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Isolation and identification of lipolytic bacteria from the digestive tract of eel (*Anguilla bicolor bicolor*)

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Abstract. *Hapsari RT, Susilowati A, Pangastuti A. 2020. Isolation and identification of lipolytic bacteria from the digestive tract of eel (*Anguilla bicolor bicolor*). Bonorowo Wetlands 10: 44-50. Anguilla bicolor bicolor* is commonly found in the waters of western Indonesia. Currently, the demand for eel is increasing, causing an increase in the economic value of eel and being used as an export commodity. This study aims to isolate and identify lipolytic bacteria found in the digestive tract of eel (*A. bicolor bicolor*). Lipolytic bacteria isolated from the digestive tract of eel (*A. bicolor bicolor*). Bacterial isolation uses minimal media enriched with olive oil. When lipolytic bacteria were screened using Rhodamine B agar media, the presence of an orange glow in the colony upon exposure to 350 nm UV light indicated lipolytic activity. Identification of lipolytic bacteria was based on observing the morphological characters of bacterial colonies (edges, elevation, colonies, and colors) and the gene sequences encoding 16S rRNA. DNA sequence characteristics of lipolytic bacteria were analyzed by BLAST Nucleotide on the NCBI website (www.blast.ncbi.mlm.nih.gov/blast.cgi). Based on the screening results, 7 isolates of lipolytic bacteria were obtained from 30 successfully isolated isolates. The DNA sequence characteristics of the bacterial isolates were analyzed by BLASTN and found 5 species, namely *Pseudomonas azotoformans, Providencia vermicola, Providencia* sp., *Aeromonas veronii,* and *Aeromonas hydrophila.*

Keywords: *Anguilla bicolor bicolor,* identification, isolation, lipase enzyme, lipolytic bacteria

INTRODUCTION

Indonesia is a country that is rich in eel species. According to Sasongko et al. (2007) and Fitri et al. (2019), there are 6 species of eel in Indonesia, namely *Anguilla marmorata*, *A. borneoensis*, *A. bicolor,* and *A. ancertralis*, *A. mauritinia,* and *A. celebesensis.* In the species *A. bicolor,* there are 2 subspecies, namely *A. bicolor bicolor* and *A. bicolor pacifica*. *Anguilla bicolor bicolor* is mostly found in the waters of western Indonesia, while *A. bicolor pacifica* is mostly found in the waters of eastern Indonesia (Sugeha and Suharti 2008). Today eel is increasingly popular for consumption, thus increasing its economic value and potential for export.

The increasing popularity of eels for consumption has encouraged researchers to research eels, one of which is the study of normal microbiota found in the digestive tract of eels. Naturally, every fish has a microbiota in its body. The microbiota can be in the form of bacteria that can be found on the skin, gills, digestive tract, and light-emitting organs (Austin, 2006; Ridwan et al., 2019). These bacteria have various functions, such as self-protection, digestive processes, and preventing diseases in the fish's body. In the digestive tract, eels have various bacteria used in the digestion process of food and protection from pathogenic bacteria that enter the body.

According to Aslamyah (2006), a fish's digestive system is simpler than that of land animals, so the digestive mechanism of fish is very limited. It affects the availability of digestive enzymes, such as amylase and lipase enzymes, in lower amounts because they have little secretion. In contrast, the protease enzyme is secreted more because the eel is a carnivorous fish. One of the enzymes that play an important role in the digestive process, namely lipase, plays a role in breaking down triacylglycerol into fatty acids and glycerol.

The lipase enzyme in the digestive tract of the eel produced by bacteria is usually extracellular (Pramiadi et al., 2014). Lipases, also known as triacylglycerol hydrolases (triacylglycerol acyl hydrolases, EC 3.1.1.3), are naturally occurring enzymes that catalyze the hydrolysis of triacylglycerol (fats/oils) into fatty acids, monoacylglycerol, diacylglycerol, and glycerol (Poedjiadi and Supriyanti 2009). According to Kurniasih et al. (2013), high enzymatic activity in the digestive tract will increase the digestibility of food by releasing enzymes that help the digestive process. The enzyme-producing bacteria will also regulate the condition of microorganisms in the intestine and suppress the growth of pathogenic bacteria in the digestive tract of fish. Increasing the enzymatic activity of the digestive tract of the eel through microbiological development can improve the quality of the eel. However, to develop and increase eel production, it is necessary to know the types of bacteria that have lipolytic activity in the digestive tract of eels.

