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Characterization and Identification of Halophilic Bacteria Isolated from *Rastrelliger* sp. as Extracellular Lipase Producers

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ABSTRACT

Halophilic bacteria represent as potential producer of lipase enzymes. This study aims to characterize and identify lipase-producing halophilic bacterial isolates from salted fish (*Rastrelliger* sp.) in Beringharjo Market, Yogyakarta. The Nutrient Agar + 3% NaCl + Tween 80 + 0.1% Rhodamine-B selective media to isolate lipase-producing halophilic bacteria. Identification was carried out by the matching profile method based on Bergey's Manual of Determinative Bacteriology. The result revealed that nine bacterial isolates exhibited lipase-producing activity. The bacterial isolate belongs to members of the genera *Marinococcus*, *Acetobacterium*, and *Kurthia*.

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1. INTRODUCTION

Indonesia is an archipelagic country with almost two-thirds of the area in the form of the ocean. The great wealth of the sea includes various types of fish. Indonesian waters have a fishery potential of around 7.6 million tons/year. Salted fish is a fresh fish ingredient product that is preserved with the addition of salt (Marliza et al., 2019). Puffer fish and mackerel fish which is an economical fish and has the potential to increase its catch every year (Thariq et al., 2014). Safrida & Devira (2012) said that bacteria in fish can be found on external body surfaces and the digestive tract. Some bacteria are pathogenic, while others are beneficial for fish because they help digestion, synthesize vitamins and decompose organic matter in the waters. Haophilic bacteria can be classified based on the salt content needed, including low halophilic types that grow optimally at 2 – 5% NaCl, medium halophilic types that grow optimally at 5 – 20 NaCl and extreme halophilic types (high salt content) grow optimally at salt levels around 20 – 30% NaCl (DasSarma, 2012).

Halophilic bacteria can produce hydrolytic enzymes, one of which is lipase enzymes. Lipase enzyme is an extracellular enzyme because it is an enzyme produced by cells and then secreted through the cell wall. It is free in the medium that surrounds the cell and reacts to break down organic matter without depending on the cell that releases it (Kasipah et al., 2013). The test of lipase enzyme production activity was carried out by a procedure according to Bairagi et al., (2002) which was enriched with tween 80 and incubated at room temperature for 24 hours then the lipase activity was shown by the formation of cloudy

white fatty acid deposits around the media. According to Ari (2012) research, the activity of hydrolytic enzymes on lipids is shown by the formation of fatty acid deposits that have the lowest enzyme activity compared to other hydrolytic enzymes.

Lypolytic bacteria are bacteria that require a certain minimum concentration of lipid for their growth. The group of lipolytic bacteria produces lipase, which is an enzyme that catalyzes the hydrolysis of fats into fatty acids and glycerol. The bacterial genera such as *Pseudomonas*, *Alcaligenesis*, *Serratia* and *Micrococcus* are lipolytic bacteria. One example of a strong lipoly is *P. fluorescens* (Chairunnisa et al., 2019). The characterization carried out is testing in both macroscopic and microscopic and the growth of bacterial isolates at different NaCl levels for further identification purposes. The results of observation of protease-producing halophilic bacteria were identified by the matching profile method based on Bergey's Manual of Determinative Bacteriology. Selected isolates of protease-producing halophilic bacteria from layur saltfish were analyzed by numerical phenetic taxonomy method using OTU (Operational Taxonomy Unit). Based on the SSM (Simple Matching Coeficient) value, dendogram construction is used to describe the classification of OTU (Operational Taxonomy Unit). The construction of the dendogram was carried out using the Average Linkage (UPGMA/Unweighted Pair Group Method With Aritmatic Average) clustering algorithm (Sari et al., 2018).

