enzymatic activity testing is performed to determine the ability of bacterial isolates to produce extracellular enzymes that play a role in the host's nutrient digestion process (Agustina et al., 2022). Testing includes amylase, lipase, and protease activity using the paper disk diffusion method. In the amylase test, 10 µL of pure bacterial culture is inoculated onto a sterile paper disk placed on a starch medium, then incubated at 37°C for 24 hours. After incubation, a drop of iodine solution is applied to the surface of the medium. Amylase activity is indicated by the formation of a clear zone around the bacterial colonies due to starch hydrolysis, while unhydrolyzed media will turn blue-black. The lipase activity test is performed by placing a paper disk containing a drop of bacterial culture on TSA medium containing olive oil, then incubating it at 37°C for 24 hours. Lipase activity is indicated by the formation of a clear zone around the bacterial colonies due to fat hydrolysis. Meanwhile, protease activity testing was performed using skim milk agar. A 10 µL bacterial culture was inoculated onto a sterile paper disk and incubated at 37°C for 24 hours. Protease activity was demonstrated by the formation of a clear zone around the bacterial colony, indicating protein degradation by the protease enzyme.

Identification of Probiotic Candidates

Bacterial isolates were characterized both macroscopically and microscopically. Macroscopic observations included the color, shape, margin, elevation, and surface of bacterial colonies growing on agar media. Microscopic observations were conducted using the Gram staining method to determine the cell shape and cell wall type of the bacteria. The Gram staining procedure was performed aseptically by adding crystal violet, Lugol's solution, 95% alcohol, and safranin to the bacterial slide, followed by microscopic observation. Isolates that appeared purple were categorized as Gram-positive bacteria, while those that appeared red were categorized as Gram-negative bacteria.

Biochemical tests included catalase, motility, and oxidative/fermentative (O/F) tests. The catalase test was performed using a 3% H_2O_2 solution to determine the bacteria's ability to produce the catalase enzyme, which is indicated by the formation of gas bubbles. The motility test was performed by inoculating the bacteria onto semi-solid media to observe the bacteria's motility based on the spread of growth around the media puncture. The O/F test was performed using two tubes of glucose media, one of which was sealed with liquid paraffin, to determine the bacteria's ability to utilize glucose oxidatively and fermentatively.

Molecular identification of selected bacterial isolates was performed through 16S rDNA gene sequence analysis at PT. Indolab Utama, Cengkareng, West Jakarta. Sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST) in the NCBI GenBank database to determine bacterial species based on the level of nucleotide sequence similarity. Identification was determined based on query cover, e-value, and identity values, with the best match indicated by query cover and identity values approaching 100% and an e-value approaching 0.0 (Madden, 2013).

Data Analysis

The data obtained in this study were tabulated using Microsoft Excel 2010, analyzed descriptively, and presented in figures and tables.

RESULT

Probiotic Candidate Bacterial Isolates

Twelve bacterial isolates, coded UNR (Usus Nila Ranau).1, UNR.2, UNR.3, UNR.4, UNR.5, UNR.6, UNR.7, UNR.8, UNR.9, UNR.10, UNR.11, and UNR.12, were obtained from the gastrointestinal tract of Ranau tilapia. These isolates were able to grow on de Man Rogosa Sharpe agar (MRSA) media. Bacteria were screened for enzymatic activity, including amylase, lipase, and protease activity.

Enzymatic Activity

Measurements of the enzymatic activity of probiotic candidate bacteria from the gastrointestinal tract using a caliper are presented in Table 1.

Table 1. Clear Zone Diameter Values for the Three Enzymatic Activities

Isolate Code	Clear Zone Diameter (mm)		
	Amylase	Lipase	Protease
UNR.1	16.7 ± 1.41	9.13 ± 1.75	15.3 ± 0.76
UNR.2	-	12.3 ± 1.83	17.5 ± 3.51
UNR.3	-	11.7 ± 1.83	15.7 ± 1.77
UNR.4	23.8 ± 1.61	15.8 ± 6.16	15.3 ± 3.60
UNR.5	22.2 ± 3.17	12.8 ± 4.67	21.1 ± 0.41
UNR.6	17.1 ± 4.83	15.6 ± 1.06	17.7 ± 1.23

Isolate Code	Clear Zone Diameter (mm)		
	Amylase	Lipase	Protease
UNR.7	16.7 ± 4.80	16.4 ± 0.55	16.7 ± 5.92
UNR.8	10.3 ± 1.60	11.2 ± 1.62	12.3 ± 4.13
UNR.9	16.6 ± 1.83	14.4 ± 1.51	10.3 ± 1.28
UNR.10	23.7 ± 5.65	11.2 ± 2.35	18.7 ± 5.71
UNR.11	25.5 ± 2.80	20.4 ± 1.2	22.7 ± 4.06
UNR.12	23.7 ± 3.58	15.9 ± 4.51	21.5 ± 4.80

Identification of Probiotic Candidate Bacteria

Bacteria were identified macroscopically to determine colony morphology, including color, shape, margin, and elevation, which served as initial species identification. The results of colony morphology observations are presented in Table 2.