Knowledge of lipolytic bacteria in eel can be applied as a candidate for probiotic bacteria to increase feed digestibility. It is expected to increase the quality and weight of eel. According to Fuller (1992), probiotics are food additives in the form of live microbes that benefit the

host by improving the balance of microbes in the digestive tract. The use of lipolytic bacteria as probiotic candidates can also suppress the growth of pathogenic bacteria because probiotic candidate bacteria can produce antibacterial compounds. With the addition of probiotics, the mortality of eels during cultivation can be reduced be suppressed. According to Telussa (2013), the development of microorganisms is currently very intensively carried out to be applied in all fields because it is considered more environmentally friendly and more economical.

Based on this description, it is necessary to study the microbiota of eel (*A. bicolor bicolor*) through the isolation and identification of lipolytic bacteria present in the digestive tract of eel. This study will be known what lipolytic bacterial microbiota is contained in the digestive tract of eels that can be used as probiotic candidates and help digest food.

The objectives of this study were (i) to isolate lipolytic bacteria from the digestive system of an eel (*A. bicolor bicolor*) and (ii) to identify lipolytic bacteria isolated from the digestive tract of an eel (*A. bicolor bicolor*).

MATERIALS AND RESEARCH

Materials

The lipolytic bacteria isolated and identified in this study were sourced from the digestive tract of *A. bicolor bicolor*, which was taken from the eel farm at Universitas Sebelas Maret, Surakarta, Central Java, Indonesia in August-November 2018 with a length of 27-32 cm.

Ways of working

Isolation of lipolytic bacteria

The eels used as samples are fasted for 24 hours to clean the digestive organs from food debris. *Anguilla bicolor bicolor* was dissected by cutting the lower part of the abdomen from the anterior of the body to the ventral fin, then cutting towards the dorsal of the eel to the lateral line, and then cutting towards the anal part of the fish. The stomach and intestines are taken, and then the intestinal contents are removed to reduce impurities from the fish's stomach contents. Then the organs were washed with physiological saline (0.85% NaCl). Then the fish digestive tract samples were homogenized in 0.85% sterile NaCl and then made serial dilutions of 10^{-1}-10^{-4}. A total of 0.1 mL of the 10^{-3}-10^{-4} dilution series was spread on minimal media with olive oil and incubated at 27ºC for 48-72 hours using the spread plate method with 2 repetitions. Colonies growing on the surface of the media and showing different morphologies can be used as candidates for lipolytic bacteria that can be stored in slanted agar at 4ºC (Gayathri et al., 2013).

Screening for lipase producing bacteria

The bacterial screening was carried out on bacterial isolates purified by taking 1 ose of isolate and then streaking on rhodamine B agar media, then incubation at 27ºC for 48 hours. Bacteria with lipolytic activity are characterized by bacterial colonies that glow pink to orange when observed under UV light with a wavelength of 350 nm (Carissimi et al., 2007).

Identification of lipolytic bacteria

Observation of the colony morphology of lipolytic bacteria. Observation of the morphology of lipolytic bacterial colonies was carried out by observing bacterial colonies growing on NA media. Aspects observed included color, shape, elevation, and the edge of the bacterial colony.

Gram stain. Bacterial isolates were taken and scratched on a glass object, sterilized, and then fixed for fixation. Furthermore, 1 drop of crystal violet was added and allowed to stand for 1 minute, and then rinsed with distilled water until the dye faded. After drying, 1 drop of iodine solution was dripped on the bacterial preparation for 1 minute and then washed with distilled water. Furthermore, the preparations were dripped with 96% alcohol for 20 seconds and then flowed again with distilled water. Then after drying, the preparation was dripped with 1 drop of safranin for 45 seconds, then drained with distilled water and dried. The preparations were observed under a microscope with 1000x magnification (Pratita and Putra 2012).