2. RESEARCH METHOD

The research sample of salted fish (*Rastrelliger* sp.) were collected at Beringharjo Market, Yogyakarta. Culture media for bacterial growth include Nutrient Broth (NB) media containing glucose, sucrose, maltose, lactose, fructose, mannitol, dulcitol, ramnose, raffinosa, arabinosa, and sorbitol, malonate broth media, Indole Motility (SIM) sulfide media, Nutrient Gelatin media, Skim Milk Agar (SMA) media, Starch media Agar, Methyl Red (MR) media, Voges-Proskauer (VP) media, Triple Sugar Iron Agar (TSIA) media, Simmons Citrate agar media, Urea Base agar media, Peptone Water (AP) media, 3% Tween 80, 0.1% Rhodamin-B, sterile aquadest, NaCl, crystal violet, iodine, ethyl alcohol 95%, safranin, malachite green, Kovac's reagent, 3% H_2O_2, ethanol 70%, methyl red, phenol red, oxidase paper, α-naphthol solution, and KOH solution.

Sample isolation is carried out by the Pour Plate method. Aseptically, 1 g of a sample of salted fish was added to 9 ml of aquadest then homogenized and became a 10^{-1} dilution. Then 1 ml of dilution suspension is first added to 9 ml of aquadest then homogenized so that it becomes a 10^{-2} dilution up to 10^{-7} dilution. Next, 1 ml of sample suspension was taken at dilution 10^{-5}, 10^{-6} and 10^{-7} and then put into the petridish followed by Nutrient Agar + NaCl+ 3% Tween 80 + 0.1 % Rhodamine-B medium that had been sterile and then shake the petridish so that the sample was evenly distributed (Puspito Rini et al., 2017) The petridish was incubated at 37°C for 2-3 days. Moreover, the lipase-producing bacterial isolates is purified and then characterized morphologically, physiologically (biochemical), growth at different NaCl levels and identified.

3. RESULTS AND ANALYSIS (11 Pt)

This study shows a colony of lipase-producing bacteria grown on selective media (Figure 1). Lipase activity is characterized by the larger the turbid zone that is formed, the greater its lipolytic ability. To induce lipase enzymes, Tween 80 is used as a lipid substrate (Damaso et al., 2008). The lipolytic bacterial isolates found in this study were 9 isolates with the largest lipase activity of 10 mm.

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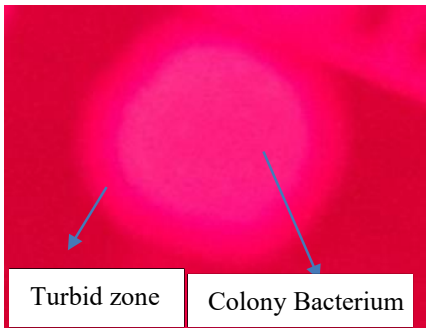

Figure 1. Colonies of Lipase-producing Halophilic Bacteria on Selective Media

Research related to the isolation of lipolytic enzymes has been widely conducted. However, most studies related to lipase enzymes do not include or the lipolytic index ranges from 1 to 2 mm only. For example, the research of Nurzhulian et al., (2021) obtained a lipolytic isolate forming a calcination zone with the highest lipolytic index of 2.95. Mazhar et al., (2018), reported a number of strains of *Bacillus cereus* lipolytic bacteria isolated from soil using tributyrin media that have a range of values the lipolitik index is 0.53 – 1.97. Kurniasih et al., (2014), isolated lipolytic bacteria in the digestive organs of catfish but the bacterial lipolytic index obtained was not measured.

Table 1. Microscopy Characteristics of Lipolytic Heterophilic Bacterial Isolates.

No.	Isolation Code	Gram Properties	Cell Shape	Cell Arrangement	Endospora
1.	A62	Positive	*Coccus*	*Staphylococcus*	-
2.	A63	Positive	*Bacillus*	*Monobacillus*	-
3.	A72	Positive	*Coccus*	*Staphylococcus*	-
4.	A83	Positive	*Bacillus*	*Monobacillus*	-
5.	A85	Positive	*Bacillus*	*Monobacillus*	-
6.	B61	Positive	*Bacillus*	*Monobacillus*	-
7.	C72	Positive	*Bacillus*	*Monobacillus*	-
8.	C75	Negative	*Coccus*	*Staphylococcus*	-
9.	C78	Positive	*Bacillus*	*Monobacillus*	-

Table 1 shows the cell character of lipase-producing halophilic bacteria. Gram staining included in differential painting which can divide bacteria into two groups, Gram-positive bacteria and Gram-negative bacteria. Bacteria that have gram-positive properties will be purplish-blue while bacteria that have gram-negative properties are red. The cause of the difference in gram staining is likely to occur because there are differences in the composition and structure of the bacterial cell wall (Geo.F.Brooks, Karen C. Carroll, Janet S. Butel, Stephen A. Morse, 2013).

