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Isolation and identification of lipolytic bacterial from the digestive tract of ricefield eel (*Monopterus albus*)

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Abstract. *Berlian IN, Susilowati A, Pangastuti A. 2020. Isolation and identification of lipolytic bacterial from the digestive tract of ricefield eel (*Monopterus albus*). Bioteknologi 17: 27-36.* Lipolytic bacteria are bacteria that produce lipase enzymes. These bacteria can be isolated from various sources containing fat. Lipid is one of the nutrients needed by rice field eel (*Monopterus albus* Zuiew, 1973) to live and grow. In the digestive system of fish, typhoid bacteria are used to hydrolyze lipids and oils into fatty acids and glycerol, which are needed in metabolic processes. This study aims to obtain isolates of lipolytic bacteria from the digestive tract of rice field eels and to determine the identity of lipolytic bacteria that can be isolated from the digestive tract of rice field eels. The obtained rice field eels were rested and fasted for 8 hours. Then, the eels were dissected. The digestive tracts were cleaned and used. Isolation of bacteria used minimal media enriched with olive oil. Lipolytic bacteria screening used Rhodamine B Agar. Macroscopic morphological characterizations are colony shape, edge, color, and elevation. Microscopic morphology is cell shape, cell size, and gram of bacteria. The hemolysis test aimed to determine hemolysis activity using Blood Agar (BA). Molecular characterization was based on the sequence of genes encoding 16S rRNA. The results of lipolytic bacterial DNA sequences were analyzed using the NCBI BLAST Nucleotide Website (www.blast.ncbi.nlm.nih.gov/blast.cgi). Based on the isolation results, 63 isolates were obtained with 11 isolates of positive lipolytic bacteria. Positive lipolytic bacteria were orange glow on the colony when exposed to UV light at 350 nm. Based on morphological characterization, 4 isolates were cocci cells, and 7 isolates were bacilli cells. In addition, 8 gram-negative isolates and 3 gram-positive isolates were identified. The identified isolates were *Staphylococcus saprophyticus* strain L29 98%, *Stenotrophomonas maltophilia* 99%, *Acinetobacter junii* F-27 100%, *Paenibacillus lactis* PF4J 1-2 96%, *Aeromonas dhakensis* WWi303 99%, *S. saprophyticus* P0081Karwar 99%, *Aeromonas caviae* strain R25 - 6 98%, *Acinetobacter pittii* strain BJ6 99%.

Keywords: Digestive tract, isolation and identification, lipase, lipolytic bacteria, *Monopterus albus*, rice field eel

INTRODUCTION

Enzymes are very effective biocatalysts because they can significantly increase the speed of specific chemical reactions (Lehninger 1995). In addition, enzymes have a role in transforming various types of energy (Winarno 1986). Nearly 400 enzymes have been identified, and about 200 enzymes are used commercially. Since 1960, the commercial use of enzymes has experienced rapid development due to the increasing public understanding of the use of enzymes, especially in the industrial sector (Sharma et al. 2001). Some of the commercialized enzymes include lipase, xylanase, and protease.

Lipases (*triacylglycerol acyl hydrolases*, EC 3.1.1.3) are enzymes that naturally catalyze the hydrolysis of triacylglycerol (fats/oils) into fatty acids, monoacylglycerols, diacylglycerols, and glycerol. Based on its physiological function, lipase plays an important role in the hydrolysis of fats and oils into fatty acids and glycerol, which are needed in metabolic processes. In addition, a lipase acts as a catalyst for esterification of hydrolysis reactions and transesterification, which is useful in the oleochemical and biodiesel industries (Gupta et al. 2011).

Lipases can be used as biocatalysts to produce useful biodegradable compounds, such as 2-ethyl-1-hexyl ester, which was obtained from enzymatic transesterification of rapeseed oil fatty acids, and 1-butyl oleate, which was obtained from the direct esterification of butanol and oleic acid and used to reduce the viscosity of biodiesel (Linko et al. 1998). Lipase that has been mobilized using immobead 150 from the fungus *Rizhomucor miehei* has been successfully marketed in 2010 by *Sigma Aldrich*, with the enzyme activity produced by the fungus of 381 U/g and is sold at a price of around 270 million per kilogram (Kurnia 2010). Lipase has been used for industrial activities such as the food industry, dairy industry, paper industry, textile industry, leather industry, wastewater treatment, chemical production, medicine, and cosmetics. The detergent industry uses 1,000 tons of lipase per year (Jaeger et al. 1999). The need for large enzymes encourages the exploration of these enzymes from various sources.

Currently, lipases have been isolated and purified from various sources, such as bacteria, fungi, plants, and animals (Hasan et al. 2006). Lipases from bacteria are more in demand than lipases from other organisms because they are more stable, selective, and specific on a wide range of substrates (Veerpagu et al. 2014). In addition, lipase from bacteria is more beneficial because it can work in various catalytic processes, has higher yields, genetic engineering can be carried out, is not disturbed by weather fluctuations, and has faster growth in cheap media (Hasan et al. 2006). Lipase from bacteria can also be found in the digestive tract

of animals, such as the stomach and pancreas (Saktiwansyah 2001).

Koven et al. (1994) stated that an alternative source of lipase was microbial symbiosis in the digestive tract of mammals and fish utilizing fat as a carbon source. Intestinal microbes producing lipase play a role in increasing the residence time of fat in the intestine. Bairagi et al. (2002) stated that microorganisms isolated from the digestive tract of fish could produce lipases that were beneficial in the digestive process of fish. In a study conducted by Bairagi et al. (2002), it was also found that lipolytic bacteria in the digestive tract of some fish (bacterial population per gram of the digestive tract), including *Catla catla* of $1.3x10^3$, *Labeo rohita* of $0.8x10^3$, *Cirrhinus mrigala* of $0.3x10^3$, *Hypophthalmichtys molitrix* of $5.0x10^3$, *Ctenopharyngodon idella* of $1.0x10^3$, *Cyprinus carpio* of $4.3x10^3$, *Oreochromis mossambica* of $1.6x10^3$, *Clarias batrachus* of $0.7x10^3$, and *Channa punctatus* of $0.3x10^3$. In addition, according to Dhage (1968), lipase activity in *C. mrigala* and *L. rohita* was concentrated in the anterior intestine. Lipase is the main enzyme involved in digesting triglycerides in all vertebrates. Some information states that lipase from fish intestines comes from endogenous sources (Bairagi et al. 2002).