Identification of lipolytic bacteria using the 16S rRNA coding gene sequence. The lipolytic bacterial genomic DNA obtained from the screening process was extracted with the presto^{TM} mini gDNA bacteria kit. The lipolytic bacterial 16S rRNA gene was amplified using My TaqTM HS Red Mix, utilizing a primer consisting of 63 forward primers (63f: 5'-CAGGCCTAACACAT GCAAGTC-3') and 1387 reverse primers (1387r: 5'-GGGCGGAWGTGTACAAGGC-3'). The PCR reaction was started by mixing 12.5 µl Kapa2G fast ready mix, 1.25 µl 63 forward primers with a concentration of 10 pmol, 1.25 µl 1387 reverse primer with a concentration of 10 pmol, 1 µl DNA template, and 9 µl ddh2o. The pre-denaturation process was carried out at 95ºC for 3 minutes. One PCR cycle of 30 cycles consisted of denaturation at 95ºC for 15 seconds, annealing at 56ºC for 15 seconds, and elongation at 72ºC for 30 minutes. Finalizing was carried out at 72ºC for 2 minutes, and then the PCR was stopped and stored at 4ºC. The PCR amplification products were then separated by gel electrophoresis (Marchesi et al. 1998). The PCR product for the gene encoding the 16S rRNA of lipolytic bacteria was then sequenced by 1st base Singapore.

Data analysis

The lipolytic bacteria isolates were analyzed descriptively by observing cell colony morphology in shape, color, elevation, and bacterial margins. The characteristics of lipolytic bacterial DNA sequences resulting from the extraction process were analyzed using bioinformatics techniques with the BLAST Nucleotide device on the NCBI website (www.blast.ncbi.mlm.nih.gov/blast.cgi).

RESULTS AND DISCUSSION

Isolation and screening of lipolytic bacteria from the digestive tract of eels

Isolation of bacteria from the digestive tract of the eel was carried out by taking all parts of the stomach and intestines of the fish 3 times so that more varied and more bacterial isolates were obtained. This study resulted in 30 bacterial isolates coded sd01 to sd30 (Table 1).

As many as 30 bacterial isolates could grow and thrive on minimal media enriched with olive oil. Pure culture isolates were distinguished based on the morphological characters of the colonies. In several studies, such as that conducted by Lestari (2016), it was stated that the isolation of bacteria from the digestive tract of eel obtained as many as 8 bacterial isolates, which colony morphological characters could distinguish. Another study conducted by Lestari et al. (2016) stated that in the digestive tract of eel, 11 isolates of bacteria could be isolated. In his research, Floris (2010) stated that the microbiota community in the digestive tract of fish is known to have an essential role in the digestive tract because it can help the process of micronutrient metabolism, synthesis of enzymes, and vitamins such as B12, which the digestive tract can directly absorb.

In the digestive tract, microbiota exists in a mixture of various microorganisms that play a role according to their function in the digestive process. Separating bacteria to form a pure culture consisting of single cells must be carried out to study the morphological characters, growth properties, physiological properties, and their role in the digestive tract (Fardiaz 1992). The obtained bacterial isolates were then screened for lipolytic ability (Figure 4).

Based on the screening results, six bacteria had lipolytic activity in the digestive tract of eels. The isolates were SD01, SD09, SD12, SD13, SD22, SD29. The presence of bacterial isolates with lipolytic activity indicates the presence of microbiota in the digestive tract of fish that functions in the digestive process of food in the breakdown of lipids into fatty acids and glycerol. Rani et al. (2005) explained that the orange glow occurs when the lipids in the medium are hydrolyzed with a lipase catalyst from bacteria into fatty acids and glycerol. Then the fatty acids enter the bacterial cell through an assisted diffusion process facilitated by a helper protein, and the indicator rhodamine B enters the bacterial cell through simple diffusion. Fatty acids in cells will bind to rhodamine b to form complex bonds, which occur due to the reaction between cationic rhodamine b with uranyl ions from fatty acids (Carissimi et al. 2007).