Table 2. Bacterial Colony Morphology

Isolate Code	Colony Morphology			
	Color	Shape	Edge	Elevation
UNR.1	White	Round	Smooth	Raised
UNR.2	White	Round	Smooth	Flat
UNR.3	White	Round	Smooth	Raised
UNR.4	Transparent	Irregular	Lobular	Flat
UNR.5	Cream White	Round	Smooth	Flat
UNR.6	White	Round	Smooth	Flat
UNR.7	White	Round	Smooth	Flat
UNR.8	Cream White	Round	Smooth	Raised
UNR.9	White	Round	Smooth	Raised
UNR.10	White	Round	Smooth	Flat
UNR.11	White	Round	Smooth	Raised
UNR.12	Pink	Round	Smooth	Convex

Microscopically, bacteria were observed to determine cell morphology, including shape and color. Gram staining was performed using a microscope at 100x magnification. The results of the microscopic observations, including cell shape and Gram stain, are presented in Table 3.

Table 3. Bacterial Cell Morphology

Isolate Code	Gram	Cell Shape
UNR.1	Positive	Rod
UNR.2	Positive	Round
UNR.3	Positive	Rod
UNR.4	Positive	Round
UNR.5	Positive	Round
UNR.6	Positive	Round
UNR.7	Positive	Rod
UNR.8	Positive	Round
UNR.9	Positive	Rod
UNR.10	Positive	Rod
UNR.11	Positive	Rod
UNR.12	Positive	Rod

Biochemical tests on 12 bacterial isolates, including a catalase test, a motility test, and an O/F (oxidative/fermentative) test, are presented in Table 4.

Table 4. Biochemical Tests of Bacterial Isolates

Isolate Code	Biochemical Test		
	Catalase	Motility	O/F
UNR.1	-	Motile	F
UNR.2	+	Non motile	F
UNR.3	+	Non motile	F
UNR.4	-	Motile	F
UNR.5	+	Motile	F
UNR.6	+	Motile	F
UNR.7	+	Non motile	F
UNR.8	-	Motile	F
UNR.9	+	Motile	F
UNR.10	-	Motile	F
UNR.11	+	Motile	O
UNR.12	+	Non motile	O

Molecular identification was performed on selected bacteria that produced the largest clear zones in three enzymatic tests. The results of 16S rDNA sequence analysis of the bacterial isolate with the code UNR.11. Based on the analysis, isolate UNR.11 had the highest level of similarity to *Bacillus cereus* with accession code MZ496502.1. The nucleotide length alignment value (query cover) is 48%, the expected value (e-value) is 0.0, and the identity percentage is 99.00%.

DISCUSSION

The enzymatic activity of bacterial isolates was observed based on the isolate's ability to produce a clear zone in a specific medium, indicating the occurrence of substrate hydrolysis. Amylolytic activity was indicated by the formation of a clear zone after the addition of Lugol's solution to the starch medium. This clear zone indicated that the bacterial isolate was capable of producing the amylase enzyme, which hydrolyzes starch into simple compounds such as maltose, glucose, and dextrin (Benson, 2001). The larger the diameter of the clear zone formed, the higher the amylolytic activity of the bacterial isolate (Zubaidah et al., 2019). In the lipase activity test, the ability of bacteria to hydrolyze fat was indicated by the formation of a clear zone in media containing olive oil. The lipase enzyme plays a role in breaking down fat molecules into glycerol and fatty acids, which are then utilized by bacteria as an energy source and cell building component (Benson, 2001). Lipolytic activity in probiotic bacteria has the potential to increase the efficiency of fat utilization in feed, thus optimizing the conversion of nutrients into fish biomass (Dhanalaksmi et al., 2015). Meanwhile, proteolytic activity is demonstrated by the formation of a clear zone on skim milk agar media due to protein degradation by extracellular protease enzymes. The larger the clear zone formed, the higher the isolate's ability to produce protease enzymes (Wilson & Remigio, 2012). Protease enzymes function to break down complex proteins into simple peptides and amino acids, making them more easily absorbed by the fish (Islamiah et al., 2017).