Monopterus albus (Zuiew, 1973) is a type of fish that is easy to find, especially in rice fields, easy to cultivate, and relatively inexpensive. *M. albus* is a carnivorous fish with a hormonal and enzymatic digestive system (Roy 2009). Fat is one of the nutrients needed by *M. albus* to live and grow. Carnivorous fish can digest and utilize fat more efficiently than omnivorous and herbivorous fish (Buwono 2000). The fat needed by eels is 3-4% in the feed. The high fat consumed by fish and which is not used as an energy source is stored as body fat (Haryati 2011). In the study of Li et al. (2011), the fat content in *M. albus* (1.71 ± 0.04 per 500 grams) was higher than the fat content in *Monopterus cuchia* ($0.695 \pm 0.05\%$ per 100 ± 7.25 grams). Therefore, the digestive tract of *M. albus* allows the presence of lipolytic bacteria that have the potential as a source of lipase production. This enzyme can be used as a feed probiotic for *M. albus*. From this, efforts to isolate lipolytic bacteria need to be carried out, especially for biotechnology applications (Mubarak et al. 2011). In addition, efforts to identify the obtained bacteria are important to obtain information about these bacteria. Previously, Ridwan et al. (2019) found lactic acid bacteria, *Lactococcus lactis*, from the digestive tract of *M. albus*.

The aims of this study were (i) to obtain isolates of lipolytic bacteria from the digestive tract of *M. Albus*; (ii) to obtain the identity of the type of lipolytic bacteria isolated from the digestive tract of *M. albus*.

MATERIALS AND METHODS

Research time and place

This research was conducted in April-August 2018 at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia.

Materials

The lipolytic bacteria isolated and identified in this study were sourced from the digestive tract of *M. albus* with body lengths ranging from 30-40 cm taken from 2 places, namely the cultivation of *M. albus* in Bendosari, Sukoharjo, which is an artificial habitat and the rice fields of Waru Village, Baki, Sukoharjo, Central Java, Indonesia, which are natural habitats.

Procedure

The obtained *M. albus* were rested and fasted for 8 hours to minimize the work of enzymes and to minimize food entering the digestive tract. After that, the eel 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 eel to the lateral line, and then cutting towards the anal part of the fish. Next, the stomach and intestines are taken, then the contents of the intestine are removed to maximize the yield of obtained enzymes. Next, the stomach and intestines were cut, then mashed and homogenized by adding sterile 0.85% NaCl then vortexed to produce a homogenate (Kurniawan 2016).

Lipolytic bacteria isolation

One gram of homogenate was taken, crushed, and dissolved in 9 mL of Physiological Saline Solution 0.85%. Next, the solution is vortexed evenly, and the solid is allowed to settle. The dilution was carried out up to a dilution series of 10^{-4}, but for isolation, samples were taken with a dilution series of 10^{-3} and 10^{-4}. Then the microorganism was planted on minimal agar enriched with olive oil using the spread plate method. It was done by inserting 0.1 mL of the microorganism source in each petri dish and then leveling it using a drigalsky with two repetitions of each dilution series (Susanty et al. 2013). The resulting isolates were incubated for 48-72 hours at a temperature of 27^0C. Finally, each colony with different morphology was selected as a candidate for lipolytic bacteria and stored in agar slanted at 4^0C (Gayathri et al. 2013).

Lipolytic bacteria screening

According to Ray et al. (2012), bacterial screening of pure bacterial isolates was carried out by taking 1 ose of isolate and then streaking it on Rhodamine B agar media. Then it was incubated at 27^0C for 48 hours. Positive results were seen in the hydrolysis of olive oil into fatty acids, which then interacted with Rhodamine B so that pink colonies appeared and could glow when irradiated using an Ultra Violet lamp with a wavelength of 350 nm.

Colony morphology characterization of lipolytic bacteria

Morphological observation

The morphological observation of lipolytic bacteria was by observing the colonies growing on NA media. The morphological characterizations of the colonies included color, shape, margins of bacterial colonies, and elevation. Based on the morphological character of each bacterial isolate that has lipolytic activity, it was identified using *Bergey's Manual of Systematic Bacteriology Second*

Edition Volume Two, The Proteobacteria (Garrity 2009) and *Bergey's Manual of Systematic Bacteriology Second Edition Volume Three, The Firmicutes* (Whitman 2009).

Gram stain

One ose of the bacterial isolate was taken and scratched on the surface of a sterile preparation and then fixed. One drop of crystal violet was added to the surface of the preparation containing the bacterial layer and allowed to stand for one minute. After one minute, the preparations were rinsed using distilled water until the dye faded. The preparations were dried over the fire of denatured alcohol. Then one drop of iodine solution was added to the surface of the preparations and allowed to stand for one minute. After one minute, the preparations were rinsed using distilled water. The preparations were rinsed with 96% alcohol until all the dye had faded. Then the preparations were rinsed using distilled water. The preparations were dried over the fire of denatured alcohol, and then one drop of safranin was added to the surface of the preparations and allowed to stand for 45 seconds. Finally, the preparations were washed using distilled water and dried. Furthermore, the preparations were observed using a light microscope with a magnification of 1000x to observe cell shape and determine the gram in bacteria (Pratita and Putra 2012).

Hemolysis test

Isolates were tested to determine their pathogenicity by hemolysis test. The isolates were grown on Blood Agar media with the addition of 5% sheep blood and then incubated for 18-24 hours at 37^0C. The appearance of a clear zone around the colony after 18 hours of incubation at 37^0C was considered a positive result of hemolysin production (Osek 2004).

Characterization of lipolytic bacteria with sequences of gene encoding 16S rRNA

DNA extraction

Extraction of lipolytic bacterial genomic DNA obtained from the screening process was carried out using the Presto^{TM} Mini gDNA Bacteria Kit.

Amplification

Amplification of the gene encoding 16s rRNA for lipolytic bacteria used 63 forward primer (63f: 5'CAGGCCTAACACATGCAAGTC-3'); and 1387 reverse primer (1387r:5'-GGGCGGAWGTGTACAAGGC-3'). The PCR reaction was carried out by mixing 1 μL of 63 forward primers with a concentration of 10 pmol, 1 μL of 1387 reverse primer with a concentration of 10pmol, 25 μL of MyTaq^{TM} HS Red Mix 2x, and 2 μL of DNA template, and 21 μL of ddH_2O. Pre-denaturation was carried out at 95^0C for 3 minutes. One PCR cycle of 30 cycles consisted of denaturation at 95^0C for 15 seconds, annealing at 56^0C for 15 seconds, elongation at 72^0C for 30 seconds, and finalizing at 72^0C for 2 minutes. Next, the PCR was stopped, and the PCR amplification product was stored at 4^0C. The PCR amplification products were transferred using gel electrophoresis (Marchesi et al. 1998).

Sequencing

Sequencing the PCR amplification of the gene encoding 16S rRNA for lipolytic bacteria was carried out at 1st Base Singapore.

Data analysis

The lipolytic bacterial isolates were obtained by morphological observations. They included the observations of macroscopic morphology (colony shape, color, margins, and elevation) and microscopic morphological observations (cell shape, gram bacteria, cell size). The hemolysis test results were analyzed descriptively. The identity of lipolytic bacterial DNA sequences resulting from the extraction process was 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 gastrointestinal tract of *M. albus*

In this study, the sources of lipolytic bacteria were the digestive tract of wild and cultivated eels. Therefore, they were taken in three repetitions. The repetition was done to obtain varied results so that various species of lipolytic bacteria could be compared and obtained. In this study, 63 bacterial isolates were obtained, consisting of 33 bacterial isolates from the digestive tract of wild eels (Table 1) and 30 bacterial isolates from the digestive tract of cultivated eels (Table 2). The isolate code consisted of BL or BB, which indicated that BL was from wild *M. albus* and BB was from cultivated *M. albus*; the number beside BL/BB indicates that the isolate was done at week 1, week 2, or week 3; the numbers next to it indicated the length of the isolated eel, and the letter at the end of the code indicates the sequence of isolates in one eel of the same size in the same week.