The presence of lipolytic activity in the digestive tract of eel has previously been known in a study conducted by Lestari (2016) through a lipid hydrolysis test on bacteria isolated from the digestive tract of eel (*A. bicolor*). Bacteria with lipolytic activity are microbiota that can come from fish's eating habits and the living environment of eels. In their research, Taufik et al. (2017) stated that eating habits would affect enzyme activity and microbiota communities in the digestive tract. Lipolytic bacteria are producers of extracellular lipase enzymes and are classified into three types: non-specific lipase, 1,3-lipase, and specific fatty acid lipase. Lipase synthesis by lipolytic bacteria is affected by temperature, nitrogen and carbon ratio, inorganic salts, oxygen, and lipid sources such as olive oil, lard, and fatty acids. Microbiota in the gastrointestinal tract can cause lipolysis in two ways: by contributing to TAG breakdown through bacterial performance and then by altering pancreatic lipase secretion or inactivating pancreatic lipase by protease enzymes (Ray et al. 2012).

Table 1. Bacterial isolates obtained from the digestive tract of eels obtained from each fish sample

Fish sample	Number of bacterial isolates	Isolation code
Fish 1	2	SD01, SD02
	1	SD03
Fish 2	6	SD04, SD05, SD06, SD07, SD17, SD18
	9	SD08, SD09, SD10, SD11, SD12, SD13, SD14, SD15, SD16
Fish 3	6	SD19, SD20, SD21, SD22, SD23, SD24
	6	SD25, SD26, SD27, SD28, SD29, SD30
Total isolate	30	

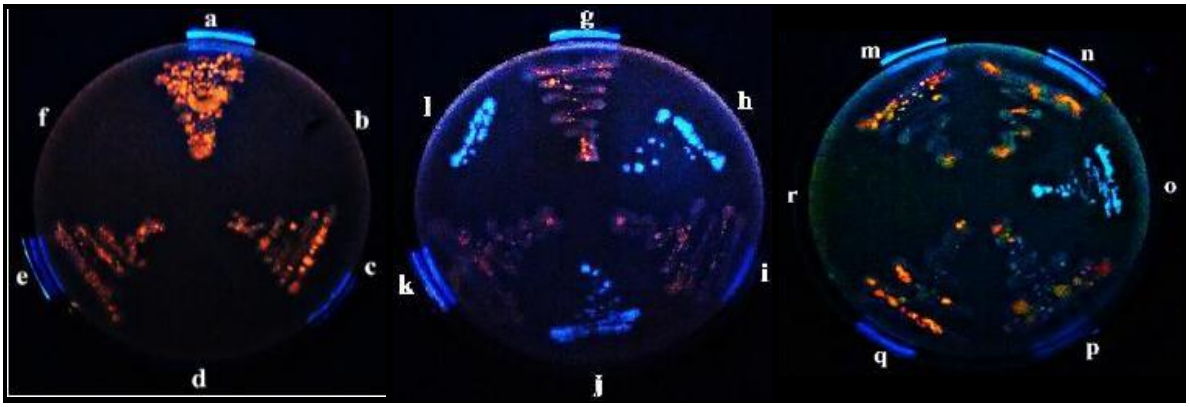

Figure 4. Colonies of lipolytic bacteria from the digestive tract of eels on Rhodamine B Agar media under UV lamp exposure of 350 nm. Bacterial isolates that had lipolytic activity were SD01 (a), SD09 (c), SD12 (e), SD13 (g), SD22 (n), SD29 (q)

Morphological characteristics of lipolytic bacteria in the digestive tract of eels

The colony and cell morphology characterized six bacterial isolates with lipolytic activity. The characteristics of bacterial colonies include the shape, color, margins, and elevation of the colonies. Bacterial cell characteristics include gram staining and cell shape (Figure 4x). The characterization can be seen in Table 2.

The physical characteristics of the lipolytic bacteria found in the digestive tract of eel revealed that the bacteria were rod-shaped and red, indicating that they were gram-negative bacteria. In the morphological characterization of the colony, the whole colony had an irregular shape, while the character of color, margin, and elevation varied in some isolates. The isolates SD01 and SD12 were yellowish-white. SD22 is beige, while the rest is white. The edges of the colonies were lobate except for isolates SD01 and SD12, which were undulate. The elevations of the colonies were flat for SD01, SD09, SD12, and SD13, whereas SD22 and SD29 had convex elevations.

