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Morphological Identification of Bacteria from Tuna Fish Isolates (Thunnus sp.) in Kondang Merak

Agus Tri Yulianto, Ade Yamindago

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Abstract: Bacteria are microscopic organisms, and a small portion of them are pathogenic or harmful to living organisms. One example is bacteria that cause damage and decay in captured Tuna fish (Thunnus sp.) off the coast of Kondang Merak, Malang. Therefore, to determine the genus of bacteria found in Tuna fish (Thunnus sp.), bacterial morphology identification was conducted. Bacterial morphology identification was performed using methods involving the identification of colony and cell morphology, as well as bacterial respiration tests to enhance genus prediction accuracy. Bacterial morphology identification involved several testing stages, including Gram-staining, cell observation and measurement, motility testing, and bacterial respiration testing. The bacterial isolation samples from Tuna fish (Thunnus sp.) on TSA media consisted of 8 samples, namely 1a, 1b, 2a, 2b, 3, 4a, 4b, and 5, which were differentiated based on bacterial colony morphology. Based on the results of colony morphology identification, cell morphology, and respiratory testing, all 8 bacterial samples were manually identified with reference to identification books. The identification results showed that several samples had similar morphological characteristics. The bacterial morphology identification results for samples 2a and 2b were identified as belonging to the genus Aeromonas; samples 4a and 4b were classified into the genus Mesophilobacter; sample 1a was categorized into the genus Carnobacterium; and samples 1b, 3, and 5 belonged to the genus Vibrio.

Introduction

Malang Regency is a region with a coastline stretching up to 77 kilometers, directly bordering the South Sea. Its geographical location gives it significant potential for capturing fisheries resources. One of the capturing fisheries commodities in Malang Regency is Bigeye Tuna (1). The catch of Tuna fish reached 1,332.1 tons per year, accounting for 32% of the Tuna catch in East Java (2). This substantial catch should ideally be used for the well-being of the coastal communities in the city of Malang. However, fish is a perishable food item that can easily spoil.

Fish spoilage occurs due to several factors, including the duration of storage, environmental conditions, and microbial contamination (bacteria). The fish's body is a suitable medium for bacterial growth (3). These bacteria typically contaminate fresh seafood without proper cooking or inadequately processed seafood, and contamination can occur during the handling and processing of fish-based food items (4). One of the bacteria commonly found on fish is Vibrio sp., which is generally aerobes and facultative anaerobes and classified as gram-negative bacteria (5). Several types of Vibrio bacteria is found in aquatic environments, such as *V. alginolyticus*, *V. charchariae*, and *V. fischeri*. These bacteria are frequently found infecting marine organisms like clams and fish, leading to vibriosis (6). Therefore, it is necessary to conduct bacterial morphology identification as an effort to provide information about the bacteria found on Tuna fish.

Morphological identification was conducted through the observation of bacterial growth from isolates obtained from Tuna fish. This observation included assessing growth results and types, Gram-staining tests, shape and size observations, and motility tests

(7). Additionally, to support the observational data, bacterial respiration tests were conducted for bacterial identification (8). The results of colony morphology identification were then compared with manual bacterial identification reference books.

Experimental Section

Material Collection

The Tuna fish (Thunnus sp.) was collected from Kondang Merak Beach, Malang. Then, the identification of bacteria was performed at the Laboratory of Fisheries Product Safety, Faculty of Fisheries and Marine Sciences, Brawijaya University, and the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Malang State University.

Isolation and Purification

Isolation of bacteria from Tuna fish (Thunnus sp.) was performed using a two-step media preparation approach. Nutrient Agar (NA) and Tryptone Soy Agar (TSA) were prepared according to standard protocols. Small tissue samples were collected from the surface of Tuna fish specimens, followed by homogenization in sterile phosphate-buffered saline (PBS). The homogenates were serially diluted, and 100 µL of appropriate dilutions were spread onto both NA and TSA plates. The plates were incubated at 37°C for 24 h to allow bacterial growth.

Gram-staining Test

Gram-staining was employed to differentiate between Gram-positive and Gram-negative bacteria. Bacterial colonies from both NA and TSA plates were selected, heat-fixed on microscope slides, and subjected to the Gram-staining procedure following the method described by Hastuti, 2014 (9). Briefly, the heat-fixed samples were stained with crystal violet and iodine solution, decolorized with acetone, and counterstained with safranin. The stained slides were examined under a light microscope, and bacterial species were categorized based on their staining characteristics

Bacterial Motility Test

Bacterial motility was assessed using semi-solid agar plates. The plates were inoculated with bacterial cultures obtained from the original NA and TSA plates and incubated at their respective optimal growth temperatures. After 24 h of incubation, the motility of the bacterial cultures was observed by the diffusion pattern from the point of inoculation. Motile bacteria displayed a diffuse pattern of growth away from the inoculation point, while non-motile bacteria exhibited growth only at the point of inoculation.