Vishwanath et al. (1998) stated that the fat content in the digestive tract of *M. albus* ranged from 10.74 ± 0.48% DWB. Lipolytic bacteria can be found in an environment containing fat or oil because that environment provides a good substrate for the growth of lipolytic bacteria (Swandi et al. 2015). According to Haryati (2011), fat consumed by fish but not used as an energy source will be stored as body fat so that it can trigger the growth of lipolytic bacteria.

Isolation of lipolytic bacteria in this study was carried out using minimal media enriched with olive oil. Olive oil acts as a carbon source needed for the growth of lipolytic bacteria. Therefore, the minimal media was chosen so that the bacteria only got a carbon source from olive oil so that lipolytic-specific bacteria were obtained. Furthermore, olive oil was chosen because it is easy to obtain and is a substrate that is easy to use by bacteria (Carissimi et al. 2007).

Table 1. Bacterial isolates from the digestive tract of wild *M. albus* obtained from each sampling

Sample length (cm)	Number of isolates	Isolate code
38	-	-
42	1	BL.1.42
38	8	BL.2.38.A; BL.2.38.B; BL.2.38.C; BL.2.38.D; BL.2.38.E; BL.2.38.F; BL.2.38.G;BL.2.38.H;
35	6	BL.2.35.A; BL.2.35.B; BL.2.35.C; BL.2.35.D; BL.2.35.E; BL.2.35.F
34.5	5	BL.3.34.5.A; BL.3.34.5.B; BL.3.34.5.C;BL.3.34.5.D; BL.3.34.5.E
36.5	13	BL.3.36.5.A; BL.3.36.5.B; BL.3.36.5.C; BL.3.36.5.D; BL.3.36.5.E; BL.3.36.5.F; BL.3.36.5.G; BL.3.36.5.H; BL.3.36.5.I; BL.3.36.5.J; BL.3.36.5.K; BL.3.36.5.L;BL.3.36.5.M
Total isolates		33

Table 2. Bacterial isolates from the digestive tract of cultivated *M. albus* obtained from each sampling

Sample length (cm)	Number of isolates	Isolate code
32.5	1	BB.1.32.5
33.5	2	BB.1.33.5.A; BB.1.33.5.B
27.5	16	BB.2.27.5.A; BB.2.27.5.B; BB.2.27.5.C; BB.2.27.5.D; BB.2.27.5.E; BB.2.27.5.F; BB.2.27.5.G; BB.2.27.5.H; BB.2.27.5.I; BB.2.27.5.J; BB.2.27.5.K; BB.2.27.5.L; BB.2.27.5.M; BB.2.27.5.N; BB.2.27.5.O;BB.2.27.5.P
27	-	-
31	8	BB.3.31.A; BB.3.31.B; BB.3.31.C; BB.3.31.D; BB.3.31.E; BB.3.31.F; BB.3.31.G; BB.3.31.H
29	3	BB.3.29.A; BB.3.29.B; BB.3.29.C
Total isolates		30

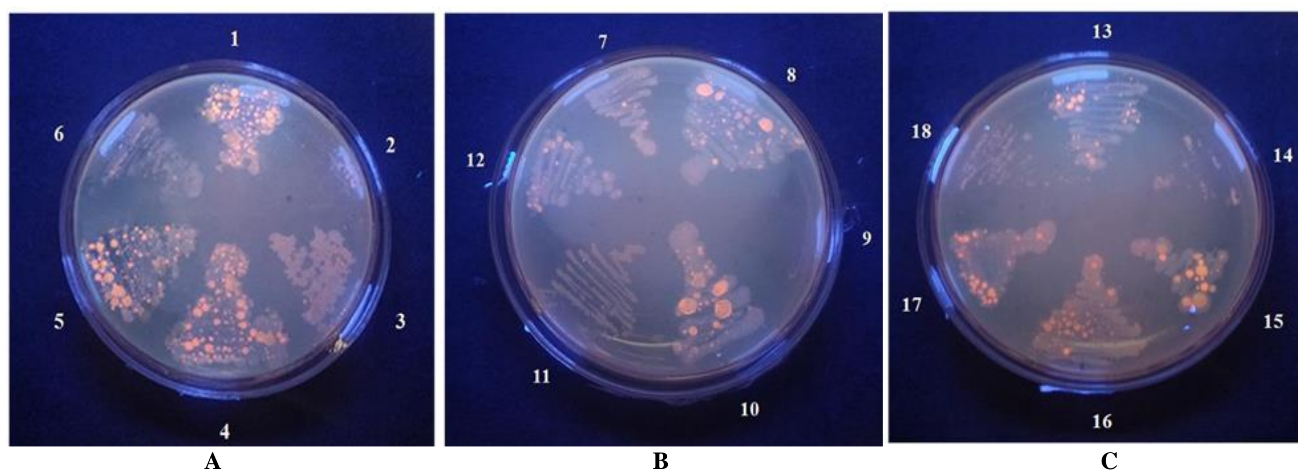

Figure 1. Colonies of lipolytic bacteria on Rhodamine B agar media under exposure to 350 nm UV lamp. Lipolytic positive isolates (BB.1.33.5.A (1); BB.1.32.5.A (4); BL.2.38.F (5); BB.2.27.5.C (7); BB.3.31. A (8); BB.3.31.D (10); BB.3.31.F (12); BL.3.36.5.A (13); BL.3.36.5.D (15); BL.3.36.5 .J (16); BL.3.34.5.A (17)) were shown by fluorescent orange under a 350 nm UV lamp

The lipolytic bacteria screening used Rhodamine B Agar media. Lipolytic activity was indicated by the presence of isolates that glowed orange when exposed to UV light with a wavelength of 350 nm (Figure 1). Rhodamine B Agar was chosen because it is a sensitive fluorescent indicator for lipase. A fluorescent indicator in the form of lipase-producing bacteria will produce an orange complex on Rhodamine B Agar media when exposed to UV light with a wavelength of 350 nm (Telussa 2013). The formation of the orange complex is based on the change of the acid to the cation form and the formation of a complex with uranyl fatty acid ions which are inversely proportional to the fatty acid long chain to produce an orange glow excitation on exposure to UV light with a wavelength of 350 nm (Carissimi et al. 2007).