Identification of lipolytic bacteria by gene sequences encoding 16S rRNA

Bacterial isolates that were detected to have lipase activity were identified molecularly based on the gene sequences encoding 16S rRNA. Lipolytic bacteria genomic DNA samples were amplified using PCR with 63F and 1387R. Marchesi et al. (1998) stated that the two primers could amplify the 16S rRNA coding gene sequence with a size of about 1300 base pairs. Based on the visualization of the PCR product (Figure 5), it can be seen that the six isolates had sizes between 1000 and 1500 bp.

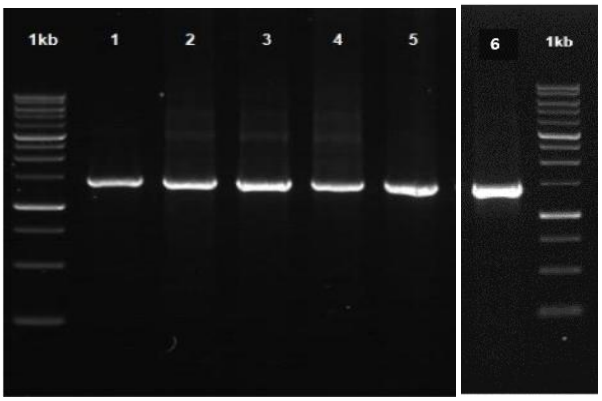

Figure 5. Electrophorogram of Lipolytic Bacteria PCR Products. Description: 1kb= Marker, 1= Isolate SD01, 2= Isolate SD09, 3= Isolate SD12, 4= Isolate SD13, 6= Isolate SD29

Table 2. Cell morphology and colony characteristics of lipolytic bacteria in the digestive tract of eels

Isolate code	Characteristics					
	Cell morphology			Colony morphology		
	Gram	Cell shape	Shape	Color	Edge	Elevation
SD01	-	Rod-shaped	Irregular	White	Undulate	Flat
SD09	-	Rod-shaped	Irregular	Yellowish white	Lobate	Flat
SD12	-	Rod-shaped	Irregular	White	Undulate	Flat
SD13	-	Rod-shaped	Irregular	Yellowish white	Lobate	Flat
SD22	-	Rod-shaped	Irregular	beige	Lobate	Convex
SD29	-	Rod-shaped	Irregular	White	Lobate	Convex

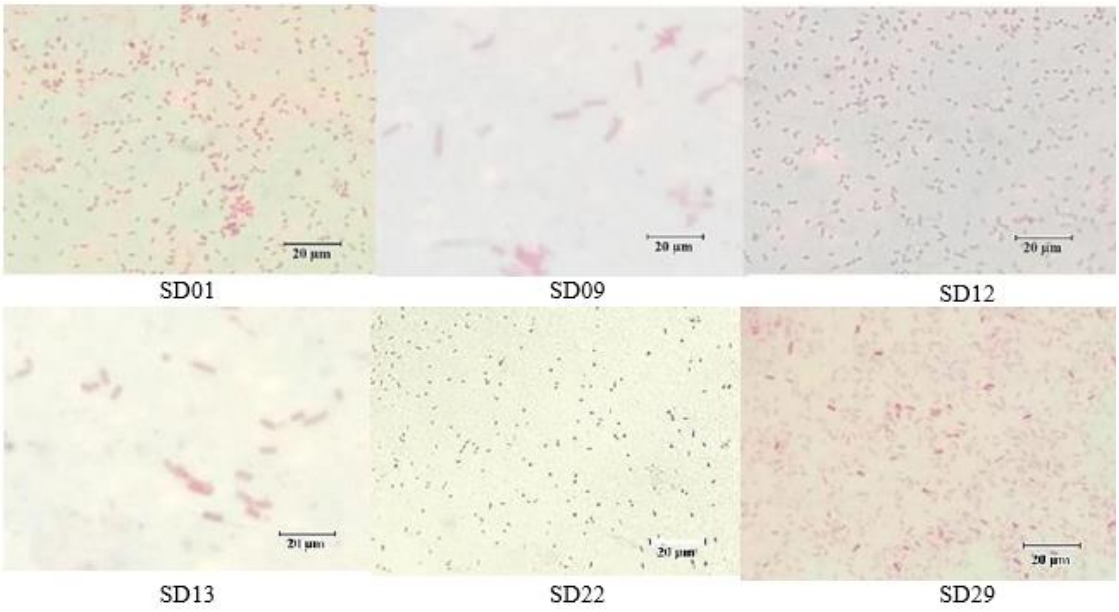

Figure 4x. Gram stain of lipolytic bacteria from the digestive tract of eel (*A. bicolor bicolor*). 1000x magnification