Bacterial Respiration Test

The bacterial respiration test, using resazurin solution, evaluates a bacterium's aerobic respiratory activity. Bacterial cultures are placed in labeled test tubes and mixed with resazurin solution before incubation. After 24-48 h, a color change from blue to pink or colorless indicates active aerobic respiration, while minimal change suggests limited aerobic respiration or facultative anaerobiosis. This test provides insights into a bacterium's oxygen-dependent metabolic activity.

Result

The growth results on NA media showed distinct bacterial colonies in several samples that had been cultured. Consequently, bacterial isolation was performed from each colony onto TSA media. Initially, on NA media, there were 5 bacterial samples based on isolates, which included (1) Tuna fish meat; (2) Tuna fish storage water; (3) Steamed Tuna fish; (4) Grilled Tuna Satay over an open flame; and (5) Steamed Tuna Satay. After isolation, 8 bacterial colonies were identified based on the morphology of the bacterial colonies that grew. Subsequently, these isolates were cultured on TSA media, designated as 1a, 1b, 2a, 2b, 3, 4a, 4b, and 5. Observations of bacterial purification growth based on morphology are presented in table 1.

Table 1. Observation of purified bacterial samples.

Bacterial code	1a	1b	2a	2b	3	4a	4b	5
Colony Shape	Ir	Ir	Ir	Rl	Ir	Rl	Rl	Rl
Colony Surface	F	Rf	F	F	C	F	Rf	F
Colony Edge	W	W	W	S	W	S	S	S
Color	WY	Y	WY	Y	Y	Y	WY	Y

Note: Ir = Irregular, Rl = Root-like, F= Flat, RF= Raised flat, C= Convex, W= Wavy, S= Stringy, Y= Yellow, WY= Whitish Yellow

Testing the staining of samples will be categorized as either gram-positive or gram-negative based on the appearance of bacterial colors under a microscope after specific treatment with various Gram-staining fluids. In Gram-positive staining, the samples will exhibit a purple color due to the absorption of crystal violet and iodine as the primary staining agents. This indicates that the bacteria possess thick peptidoglycan layers, allowing the primary stain to be absorbed and trapped within. Conversely, in Gram-negative staining, the samples will appear red due to the absorption of the secondary stain, safranin (9).

In addition to morphology observations through Gram-staining and growth pattern assessments, a motility test is conducted for enhanced results. To further refine the analysis, a motility test is employed using the drop-by-drop method. This method involves placing a drop of bacterial culture onto a slide and observing it under a microscope to detect any movement. The results of both the Gram-staining and motility tests are presented in Table 2.

Table 2. Observation of gram-staining and motility test.

Bacterial code	Gram-staining				Growth Form	Motility Test
	Gram	Size (µm)		Shape Observation		
		Length	Width			
1a	+	1,25	0,5	B	S	-
1b	-	1,25	0,25	B	S	-
2a	-	1,00	0,5	B	T	-
2b	-	1,50	0,25	B	T	-
3	-	1,25	0,5	B	S	-
4a	-	1,00	0,5	B	S	-
4b	-	1,00	0,25	B	Rl	-
5	-	1,25	0,24	B	S	-

Note: B= Bacillus, S= Sword, T= Thorny, Rl= Root-like.

The results of bacterial respiration can be determined based on the observation of the distribution of bacterial cells within the reaction tube. Aerobes bacteria can be identified by the distribution of cells on the surface of the medium, microaerophilic bacteria have limited distribution on the surface of the medium, while anaerobes bacteria exhibit bacterial cell distribution within (at the bottom) of the liquid medium. Facultative anaerobes bacteria have an even distribution throughout the liquid medium, as evident from the overall turbidity in the medium (10). The results of bacterial sample respiration are as follows.

Table 3. Respiration test result.

Bacterial code	1a	1b	2a	2b	3	4a	4b	5
Respiration type	FA	FA	FA	FA	FA	A	A	FA

Note: A= Aerobes, FA= Facultative Anaerobes

Based on the results of colony morphology identification, cell morphology, and respiratory testing, manual identification using a bacterial identification guide was conducted for the 8 bacterial samples. The identification results revealed that several samples exhibited similar morphological characteristics. The morphological identification results of bacterial samples 2a and 2b were identified as belonging to the genus Aeromonas; samples 4a and 4b were categorized into the genus Mesophilobacter; sample 1a was placed in the genus Carnobacterium; and samples 1b, 3 and 5 were classified within the genus Vibrio.