Table 2. Physiological and Biochemical Characteristics of Lipase-producing Halophilic Bacteria

No	Isolation Code	Physiological Character of Biochemistry						
		Catalase	Oksidase	Motil	Citrate	H_2S	Indole	Malonat
1.	A62	+	+	-	-	-	-	-
2.	A63	+	+	-	+	-	-	-
3.	A72	+	+	-	-	-	-	-
4.	A83	+	+	-	-	-	-	-
5.	A85	+	+	-	+	-	-	-

6.	B61	+	+	-	-	-	-	-
7.	C72	+	+	-	-	-	-	-
8.	C75	+	+	-	-	-	-	-
9.	C78	+	+	-	-	-	+	+

Table 2 shows the physiological and biochemical characteristics of lipolytic halophilic bacteria consisting of catalase, oxidase, motility, citrate, H_2S, indole, and malonate tests. Facultative aerobic and anaerobic bacteria use oxygen and produce hydrogen peroxide which is toxic to the bacteria's own enzyme system. The accumulation of hydrogen peroxide causes death for the bacteria themselves. The catalase enzyme functions to decompose hydrogen peroxide (H_2O_2) into water and oxygen (Indriani, 2012). The positive reaction of the catalase test was shown by the formation of oxygen gas bubbles on the surface of the colony on the glass of the object after being dripped with 3% H_2O_2. The formation of gas bubbles indicates the decomposition of H_2O_2 by the catalase enzyme into water and oxygen (Aminullah, Fida Rachmadiarti, 2015). Motility tests were carried out with the aim of determining the movement of bacterial cells (Aminullah, Fida Rachmadiarti, 2015). Bacterial motility is supported by the presence of structures that resemble long threads called flagellates that originate from the cell membrane. Energy to move flagellates is obtained from ATP changes decomposed by the ATP-ase coenzyme (Raihana, 2011). Simmon's citrate test is one of the main components in the Krebs cycle which is the result of a reaction between acetyl coenzyme A (CoA) and oxaloacetic acid (4C). The utilization of citrate involves the enzyme citrate permease, which breaks down citrate into oxaloacetic acid and acetic acid. Oxaloacetate is further broken down into pyruvate and CO_2. The production of Na_2CO_3 and NH_3 from the utilization of sodium citrate and ammonium salt produces alkaline pH, respectively. This causes a change in the color of the medium from green to blue (Hemraj, 2013). Indole production test aimed at determining the ability of microbes to degrade the amino acid tryptophan. Negative test results showed that the isolate obtained did not have the ability to hydrolyze tryptophan (Gergonius & Sine, 2016). Meanwhile, the positive test results showed that the bacteria contained the tryptophanase enzyme which is a catalyst for the decomposition of the indole group contained in the amino acid tryptophan (Ulfa et al., 2016). According to Pedraza (2014) the malonate test is usually positive in several species of the genus *Enterobacter*, *Klebsiella*, and Citrobacter. While most species of the genus Escherichia, Salmonella, Shigella, Edwardsiella, Yersinia, *Serratia*, *Morganella*, *Proteus* and *Providencia*, give negative reactions.

Table 3. Physiological and Biochemical Characteristics of Lyptic Halophilic Bacteria

No	Isolate Code	Physiological Character of Biochemistry					
		MR	VP	Urea	TSIA	Gelatin Hydrolysis	Strach Hydrolysis
1.	A62	+ weak	+	+	A/K	+	-
2.	A63	+ weak	+	+	K/K	+	-
3.	A72	+ strong	+	+	A/K	-	-
4.	A83	+ weak	+	+	K/K	+	-
5.	A85	+ weak	+	+	K/K	+	-
6.	B61	+ weak	+	-	K/K	+	-
7.	C72	+ weak	+	+	K/K	-	-
8.	C75	+ weak	+	+	K/K	+	-
9.	C78	+ weak	+	+	A/K	-	+