Table 3. BLASTN analysis of gene sequences encoding 16S rRNA for lipolytic bacteria in the digestive tract of eel

Isolate code	Identity	Query cover	Closest relatives
SD01	99%	96%	*Pseudomonas azotoformans* KGGI28
SD09	93%	92%	*Providencia vermicola* 79C1
SD12	99%	98%	*Pseudomonas azotoformans* KGGI28
SD13	94%	73%	*Providencia*
SD22	98%	92%	*Aeromonas hydrophila* M_81
SD29	99%	93%	*Aeromonas veronii* FC951

The amplicon of the 16S rRNA gene is then sequenced and will produce a sequence of nucleotide bases. The characteristics of DNA sequences were analyzed using the BLASTN program (Table 3). Janda and Abbott (2007) stated that a bacterial species is the same as the reference in GenBank if it has a minimum similarity of 97%; if the base sequence similarity is <97%, it can indicate a new species. In contrast, Hagstrom et al. (2000) stated that if the sequence of bases had a similarity of 93%-97%, it was said that the isolates had similarities at the genus level.

The BLASTN analysis that has been presented shows that of the 6 identified isolates, 4 of them had 98-99% identities, while two other isolates, namely SD09 and SD13 isolates, had 93% and 94% identities, respectively. The presence of microbiota in the digestive tract of eels in this study (Table 3) is supported by research conducted by Esteve and Garay (1991) which found much microbiota associated with *Anguilla anguilla* in freshwater, including *Aeromonas hydrophila*, *A. sobria*, *Pseudomonas azotoformans*, and *Plesiomonas shigelloides*. In his research, Huang et al. (2018) stated that the most commonly found bacteria associated with the digestive tract of *A. anguilla* eels were from the genus *Aeromonas* (52.18±23.81%). Huang et al. (2018) also stated that bacteria from the genus *Aeromonas* were more commonly found in adult eels (81.03±2.99%) than in eels in the previous phase.

The genus *Pseudomonas* has been widely known as a microorganism isolated from various natural sources, such as soil, plants, water, and aquatic animals. Fendri et al. (2010) stated that bacteria in this genus have capabilities in food technology, medicine, environmental microbiology, and natural degradation agents and produce extracellular lipase enzymes. *Pseudomonas azotoformans* is a gram-negative bacterium with rod-shaped cells measuring 0.6-0.8 x 1.4-2.0 µm, does not produce spores, is obligate aerobes, and is motile with a polar flagellum. These bacteria produce fluorescent pigments with an optimum temperature for growth in the range of 25-30º C and an optimum pH of 7 (Feliatra et al. 2004).

Research conducted by Fendri et al. (2010), *P. azotoformans* is said to be able to be used as a lipid degrading agent. This bacterium is grown in media containing 2% triacylglycerol, and it produces hydrolysis products in diacylglycerol, monoacylglycerol, and fatty acids with an optimum temperature of 30ºC and pH 6. Another study conducted by Gram et al. (2001) stated that *P. azotoformans* bacteria could play a role in suppressing the death of aquatic animals due to vibriosis in rainbow trout, and in vitro is antagonistic to *Aeromonas salmonicida*. According to research by Haba et al. (2000), the genus *Pseudomonas* is a producer of lipase enzymes with the highest lipase activity among the genera *Bacillus*, *Rhodococcus*, and *Staphylococcus*, which is 1.703 U/L, *P. azotoformans*, a lipase producer with olive oil as a substrate, has lipase activity of 4.4 units/L. L.