Discussion

Carnobacterium, a bacterial genus commonly found in meat and seafood products, exhibits distinct morphological features (11). Typically, these bacteria present as rod-shaped or spherical cells, with size ranging from 0.5 to 1.5 micrometers in width and 1 to 3 micrometers in length. They are Gram-positive, indicating a thick peptidoglycan cell wall and typically facultative anaerobes (12). However, Carnobacterium species are generally non-motile, lacking flagella for active movement. These species are vital for food preservation, fermenting lactic acid to deter spoilage and pathogens, prolonging meat and seafood shelf life. Some also act as biocontrol agents, enhancing food safety by inhibiting harmful bacteria (13).

Vibrio is a diverse genus of Gram-negative bacteria, characterized by their curved or comma-shaped morphology. These bacteria are typically facultative anaerobes, capable of thriving in environments with or without oxygen (5). They are commonly found in aquatic habitats, both free-living in marine and freshwater environments and forming symbiotic relationships with various marine organisms (6). While some Vibrio species play essential roles in nutrient cycling and ecological processes, others, like *V. cholerae*, are notorious human pathogens. *V. cholerae* is responsible for cholera, a severe diarrheal disease, and it is considered an aerobes bacterium, primarily flourishing in oxygen-rich environments (14).

Aeromonas is a diverse group of gram-negative bacteria primarily inhabiting aquatic environments. These rod-shaped bacteria are facultative anaerobes and play ecological roles in nutrient cycling and decomposition in aquatic ecosystems, some species can pose health risks as opportunistic pathogens (15).

Table 4. Observation of bacterial morphology.

Species	Bacterial code	Gram	Bacterial classification	Growth form	Size (µm)	
					Length	Width
Carnobacterium	1a	+	Facultative anaerobes	Sword	1,25	0,5
Vibrio	1b	-	Facultative anaerobes	Sword	1,25	0,25
	3	-	Facultative anaerobes	Sword	1,25	0,5
	5	-	Facultative anaerobes	Sword	1,25	0,24
Aeromonas	2a	-	Facultative anaerobes	Thorny	1,00	0,5
	2b	-	Facultative anaerobes	Thorny	1,50	0,25
Mesophilobacter	4a	-	Aerobes	Sword	1,00	0,5
	4b	-	Aerobes	Root-like	1,00	0,25

Aeromonas infections in humans can result in gastroenteritis, wound infections, or severe systemic illnesses, especially in immunocompromised individuals (16). Due to their prevalence in water sources used for drinking and recreation, Aeromonas species are of interest in public health and environmental microbiology, with ongoing research to understand their pathogenicity and antibiotic resistance mechanisms (15, 17).

Mesophilobacter is a newly proposed genus and species of gram-negative coccobacilli found in seawater environments. These microorganisms are aerobes, nonmotile, and moderately halophilic, with optimal growth temperatures ranging from 33 to 37°C. They exhibit pleomorphism, with varying cell shapes in different stages of their growth. Its colonies on nutrient agar are smooth, circular, and pale yellowish-brown, and its growth in nutrient broth is moderate and turbid. These characteristics distinguish Mesophilobacter as an intriguing marine bacterium (18).

Conclusion

The bacterial identification process based on morphological characteristics allowed for the classification of the tested bacterial samples into different genera. This method provides a valuable initial step in identifying bacteria, although it is limited to the genus level. Carnobacterium (1a) Vibrio (1b, 3 and 5), Aeromonas (2a and 2b) and Mesophilobacter (4a and 4b). Further molecular and biochemical tests would be necessary to achieve species-level identification and gain a more comprehensive understanding of these bacterial samples.

Declarations

Author Informations

Agus Tri Yulianto
Affiliation: Department of Marine Science, Faculty of Fisheries and Marine Science, Brawijaya University, Malang – 65145, Indonesia.
Contribution: Investigation, Writing - Original Draft.

Ade Yamindago
Affiliation: Department of Marine Science, Faculty of Fisheries and Marine Science, Brawijaya University, Malang – 65145, Indonesia.
Contribution: Conceptualization, Project administration, Supervision.

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Conflict of Interest

The authors declare no conflicting interest.

Data Availability

The unpublished data is available upon request to the corresponding author.

Ethics Statement

Not applicable.

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Not applicable.

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