Based on the results of lipolytic bacteria screening, there were 11 isolates that had lipolytic activity, consisting of 6 isolates from cultivated *M. albus* and 5 isolates from wild *M. albus*. The isolates were BB.1.33.5.A; BB.1.32.5.A; BL.2.38.F; BB.2.27.5.C; BB.3.31.A; BB.3.31.D; BB.3.31.F; BL.3.36.5.A; BL.3.36.5.D; BL.3.36.5.J; BL.3.34.5.A. The results showed that several bacterial isolates with lipolytic activity were found in the digestive tract of wild and cultivated *M. albus*. This result is in accordance with the statement of Bairagi et al. (2012) that lipolytic bacteria can be isolated from the digestive tract of fish. According to Hungate (1996), lipolytic bacteria in the digestive tract play a role in the hydrolysis of fats into glycerol and fatty acids.

Morphological characteristics of lipolytic bacteria in the digestive tract of *M. albus*

The observed morphological characters of 11 bacterial isolates with lipolytic activity included macroscopic and microscopic characters. Macroscopic characters included colony shape, edge, color, and elevation. At the same time, microscopic characters included gram, cell shape, and cell size. The macroscopic morphological characteristics of lipolytic bacterial colonies are listed in Table 3, while the microscopic morphological characteristics of lipolytic bacterial colonies are shown in Table 4 and Figure 2.

Based on microscopic morphological characters, isolate BB.1.33.5.A (1) and isolate BB.3.31.D (6) had round cells (coccus) with purple cells indicating gram-positive. Isolate BB.2.27.5.C (4) was also gram-positive but had the form of rod cells (bacillus). Isolate BB.2.38.F (3) and isolate BL.3.36.5.J (10) had a round cell shape (coccus) with red cells indicating gram-negative. While the other isolates, namely, BB.1.32.5.A (2); BB.3.31.A (5); BB.3.31.F (7); BL.3.36.5.A (8); BL.3.36.5.D (9); and BL.3.34.5.A (11) had the same microscopic morphology, namely the shape of rod cells (bacillus) with red cell color indicating gram-negative. All lipolytic bacteria isolates had cell sizes ranging from 0.8-1.25 µm.

Hemolysis test on lipolytic bacterial isolate

Isolates of lipolytic bacteria were tested for hemolysis using BA (Blood Agar) media to determine the pathogenic potential. After incubation for 1x24 hours, the results of the hemolysis test were obtained, as shown in Table 5 and Figure 3.

Table 3. Macroscopic morphology of lipolytic bacterial colonies on NA media

No.	Isolation code	Shape	Edge	Color	Elevation
1	BB.1.33.5.A	*Circulair*	*Entire*	Beige	*Raised*
2	BB.1.32.5.A	*Irregular*	*Undulate*	Yellow	*Raised*
3	BL.2.38.F	*Circulair*	*Entire*	Beige	*Convex*
4	BB.2.27.5.C	*Filamentous*	*Lobate*	White	*Convex*
5	BB.3.31.A	*Circulair*	*Entire*	White	*Convex*
6	BB.3.31.D	*Circulair*	*Entire*	Beige	*Raised*
7	BB.3.31.F	*Circulair*	*Entire*	White	*Convex*
8	BL.3.36.5.A	*Circulair*	*Entire*	White	*Convex*
9	BL.3.36.5.D	*Circulair*	*Entire*	White	*Convex*
10	BL.3.36.5.J	*Circulair*	*Entire*	Beige	*Convex*
11	BL.3.34.5.A	*Circulair*	*Entire*	White	*Convex*

Table 4. Microscopic morphology of lipolytic bacterial cells

	Isolate code	Gram	Cell shape	Size (± µm)
1	BB.1.33.5.A	Positive	round (*coccus*)	0.83
2	BB.1.32.5.A	Negative	rod (*bacillus*)	1.2
3	BL.2.38.F	Negative	round (*coccus*)	1.25
4	BB.2.27.5.C	Positive	rod (*bacillus*)	1
5	BB.3.31.A	Negative	rod (*bacillus*)	0.8
6	BB.3.31.D	Positive	round(*coccus*)	0.8
7	BB.3.31.F	Negative	rod (*bacillus*)	0.83
8	BL.3.36.5.A	Negative	rod (*bacillus*)	1
9	BL.3.36.5.D	Negative	rod (*bacillus*)	1
10	BL.3.36.5.J	Negative	round (*coccus*)	1
11	BL.3.34.5.A	Negative	rod (*bacillus*)	1