Providencia vermicola, belonging to the Enterobacteriaceae family, is a gram-negative bacterium. Its rod-shaped cells measure 2.14-5.0 x 0.57-0.71 µm. Colonies are round, shiny, and have a convex elevation. Bacteria species *P. vermicola* can be found as a microbiota in the digestive tract of freshwater fish (Ramkumar et al., 2014). Cultivation on NA and TSA media will produce a distinctive aroma. These bacteria can grow up to a temperature of 41ºC. *P. vermicola* can also produce acids from L-arabinose and 2-ketogluconic and L-erythritol, D-glucosamine, and D-glucuronic acid (Somvanshi et al. 2006). Tanu et al. (2012) reported that these bacteria could be isolated from the digestive tract of seahorses, and in some freshwater fish, these bacteria are pathogenic. *P. vermicola*, isolated from the digestive tract of seahorses, has a role in synthesizing extracellular lipase enzymes because seahorse feed contains a lot of unsaturated fatty acids. In his research, Bala et al. (2018) found that he could isolate *P. vermicola* bacteria from water disposal in palm oil refineries. Bala et al. (2018) also stated that the mycobacteria could be used as oil degradation agents that pollute oil refinery areas because they have lipase activity and live in areas with high lipid levels.

Aeromonas is a genus of bacteria found in aquatic areas, and several species can be found as pathogenic bacteria in aquatic animals (Ruzauskas et al., 2017). In Thenmozhi and Ahilan's (2014) research, *Aeromonas* species were isolated from *Cyprinus carpio*, namely *A. salmonicida* and *A. hydrophila*, and showed lipolytic activity. *Aeromonas salmonicida* showed lipolytic activity of 69.23%, and *A. hydrophila* showed lipolytic activity of 75%. Cahill (1990), in his research, stated that the genus *Aeromonas* is one of three bacteria other than *Pseudomonas* and *Vibrio*, which are most commonly found as microbiota in the digestive tract of freshwater fish.

Aeromonas veronii is a gram-negative, rod-shaped bacterium, ornithine decarboxylase positive, motile with polar flagella, growing at an optimum temperature of 35-37°C (Skwor et al. 2014). This bacterium is associated with leeches, found in aquatic environments and human fecal specimens, identified as a cause of disease in fish and humans by gastrointestinal tract attack (Pemberton et al. 1997). *Aeromonas veronii* is reported as a lipolytic bacterium and can produce toxins known as extracellular

products (Pramudita et al., 2013). Nawaz et al. (2010) stated that *A. veronii* has optimum lipolytic activity at 35° C and is found in the digestive tract of catfish. This bacterium can produce four different lipase enzymes that can play a role in the host infection process and damage the host cell plasma membrane.

Aeromonas hydrophila is a gram-negative, rod-shaped, facultative anaerobe, motile, resistant to tetracycline. *Aeromonas hydrophila* was isolated from fresh and marine waters, causing disease in several fish (Skwor et al. 2014). *Aeromonas hydrophila* has various sizes ranging from 1.0 to 3.5 microns in length and 0.8 to 1.0 microns in width, with the optimum temperature for growth of 28-37°C (Arwin et al. 2016). This type of bacteria is found as the main microbiota found in the digestive tract of freshwater fish. In fish cultured in ponds, this bacterium is ubiquitous due to the presence of fish feces that contaminate pond waters, which often causes disease in fish, so it can be indicated that the source of *A. hydrophila* in pond waters comes from fish feces.

Aeromonas hydrophila species were identified as decomposers of chitin in the digestive tracts of freshwater fish. Additionally, *A. hydrophila* was capable of producing extracellular enzymes capable of metabolizing cellobiose. Under anaerobic conditions with many colonies, *A. hydrophila* produced lipases with high activity (Cahill 1990). Cascon et al. (1996) stated that *A. hydrophila* could produce glycerophospholipid-cholesterol acyltransferase, which is analogous to the mammalian plasma lecithin-cholesterol acyltransferase, which is part of the lipase enzyme. Sholikhah's research (2009) stated that *A. hydrophila* produces enzymes and extracellular toxins that contain hemolytic and protease activities that cause disease in fish.

In conclusion, this study isolated 30 bacterial isolates from the digestive tract of eel (*A. bicolor bicolor*) and as many as six isolates with lipolytic activity. The identity of the lipolytic bacteria obtained from the digestive tract of eel were isolates SD01 and SD12, identified as *P. azotoformans*; isolate SD09 was identified as *P. vermicola*; SD13 was identified as *Providencia* sp.; SD22 was identified as *A. hydrophila*, and isolate SD29 was identified as *A. veronii*.

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