A/K : Glucose fermentation
K/K : Glucose, lactose, sucrose fermentation

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Table 3 shows the physiological and biochemical characteristics of lipolytic halophilic bacteria consisting of MR, VP, Urea, TSIA, gelatin hydrolysis and starch hydrolysis. The Methyl Red (MR) test, aims to detect the ability of organisms to produce and maintain stable acidic end products from glucose fermentation. Methyl red is an indicator of pH, which remains red at pH 4.4 or less (Sunarjo, 1994). The positive (+) result is characterized by the addition of methyl red, the media changes color to red, which can be interpreted that mixed acid (methylene glycone) is produced by bacteria through the glucose fermentation process in methyl red media (Kumar, 2013). The VP test aims to detect whether glucose can be converted to acetyl methyl carbinol. The positive test on the nine isolates is due to the fact that isolates A62, A63, A72, A83, A85, B61, C72, C75, and C78 can ferment carbohydrates into acidic products resulting in neutral products such as acetonin (Rahayu & Gumilar, 2017). The urease test aims to determine the ability of bacteria to convert urea into ammonia (Awaludin Prihanto et al., 2018). Discoloration can occur when the urease enzyme breaks the bonds of carbon and nitrogen to form ammonia. The presence of ammonia causes the atmosphere of the medium to become alkaline/alkaline so that the phenol red indicator will turn pink in the medium, this indicates the occurrence of a positive reaction or the production of urease (Gergonius & Sine, 2016). Based on the starch hydrolysis test, only C78 bacterial isolate can hydrolyze starch while other isolates show negative results. A negative result is characterized by the absence of a clear zone around the bacterial colony after iodine/lugol solution was dripped while the positive result was marked by the formation of a clear zone around the colony (Wahyuni et al., 2014). All isolates show negative results that indicate that no hydrolysis of starch by the amylase enzyme has occurred. The clear zone indicates that the bacteria are able to hydrolyze starch or amylum into simple sugars. The clear zone indicates the presence of exoenzyme hydrolysis activity (amylase) (Cappuccino & Sherman, 2005). The gelatin hydrolysis test was carried out to find out whether the bacteria can produce gelatinase enzymes that are able to hydrolyze gelatin. Hydrolysis of gelatin occurs because bacteria produce gelatinase to hydrolyze protein polymers, gelatin, for amino acids (Lubis et al., 2015). According to Rori et al., (2020) gelatinase production is characterized by a change in the color of the media and the melting of the media even when frozen. According to Rahmadian et al., (2018), the Triple Sugar Iron Agar (TSIA) test aims to distinguish types of bacteria based on their ability to break down dextrose, lactose, sucrose, and sulfide release. The media used has two parts, slant (slanted) and butt (base).

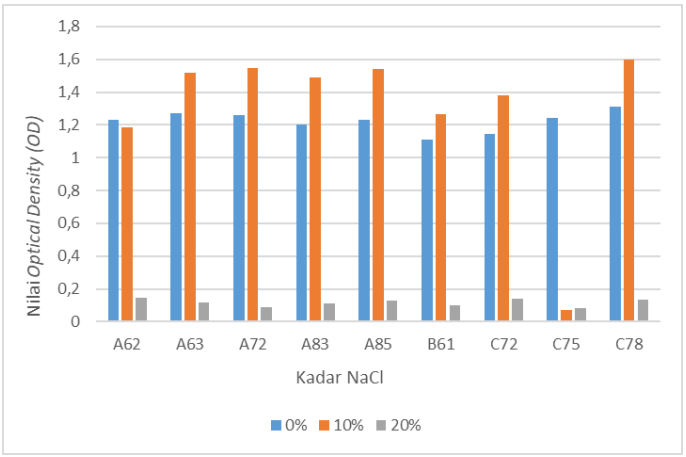

Figure 2. Growth of Haliphilic Bacteria of *Nutrient Broth* + NaCl Media at Different NaCl Levels