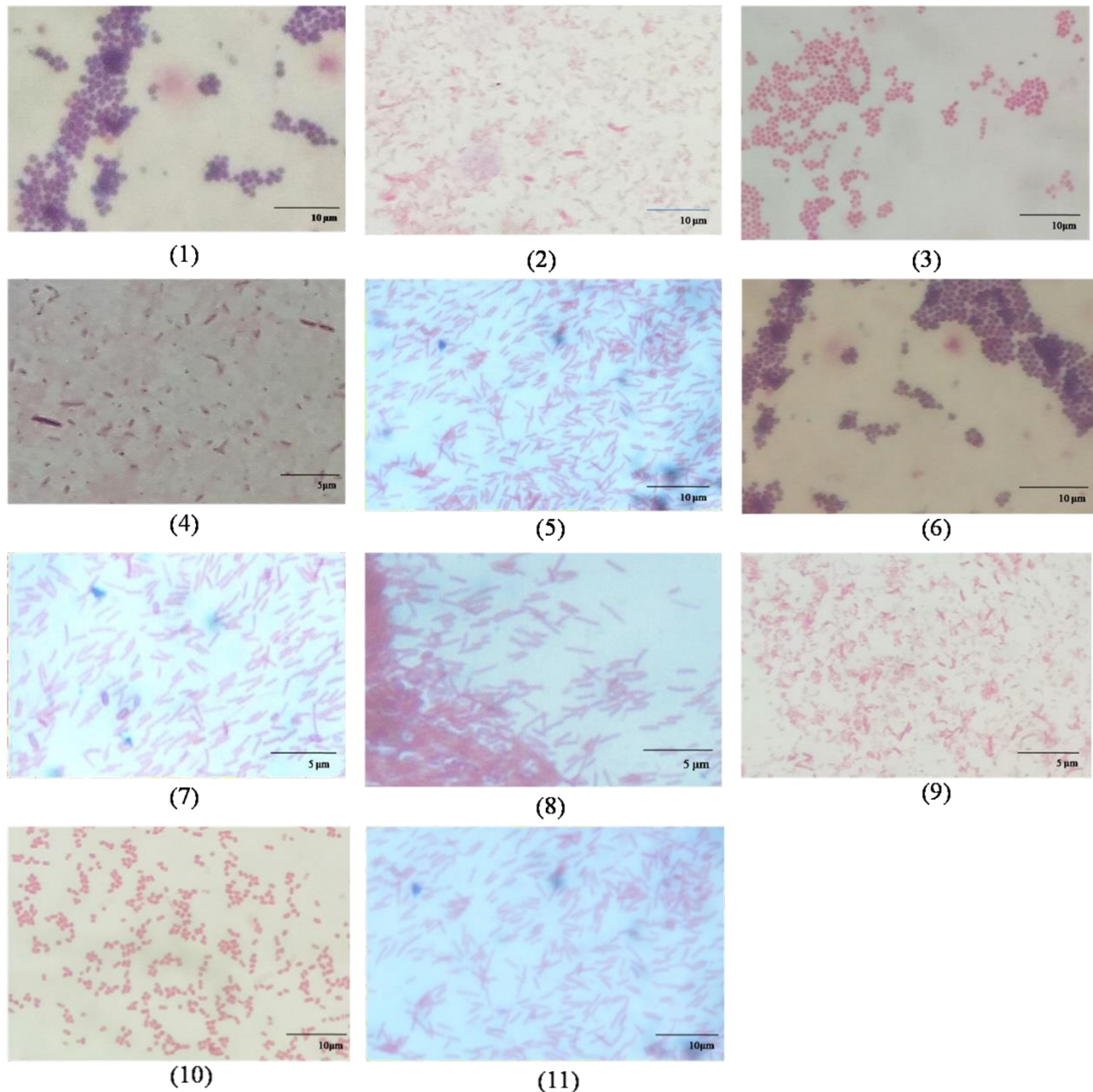

Figure 2. Macroscopic morphological character of lipolytic bacteria. Note: See Table 5 for isolate code

Table 5. Results of hemolysis test on lipolytic bacteria

No.	Isolation code	Clear zone	Hemolytic activity
1	BB.1.33.5.A	Absent	Negative
2	BB.1.32.5.A	Absent	Negative
3	BL.2.38.F	Present	Positive
4	BB.2.27.5.C	Absent	Negative
5	BB.3.31.A	Present	Positive
6	BB.3.31.D	Absent	Negative
7	BB.3.31.F	Present	Positive
8	BL.3.36.5.A	Present	Positive
9	BL.3.36.5.D	Present	Positive
10	BL.3.34.5.A	Absent	Negative
11	BL.3.36.5.J	Present	Positive

Isolate BB.1.33.5.A (1); Isolate BB.1.32.5.A (2); Isolate BL.2.27.5.C (4); Isolate BB.3.31.D (6); and Isolate BL.3.34,5.A (10) were bacteria with no hemolytic activity (gamma hemolysis (γ)) because they were colorless (clear) when they were planted in the media. They did not lyse the media, so no clear zone was formed around the colony. Meanwhile, the other 6 isolates, namely Isolate BL.2.38.F (3); Isolate BB.3.31.A (5); Isolate BB.3.31.F (7); Isolate BL.3.36.5.A (8); Isolate BL.3.36.5.D (9); Isolate BL.3.36.5.J (11) were bacteria with hemolysis activity (beta hemolysis (β)) because they were cloudy when they were planted in the media and lysed the media so that a clear zone was formed around the colony. So, when viewed from the hemolytic activity of 11 lipolytic bacteria isolates, only 5 isolates could be used as candidates for industrial-scale applications. These 5 isolates consisted of 2 from the digestive tract of wild *M. albus* and 3 from the digestive tract of cultivated *M. albus*. However, it is still necessary to carry out other pathogenicity tests on these 5 isolates.

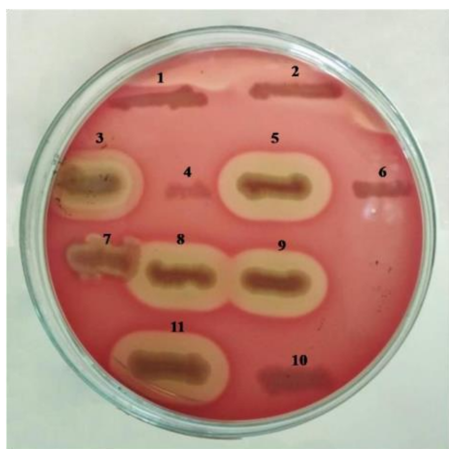

Figure 3. Hemolysis test on lipolytic bacteria. Isolate BL.2.38.F (3); Isolate BB.3.31.A (5); Isolate BB.3.31.F (7); Isolate BL.3.36.5.A (8); Isolate BL.3.36.5.D (9); and Isolate BL.3.36.5.J (11) had hemolytic activity (beta hemolysis (β)), which was indicated by the presence of a clear zone around isolate

The color change from blood red to dark color (transparent colorless) indicated growing bacterial colonies. This situation indicated incubation. The formation of the hemolysis zone was caused by bacteria secreting active glycolipid compounds in a hydrophilic substrate (Maneerat et al. 2007). In addition, some bacteria produce cytosolic, which can dissolve red blood cells. In this test, BA (Blood Agar) media was used to help the growth of microorganisms that were difficult to culture and to distinguish groups of microorganisms that did or did not lyse red blood cells (Sari et al. 2015).

Lipolytic bacterial species based on sequence analysis of the 16S rRNA encoding gene

Molecular identification of lipolytic bacterial isolates was based on the 16S rRNA encoding gene sequence (Pangastuti 2006). The lipolytic bacterial genome DNA samples were amplified using PCR (Polymerase Chain Reaction) with primers of 63F and 1387R. The primer pair 63F and 1387R can amplify the 16S rRNA coding gene sequence with a size of about 1,300 base pairs (Marchesi et al. 1998). In this study, the obtained PCR products were 11 isolates of lipolytic bacteria with almost equal size, consisting of about 1,300 base pairs (Figure 4). All 16S rRNA gene amplicons were sequenced to produce a sequence of nucleotide bases for the 16S rRNA encoding gene (Appendix 1). The results are processed using the BLASTN program and then synchronized with the database in the Gene Bank so that the percentage of similarity is obtained.

The obtained identity value determines the level of isolates similarity with the database on the Gene Bank. Hall (2001) stated that the higher identity value indicated higher similarity with the reference sequence in the Gene Bank. According to Janda and Abbot (2007), a bacterial species is said to be the same if it has a homology of more than or equal to 97%. Based on BLASTN analysis, 10 isolates of lipolytic bacteria had a similar percentage of 98% - 100% with the database in the Gene Bank, while 1 other isolate (BL.2.38.F) had a percentage similarity of 96% with the database in the Gene Bank (Table 6).

Staphylococcus is a group of gram-positive bacteria, and most of them are facultative anaerobic bacteria. This genus can be isolated from water, air, sand, soil, and various animal products such as meat, milk, and cheese. Some species are opportunistic pathogenic bacteria for humans and/or animals (Whitman 2009). In a research conducted by Tanasupawat et al. (1991) in the digestive tract of fish, there are *Staphylococcus* species that have lipolytic activity using *Tween 80* media, namely *Staphylococcus gallinarum*. Kurniasih et al. (2014) stated that *Staphylococcus* has lipolytic activity and is a candidate for probiotic bacteria in catfish feed because it can increase digestibility. Isolate BB.1.33.5.A and isolate BB.3.31.D were identified as *Staphylococcus saprophyticus*. This species is a gram-positive bacterium that belongs to the facultative anaerobic bacteria of the genus Staphylococcus. This bacterium grows optimally at a temperature of 28-35^{0}C. According to Sakinc et al. (2007), *S. saprophyticus* shows optimum lipolytic activity at a temperature of 30^{0}C and pH 6 but can lose its activity at a pH of 4.8-5. Isolate BB.1.33.5.A and Isolate BB.3.31.D showed non-pathogenic in the hemolysis test.