Based on test results, isolate UNR.11 demonstrated the highest enzymatic activity compared to other isolates and was able to produce all three types of digestive enzymes: amylase, lipase, and protease. The ability to produce multiple types of extracellular enzymes indicates that the isolate possesses multi-enzymatic properties, an important criterion for

candidate probiotic bacteria (Bairagi et al., 2002). This multi-enzymatic activity plays a role in increasing feed digestibility by hydrolyzing carbohydrates, fats, and proteins into simpler compounds that are more easily utilized by fish (Andriani et al., 2019). Research by Marlida & Elrifadah (2017) also showed that bacteria capable of producing amylase, lipase, and protease enzymes have the potential to be used as probiotic agents to support feed efficiency in fish farming.

Physiological and biochemical characterizations showed that most isolates exhibited positive catalase activity, were motile, and capable of glucose fermentation. The catalase test was conducted to determine the bacteria's ability to produce the enzyme catalase, which decomposes hydrogen peroxide (H_2O_2) into water and oxygen. Hydrogen peroxide is a toxic compound produced by aerobic metabolism that can damage bacterial cells if not broken down (Djide & Sartini, 2006). A positive result in the catalase test is indicated by the formation of gas bubbles due to the release of oxygen (Kosasih et al., 2019). The ability to produce the enzyme catalase indicates that the isolate is able to withstand oxidative stress during metabolic processes (Rahmawati et al., 2021). The motility test demonstrated the bacteria's ability to move, as indicated by the spread of growth around the media puncture. The motile nature of bacteria is related to the presence of flagella as a means of movement (Rahmawati & Isnaeni, 2016). In the oxidative/fermentative (O/F) test, most isolates showed fermentative properties, indicating the bacteria's ability to utilize glucose without requiring oxygen. Meanwhile, some isolates showed oxidative properties, indicating the need for oxygen in the metabolic process (Sarbaini et al., 2016).

Based on colony morphology, Gram staining, and biochemical test results, isolate UNR.11 exhibits spherical, white colonies with a smooth surface, raised ridges, Gram-positive colonies, catalase-positive colonies, and motile colonies. These characteristics are consistent with those of the Bacillus genus, which are generally Gram-positive, motile, and capable of producing various extracellular enzymes (Yuka et al., 2021; Andriyanto & Yulianti, 2020). Molecular identification using 16S rDNA gene sequence analysis indicated that isolate UNR.11 was identified as *Bacillus cereus*. 16S rDNA analysis is a widely used molecular identification method due to its high accuracy in determining phylogenetic relationships between bacterial species (Bartram et al., 2011). Identification results were obtained through BLAST analysis of the NCBI GenBank database based on query cover, e-value, and identity values.

Bacillus cereus is known to have potential as a probiotic in fish farming because it can produce digestive enzymes such as amylase, protease, and lipase that can increase feed utilization efficiency (Kurniasih et al., 2013). The addition of *B. cereus* to feed has been reported to increase fish growth, digestive enzyme activity, and feed efficiency (Simanjuntak et al., 2019). Furthermore, this bacterium is capable of forming endospores that allow it to survive extreme environmental conditions, such as temperature changes, low pH, and exposure to digestive enzymes (Wijnands et al., 2006). The ability to form endospores makes *B. cereus* more stable and easily applied as a probiotic in fish farming systems, both through feed and maintenance media.

CONCLUSION

Based on the research results, 12 isolates of probiotic candidate bacteria were successfully isolated from the gastrointestinal tract of tilapia (*Oreochromis niloticus*) in Lake Ranau. The results of enzymatic activity testing showed that 10 isolates had amylolytic activity, while all isolates showed proteolytic and lipolytic activity. Isolate UNR.11 was the isolate with the highest enzymatic activity as indicated by the clear zone diameter of 25.5 ± 2.80 mm in the amylase test, 20.4 ± 1.2 mm in the lipase test, and 22.7 ± 4.06 mm in the protease test. Based on morphological, biochemical characterization, and 16S rDNA molecular analysis, the isolate

was identified as *Bacillus cereus* UNR.11 which has the potential as a probiotic candidate because it is able to produce important digestive enzymes. However, further research is needed regarding the pathogenicity aspects and the application of *Bacillus cereus* UNR.11 in freshwater fish feed to ensure its safety and effectiveness in supporting the growth and health of farmed fish.

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