Figure 2 is the average value of the absorption of the growth of lipolytic halophilic bacteria at different levels of NaCl. The results obtained were that the average optimal growth of each isolate was at a NaCl level of 10% and not optimal at a NaCl level of 20%. The non-optimal growth of halophilic bacteria was characterized by no increase in absorbance value at the 72nd hour measurement. All isolates grown on

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NB media optimally grew at NaCl levels of 0% - 10% and were not optimal at NaCl levels of 20% except for A72 and C75 isolates grown at 20% NaCl. Meanwhile, C75 isolate does not grow at a NaCl level of 10% and grows at a NaCl level of 20%. This is because at the time of measurement in the cuvette, it is possible that bacterial isolates, nutritional deficiencies, pH changes and other unknown factors will urge and interbreed bacteria resulting in a decrease in growth rate. According to (Ansori, 2015), In some cases, cells contained in a culture whose cell population is not growing may be elongated, abnormally swollen, or abnormal, a manifestation of unbalanced growth. Judging from the ability of bacterial isolates, it is the basis that all lipolytic halophilic bacterial isolates include moderate halophilic bacteria.

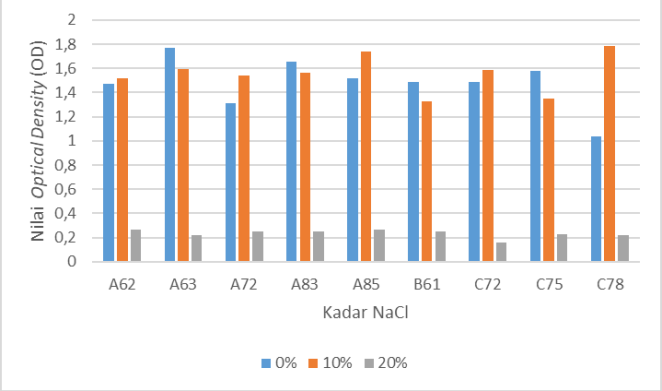

Figure 3. Growth of Halophilic Bacteria of Nutrient Broth + Tween 80 + NaCl Media at Different NaCl Levels.

Figure 3 shows the average absorbance value of the growth of lipolytic halophilic bacteria at different levels of NaCl grown on Nutrient Broth + Tween 80 (NBT) medium. The result obtained is that isolates on NBT media can grow at NaCl levels of 0% to 20% with the highest growth at 10%. The OD values of NaCl levels of 10% and 20% are not much different. Isolates A62, B61, C75 and C78 can grow at a NaCl level of 20% while isolates A63, A72, A83, A85 and C72 are not optimal to grow at a NaCl level of 20%. This is likely because the lymic bacteria do not consume nutrients to adapt to tween 80 so as to increase the absorption of tilapia. Based on the results of the absorption of isolate growth on NBT media, it can be concluded that all lipolytic halophilic bacterial isolates belong to the group of moderate or moderate halophilic bacteria. According to (Sari, 2013), in a certain phase there is a nutrient deficiency, usually the cells undergo adaptation to a less favorable environment. If the bacterial isolate has lacked nutrients, then the isolate will utilize the dead isolate cells to be used as a source of nutrients containing macromolecules such as proteins, lipids, carbohydrates and other components, so that the living isolated cells get energy to metabolize and divide the cell, with an increase in the number of cells, the absorbance value also increases. Judging from the overall absorbance value of the cultures of the nine isolates, it can be seen that the nine isolates experienced faster growth on Nutrient Broth + Tween 80 media compared to the isolates grown on Nutrient Broth media. This is because the nine isolates need to adapt to new environments such as different growth media (non-specific media). In breaking down the Tween 80 compound on Nutrient Broth + Tween 80 media, the isolated cells require a short time and energy compared to the isolate inoculated on the Nutrient Broth medium to secrete the lipase enzyme. The lipase enzyme is secreted to break down the Tween 80 compound which can then be used by bacterial cells as a source of carbon.