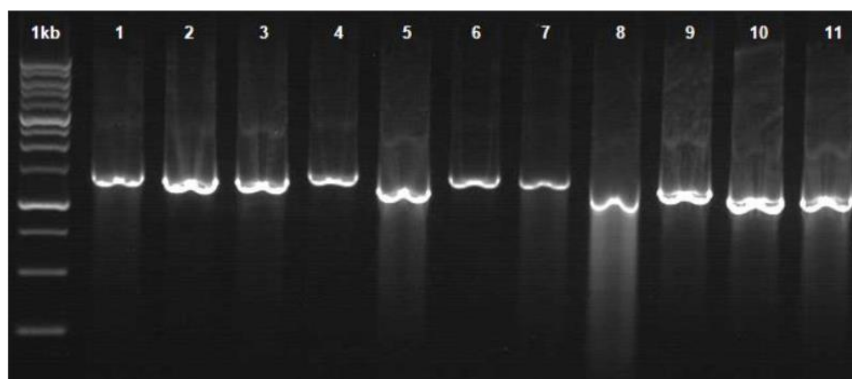

Figure 4. Electrophorogram of lipolytic bacteria PCR products. Note: M: Marker, 1: Isolate BB.1.33,5.A; 2: Isolate BB.1.32,5.A; 3: Isolate BL.2.38.F, 4: Isolate BB.2.27,5.C; 5: Isolate BB.3.31.A; 6: Isolate BB.3.31.D; 7: Isolate BB.3.31.F; 8: Isolate BL.3.36,5.A; 9: Isolate BL.3.36,5.D; 10: BL.3.34,5.A; 11: Isolate BL.3.36,5.J (Documentation: Genetics Science 2018)

Table 6. BLASTN analysis of 16S rRNA encoding gene sequences for lipolytic bacteria in the digestive tract of *M. albus*

Isolate name	Identity	Coverage	Closest relatives
BB.1.33.5.A	98%	95%	*Staphylococcus saprophyticus* strain L29
BB.1.32.5.A	99%	100%	*Stenotrophomonas maltophilia*
BB.2.38.F	100%	100%	*Acinetobacter junii* strain F-27
BL.2.27.5.C	96%	98%	*Paenibacillus lactis* strain PF4J 1-2
BB.3.31.A	99%	100%	*Aeromonas dhakensis* strain WWi303
BB.3.31.D	99%	100%	*Staphylococcus saprophyticus* strain P0081Karwar
BB.3.31.F	99%	100%	*Aeromonas dhakensis* strain WWi303
BL.3.36.5.A	99%	100%	*Aeromonas dhakensis* strain WWi303
BL.3.36.5.D	98%	99%	*Aeromonas caviae* strain R25-6
BL.3.34.5.A	99%	100%	*Acinetobacter pittii* strain BJ6
BL.3.36.5.J	99%	100%	*Aeromonas dhakensis* strain WWi303

Stenotrophomonas is a group of gram-negative bacteria that are motile using two or more flagella and are opportunistic pathogenic bacteria. These bacteria grow optimally at a temperature of 35^{0}C and have high lipolytic activity. *Stenotrophomonas maltophilia* is a species that has high lipolytic activity because it contains esterase in the outer membrane of bacteria which acts as a substrate for growth (Garrity 2009). Hasan-Beikdashti et al. (2012) succeeded in optimizing lipase production from *S. maltophilia* isolated from soil and found optimal by adding peptone, yeast extract, olive oil, and FeSO4 to the media. Isolate BB.1.32.5.A, identified as *S. maltophilia,* showed non-pathogenicity in the hemolysis test.

Acinetobacter is a genus of gram-negative bacteria and a common cause of human infections such as endocarditis, meningitis, and bacteremia. Species belonging to this genus are widely found in soil, water, and dry environments (Yakut et al. 2016). *Acinetobacter* can also be isolated from spoiled food, waste material, and contaminated soil (Musa and Adebayo-Tayo 2012). Based on Joseph et al. (2007), *Acinetobacter* is one of several lipase-producing microorganisms capable of producing lipase at a low temperature of around 5^{0}C. Musa and Adebayo-Tayo (2012) stated that *Acinetobacter* had the highest lipase activity during 7 hours of incubation compared to other bacteria such as *Arthrobacter* sp., *Brevibacterium* sp., *Staphylococcus* sp., *Yersinia* sp., *Lactobacillus* sp., *Citrobacter* sp., *Streptococcus* sp., *Acidomonas* sp., *Acetobacterium* sp., *Bacillus* sp., *Serratia marcescens*, and *Aeromonas hydrophila*. *Acinetobacter* ranks first as the bacteria that produces the highest lipase, with an average lipase production of 1.25 U/mL-8.65 U/mL. In this study, there were 2 species of the genus *Acinetobacter*, namely *Acinetobacter junii* and *Acinetobacter pittii*. The *A. junii* is an aerobic, gram-negative, opportunistic bacterial species with a size ranging from 0.9-1.6 m and grows optimally at a temperature of 15^0-35^{0}C (Garrity 2009). Isolate BL.2.38.F is a species of *A. junii*, but in the hemolysis test, this bacterium was positive for pathogens with the formation of a clear zone around the bacterial colony, so isolate BL.2.38.F was not possible to be developed for lipase production. The *A. pittii* is a gram-negative bacterium that infects humans (Atrouni et al. 2016). Isolate BB.3.34.5.A, identified as *A. pittii*, showed non-pathogenicity in the hemolysis test.

Paenibacillus lactis is a gram-positive bacterium with rod-shaped cells. These bacteria are pathogenic to insects and can be found in humus-rich soils because these bacteria play a role in the process of extracellular carbohydrate secretion and enzyme secretion. In addition, *P. lactis* can be found in waters, as shown by the research of Rawat et al. in 2018. The *P. lactis* was isolated from the Alaknada and Bhagirathi Rivers. Isolate BB.2.27.5.C, identified as *P. lactis,* did not show any hemolytic activity in the hemolysis test.

Aeromonas is a gram-negative bacterium that can grow optimally at a temperature of 22^{0}C-37^{0}C. *Aeromonas* is generally found abundantly in waters, brackish water, chlorinated water, and wastewater. Several species in this genus cause disease in humans, amphibians, freshwater fish, saltwater fish, and invertebrates (Garrity 2009). In Thenmozhi et al. (2015), Aeromonas species were isolated from *C. carpio*, namely *Aeromonas salmonicida* and *A. hydrophila,* and showed lipolytic activity. The *A. salmonicida* showed lipolytic activity of 69.23%, and *A. hydrophila* showed lipolytic activity of 75%. Divakar et al. (2012) showed that *Aeromonas caviae* AU04 is a bacterium that produces extracellular lipase and is thermostable. Lipase secretion by *A. caviae* AU04 was optimum at 31^{0}C and pH 7.0. According to the hemolysis test, isolate Bl.3.36.5.D identified as *A. caviae* was a positive bacterium with lipolytic activity, so it was not possible to develop this isolate in lipase production. Isolate BB.3.31.A; Isolate BB.3.31.F; Isolate BB.3.36.5.A; Isolate BL.3.36.5.J were identified as *Aeromonas dhakensis*. It is a subspecies of *A. hydrophila* that can grow optimally at a temperature of 28^{0}C (Garrity 2009). The *A. dhakensis* is a pathogenic bacterium that complies with the hemolysis test on Isolate BB.3.31.A; Isolate BB.3.31.F; Isolate BB.3.36.5.A; Isolate BL.3.36.5.J that all isolates were pathogenic. This condition made it impossible for these isolates to be developed in lipase production.

The *S. saprophyticus* (Isolate BB.1.33.5.A and Isolate BB.3.31.D); *S. maltophilia* (Isolate BB.1.32.5.A); *A. pittii* (Isolate BB.3.34.5.A); *P. lactis* (Isolate BB.2.27.5.C) are some of the species obtained in this study which allow it to be developed to the next stage, but other pathogenicity tests still need to be carried out to ensure these species are safe for lipase production.

In conclusion, 63 isolates of bacteria were isolated from the digestive tract of *M. albus*, consisting of 33 isolates of bacteria from the digestive tract of wild *M. albus* and 30 isolates of bacteria from the digestive tract of cultivated *M. albus*. 11 isolates had a lipolytic activity with 5 isolates being non-hemolytic, namely BB.1.33.5.A; BB.1.32.5.A; BB.2.27.5.C; BB.3.31.D; BL.3.34,5.A and 6 isolates were hemolytic, namely BL.2.38.F; BB.3.31.A; BB.3.31.F; BL.3.36.5.A; BL.3.36.5.D; BL.3.36.5.J. Based on the sequence of the gene encoding 16S rRNA, isolate BB.1.33.5.A was identified as *S. saprophyticus* strain L29 by 98%; Isolate BB.1.32.5.A was identified as 99% of *S. maltophilia*; Isolate BL.2.38.F was identified as *A. junii* F-27 by 100%; Isolate BB.2.27.5.C was identified as *P. lactis* PF4J 1-2 by 96%; Isolate BB.3.31.A, Isolate BB.3.31.F, Isolate BL.3.36.5.A, Isolate BL.3.36.5.J were identified as 99% of *A. dhakensis* WWi303; Isolate BB.3.31.D was identified as *S. saprophyticus* P0081Karwar by 99%; Isolate BL.3.36.5.D was identified as *A. caviae* strain R25-6 by 98%, and Isolate BL.3.34.5.A was identified as 99% of *A. pittii* strain BJ6.

REFERENCES

Atrouni AA, Guilolu MLJ, Hamze M, Kempf M. 2016. Emergence of NDM-1 and OXA-72 producing *Acinetobacter pittii* clinical isolates in Lebanon. New Microbes New Infect 12: 43-44. DOI: 10.1016/j.nmni.2016.04.005.

Bairagi A, Sarkar G, Sen KSK, Ray AK. 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquac Intl 10: 109-121. DOI: 10.1023/A:1021355406412.

Buwono ID. 2000. Kebutuhan Asam Amino Esensial dalam Ransum Ikan. Kanisius, Yogyakarta. [Indonesian]

Carissimi M, Stopiglia CDO, de Souza TF, Corbellini VA, Scroferneker ML. 2007. Comparison of lipolytic activity of *Sporothrix schenckii* strains utilizing olive oil-rhodamine b and tween 80. Tecno-Lógica 11 (1-2): 33-36.

Dhage KP. 1968. Studies of the digestive enzymes in the three species of the major carps of India. J Biol Sci 11: 63-74.

Divakar NVK, Nandhinidevi G, Gautam P. 2012. Lipase from *Aeromonas caviae* AU04: Isolation, purification and protein aggregation. Biocatal Agric Biotechnol 1: 45-50. DOI: 10.1016/j.bcab.2011.08.004.

Garrity GM, Brenner DJ, Staley JT. 2009. Bergey's Manual of Systematic Bacteriology. Second Edition. The Proteobacteria. Department of Microbiology and Molecular Genetics Michigan State University, USA.

Gayathri VR, Perumal P, Mathew LP, Prakash B. 2013. Screening and molecular characterization of extracellular lipase producing *Bacillus* species from coconut oil mill soil. Intl J Food Sci 2 (7): 502-509.

Gupta P, Upadhyay LSB, Shrivastava R. 2011. Lipase catalyzed-transesterification of vegetable oils by lipolitic bacteria. Res J of Microbiol 6 (3): 281-288. DOI: 10.3923/jm.2011.281.288.

Hall BG. 2001. Phylogenetic Trees Made Easy: A How to Manual for Molecular Biologist. Sinaeur Associates, Inc., Sunderland.

Haryati. 2011. Substitusi tepung ikan dengan tepung maggot terhadap retensi nutrisi, komposisi tubuh, dan efisiensi pakan ikan bandeng (*Chanos chanos* Forskal). J Iktiologi Indonesia 11(2): 185-194. [Indonesian]

Hasan F, Shah AA, Hameed A. 2006. Industrial applications of microbial lipases. Enzyme Microb Technol 39 (2): 235-251. DOI: 10.1016/j.enzmictec.2005.10.016.

Hasan-Beikdashtia M, Forootanfara H, Safiariana MS, Amerib A, Ghahremani MH, Khoshayandd MR, Faramarzi MA. 2012. Optimization of culture conditions for production of lipase by a newlyisolated bacterium *Stenotrophomonas maltophilia*. J Taiwan Inst Chem Eng 43 (5): 670-677. DOI: 10.1016/j.jtice.2012.03.005.

Hungate RE. 1966. The Rumen and its Microbes. Academic Press, London.

Jaeger KE, Djikstra BW, Reetz MT. 1999. Bacterial biocatalysts: Molecular biology, three dimensional structures and biotechnological applications of lipases. Ann Rev Microbiol J Impact 53: 315-351. DOI: 10.1146/annurev.micro.53.1.315.

Janda M, Abbot L. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory. J Clin Microbiol 45 (9): 2761-2764. DOI: 10.1128/JCM.01228-07.

Joseph B, Ramteke PW, Thomas G, Shrivastava N. 2007. Standard review cold-active microbial lipase: A versatile tool for industrial applications. Biotechnol Mol Biol Rev 2 (2): 39-48.

Koven WM, Henderson RJ, Sergent JR. 1994. Lipid figestion in turbot *(Scophthalmus maximus)*. I: Lipid class and fatty acid composition of digesta from different segments of the digestive tract. Fish Physiol Biochem 13 (1): 69-79. DOI: 10.1007/BF00004121.