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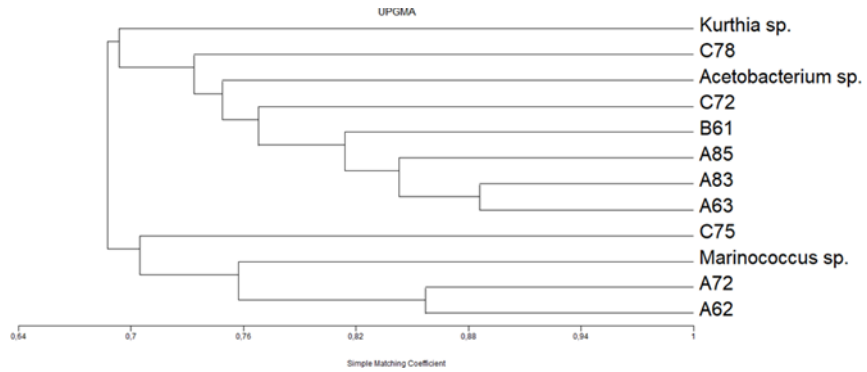

Figure 4. Dendogram of Bacterial Strains from Phenetic Numerical Classification.

Figure 4 is the result of a dendogram of clustering using the average linkage method or UPGMA for the SSM similarity matrix obtained from the results of the bacterial genus from lipase-producing halophilic bacteria samples from salted fish, namely Marinococcus, Acetobacterium, and Kurthia. Bacterial isolate with isolation codes A62, A72 and C75 is suspected to be a cluster with the genus Marinococcus sp. However, isolates A62 and A72 have a greater similarity index of 75.7% while C75 isolates have a similarity index of 70.5% which is suspected to be a cluster with the genus Marinococcus sp. Bacterial isolates with codes C72, B61, A85, A83, A63 have a similarity index of 74.9% and are suspected to be one cluster with bacteria of the genus Acetobacterium sp. The bacteriocyst isolate with the code C78 has a similarity index of 69.4%, based on the results of profile matching suspected to be of the same genus as Kurthia sp. These results show that lipase-producing halophilic bacterial isolates that have been successfully isolated from salty peda (Rastrelliger sp.) are dominated by bacterial isolates that have lipsticks with Acetobacterium sp.

References in determining bacterial strains are based on previous research. Based on the research of Andriyani, (2005), who isolated halophilic bacteria from various kinds of salted fish and found the suspected genus Marinococcus sp and Kurthia sp. Rathakrishnan & Gopalan, (2022) said that the bacteria Marinococcus sp. Included in the moderate halophilic group that produces hydrolytic enzymes, one of which is lipase enzyme. The genus Marinococcus sp. produces lipase activity isolated with Tween 80 substrate (Ventosa et al., 1998). Acetobacterium sp. according to Arslan et al., (2022) produces lipids and grows at a NaCl ability of 10-30%. This bacterium is usually found in seawater and freshwater waters because it requires minerals to grow in its habitat (Balch et al., 1977). In the study of Kurniasih et al., (2014) who isolated bacteria in the digestive tract of catfish and found Kurthia where lipolytic enzymes were found with Tween 80 substrate, an emulsifier of olive oil and a medium of blue spirit agar. Lypolitical bacteria are characterized by the presence of cloudy blue deposits around the media and it is suspected that the bacteria belong to the Kurthia genus. Kurthia is a gram-positive bacterium that can be found in fresh meat and farm animal feces (Pukall & Stackebrandt, 2015) and most of its species produce lipase enzymes isolated using Tween 40 and Tween 80 (Ruan et al., 2014).

4. CONCLUSION (11 Pt)

Based on the research conducted, it can be concluded that the results of testing on morphology, physiology and growth properties at different levels of NaCl of the 9 lipase-producing halophilic bacterial isolates, it is suspected that the bacterial isolate belongs to members of the genera *Marinococcus*, *Acetobacterium*, and *Kurthia*. Based on the phenetic diversity of lipase-producing halophilic bacterial isolates from salted fish (*Rastrelliger* sp.) in Beringharjo Market, 11 bacterial clusters were obtained. There are 8 isolates that have a similarity index of ≥70%, 3 of which are suspected to be one cluster with the genus

Marinococcus with a similarity index of 75.7% and 70.5%, the other 5 are suspected to be one cluster with the genus *Acetobacterium*. Meanwhile, there is 1 isolate that has a similarity indeks ≤70% is 69.4% which is suspected to be 1 cluster with the *Kurthia* genus.

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