Kurnia DRD. 2010. Studi Aktivitas Lipase dari *Aspergillus niger* sebagai Biokatalis pada Proses Gliserolisis untuk Menghasilkan Monoasilgliserol. [Tesis]. Program Pasca Sarjana Universitas Diponegoro, Semarang. [Indonesian]

Kurniasih T, Lusiastuti AM, Azwar ZI, Melati I. 2014. Isolasi dan seleksi bakteri saluran pencernaan ikan lele sebagai upaya mendapatkan kandidat probiotik untuk efisiensi pakan ikan. J Riset Akuakultur 9 (1): 99-109. DOI: 10.15578/jra.9.1.2014.99-109. [Indonesian]

Kurniawan MR. 2016. Identifikasi dan Prevalensi Infeksi Cacing Saluran Pencernaan pada Belut Rawa (*Synbranchus Bengalensis*) yang Dipasarkan Di Kota Surabaya, Jawa Timur. [Skripsi]. Universitas Airlangga, Surabaya. [Indonesian]

Lehninger AL. 1995. Dasar-Dasar Biokimia. Alih Bahasa: Thewijaya M. Penerbit Erlangga, Jakarta. [Indonesian]

Li G, Sinclair AJ, Li D. 2011. Comparison of lipid content and fatty acid composition in the edible meat of wild and cultured freshwater and marine fish and shrimps from China. J Agric Food Chem 59: 1871-1881. DOI: 10.1021/jf104154q.

Linko Y, Lamsa M, Wu X, Uosukainen, Seppala J, Linko P. 1998. Biodegradable products by lipase biocatalysis. J Biotechnol 66: 41-50. DOI: 10.1016/S0168-1656(98)00155-2.

Maneerat S, Phetrong K, Song K. 2007. Isolation of biosurfactant producing marine bacteria and characteristics of selected biosurfactant. J Sci Technol 29: 781-791.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. 1998. Design and evaluation of useful bacterium spesific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64 (2): 795-799. DOI: 10.1128/AEM.64.2.795-799.1998.

Mubarak E, Kermanshasi RK, Moosavi-nejad Z. 2011. Isolation and identification of novel, lipase-producing bacterium, *Pseudomonas aeruginosa* KM110. Iran J Microbiol 3: 92-98.

Musa H, Adebayo-Tayo BC. 2012. Screening of microorganism isolated from different enviromental samples for extracellular lipase production. Assumption Univ J Technol 15 (3): 179-186.

Osek J. 2004. Phenotypic and genotypic characterization of *Escherichia coli* O157 strains isolated from human, cattle, and pigs. Veterinarni Medicina Journal Impact 9: 317-326. DOI: 10.17221/5711-VETMED.

Pangastuti A. 2006. Definisi spesies prokaryota berdasarkan urutan basa gen penyandi 16s rRNA dan gen penyandi protein. Biodiversitas 7 (3): 292- 296. DOI: 10.13057/biodiv/d070319.

Pratita MYE, Putra SR. 2012. Isolasi dan identifikasi bakteri termofilik dari sumber mata air panas di Songgoriti setelah dua hari inkubasi. J Teknik Pomits 1 (1): 1-5.

Ray AK, Ghosh K, Ringo E. 2012. Enzyme-producing bacteria isolated from fish gut: A review. Aquac Nutr 18 (5): 465-492. DOI: 10.1111/j.1365-2095.2012.00943.x.

Ridwan, Retnaningrum E, Ilmi M, Daryono BS. 2019. Identification and antimicrobial activity of lactic acid bacteria from the digestive tract of eels (*Monopterus albus*). Bioteknologi 16: 5-10. DOI: 10.13057/biotek/c160102.

Roy R. 2009. Budi Daya dan Bisnis Belut. Agromedia Pustaka, Jakarta. [Indonesian]

Sakinc T, Kleine B, Gattermann SG. 2007. Biochemical characterization of the surface associated lipase of *Staphylococcus saprophyticus*. FEMS (Federation European Microbiological Societies) Microbiol Lett 274: 335-341. DOI: 10.1111/j.1574-6968.2007.00857.x.

Saktiwansyah E. 2001. Karakterisasi Enzim Lipase Intraseluler dengan Aktivitas Esterifikasi dari Kapang Rhizopus oryzae TR 32. [Tesis]. Program Pascasarjana, IPB, Bogor. [Indonesian]

Sari M, Afiati F, Kusharyoto W. 2015. Potensi bakteri lumpur minyak sebagai penghasil biosurfaktan dan antimikroba. Pros Sem Nas Masy Biodiv Indon 1 (1): 85-88. DOI: 10.13057/psnmbi/m010113. [Indonesian]

Sharma R, Christy Y, Banarjee UC. 2001. Production, purification, characterization, and applications of lipases. Biotechnol Adv 19 (8): 627- 662. DOI: 10.1016/S0734-9750(01)00086-6.

Susanty A, Fitriani, Chandra KP. 2013. Isolasi bakteri penghasil lipase dengan kemampuan esterase untuk produksi metil ester. J Riset Teknologi Industri 7 (14): 14-142. DOI: 10.26578/jrti.v7i14.1542. [Indonesian]

Swandi MK, Periadnadi, Nurmiati. 2015. Isolasi bakteri pendegradasi limbah cair industri minyak sawit. J Biologi Universitas Andalas 4 (1): 71-76. [Indonesian]

Telussa I. 2013. Isolasi bakteri penghasil enzim lipase dari coco butter subtitute dan karakterisasi lipasenya. Prosiding FMIPA Universitas Pattimura 2013: 134-143. [Indonesian]

Thenmozhi S, Vijayalakshmi P, Moorthy K, Sureskhumar BT. 2015. Virulence and molecular characterization of *Aeromonas* Spp. from fish samples using ardra technique. Intl J Pharm Bio Sci 6 (1): 593-603.

Veerpagu M, Narayanan S, Jeya AKR, Alagendran S. 2014. Isolation and identification of a novel lipase producing bacteria from oil spoiled soil. Int J Innov Res Technol Sci Eng 3 (12): 18122-18129. DOI: 10.15680/IJIRSET.2014.0312050.

Vishwanath W, Lilabati H, Bijen M. 1998. Biochemical, nutritional and microbiological quality of fresh and smoked mud eel fish *Monopterus albus*-a comparative study. Food Chem 61 (1): 153-156. DOI: 10.1016/S0308-8146(97)00108-8.

Whitman WB, Vos PD, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH. 2009. Bergey's Manual of Systematic Bacteriology. Second Edition Volume Three. The Firmicutes. Department of Microbiology 527 Biological Sciences Building University of Georgia, USA.

Winarno FG. 1986. Enzim Pangan. Gramedia, Jakarta. [Indonesian]

Yakut N, Kepenekli EK, Karaasalan A, Atici S, Akkoc G, Demir SO, Soysal A, Bakir M. 2016. Bacteremia due to *Acinetobacter ursingii* in infants: Report two cases. Pan Afr Med J 29 (193): 1-4. DOI: 10.11604/pamj.2016.23.193.8545.