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Characteristics and Antibacterial Activities of Bacteriocin Produced by Lactic Acid Bacteria Isolate LG-50 and LG-90

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Abstract
Biopreservative agent is natural food preservative agent derived from microbes such as bacteriocin produced by lactic acid bacteria (LAB). Bacteriocin is a protein compound that has a small molecular weight and has antibacterial activity because it is effective in preventing the growth of pathogenic bacteria in food and beverages. *Escherichia coli* and *Staphylococcus aureus* are pathogenic bacteria that can contaminate seafood products and they can cause infection and food toxication. Isolate LG-50 and LG-90 are LAB, bacterial collection of the Microbiology Laboratory, Faculty of Biology, isolated from mangrove sediment in Logending beach. Both isolates are not yet known for their potencies to synthesize bacteriocin and its inhibitory capability to the growth *E. coli* and *S. aureus*. The research aimed to determine the ability of LG-50 and LG-90 isolates to produce bacteriocin, to determine the ability of both isolates to inhibit the growth of *E. coli* and *S. aureus* bacteria *invitro*, and to characterize the isolate LG-50 and LG-90. The results of this research showed that the isolate LG-50 and LG-90 were able to produce bacteriocins and capable to inhibit the growth of *E. coli* with inhibition zone diameters of 13.5 mm and 13 mm, to *S. aureus* with inhibition zone diameters of 15 mm and 15.5 mm. Isolate LG-50 and LG-90 were thought to be a species of Lactobacillus.
Key Words: *Bacteriocin, Escherichia coli, Lactic Acid Bacteria (LAB), Staphylococcus aureus*

INTRODUCTION

Lactic acid bacteria (LAB) are a type of bacteria that is not pathogenic bacteria and produce chemical compound functions to extend the shelf life of food products or called as biopreservative agents. LAB can also give flavor to food, inhibit spoilage bacteria in food and feed and can also inhibit pathogenic bacteria. In the food industry, LAB is also known as food grade organism or Generally Recognized as Safe (GRAS) organism because it has a safe reputation (Wulandari *et al*., 2016). LAB as GRAS microorganisms, it can be added to food, do not affect other natural microbes present in the body, and produce antimicrobial peptides, namely bacteriocins (Sari *et al*., 2011).

The use of LAB as a natural preservative can be done in two ways, namely adding LAB cultures as a starter in food products or only using antimicrobial metabolites produced by LAB as natural preservatives. In addition to producing organic acids (lactic acid, acetic acid), fermentation also produces a type of protein, namely bacteriocins, which can inhibit the growth of pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*). Bacteriocin compounds are very useful because they inhibit pathogenic bacteria that can damage food or endanger human health so that food safety is more guaranteed. Bacteriocins can potentially act as natural food preservatives (Rustan, 2013). Bacteriocins have potencies to be used as preservative agent in food and feed by controlling spoilage and pathogens naturally, including Gram positive and Gram negative bacteria (Mataragas *et al*., 2003). In addition, bacteriocin also non-toxic and easily biodegradable. Because bacteriocin is a protein compound that does not harm the intestinal microflaura, it is easily digested by enzymes in the digestive tract, and is safe for the environment (Suganya *et al*., 2015).

Several bacteriocins are produced by the LAB group which comes from fermentation products, but not all those bacteria are capable in producing this antimicrobial protein. These bacteriocins are sensitive to proteolytic enzymes proteinase-K and papain. These bacteriocins are stable at high temperatures and pH 2-8 and produce the highest antibacterial activity against *S. aureus* (Kusmarwati *et al*., 2014). According to Andarilla *et al*., (2018), the bacteriocin antibacterial activity produced by *Lactobacillus casei* is more sensitive to Gram positive bacteria *S. aureus* when compared to Gram negative bacteria *E. coli* with an average diameter of the inhibition zone of 12.225 mm and of 8.45 mm.

Bacterial isolates of LG-50 and LG-90 are LAB, isolated from mangrove sediment in Logending beach. Both isolates were not yet known for their potencies to produce bacteriocin and their inhibitory capability to the growth of *E. coli* and *S. aureus*. The purpose of this study was to determine the ability of LG-50 and LG-90 isolates to produce bacteriocin, to determine the ability of LG-50 and LG-90 isolates to inhibit the growth of *E. coli* and *S. aureus* invitro, and to identify the isolates

MATERIAL AND METHOD

The research used LAB isolates LG-50 and LG-90, pathogenic bacteria *E. coli* and *S. aureus* (bacterial collection of Microbiology Laboratory, Faculty of Biology, Unsoed), chemicals and media for bacterial cultivation and microbiology assay.

This research was conducted using a analytical observation by testing the isolate LG-50 and LG-90 to produce bacteriocin and its ability to inhibit the growth of *E. coli* and *S. aureus*. Antibacterial activity was tested using the difussion method. The parameters measured were the inhibition zone diameter produced by LG-50 and LG-90 isolates in inhibiting the growth of *E. coli* and *S. aureus* and characters data of LG-50 and LG-90 isolates. The data obtained was analyzed descriptively. The characteristics of the isolates refer to Bergey's Manual of Systematic Bacteriology.

Isolates reculture

Isolates LG-50 and LC-90 was taken 1 loop and then inoculated on de Mann Rogosa Sharpe Agar (MRSA) medium by streak inoculation, incubated at 37ºC for 48 hours. Isolates *E. coli* and *S. aureus* was recultured on Nutrient Agar (NA) slant medium, incubated at 37°C for 24 hours.

Inhibition test of LG-50 and LG-90 Supernatants to *E. coli* and *S. aureus*

Isolate LG-50 and LG-90 were taken 1 loop and inoculated into test tube containing 10 mL of de Mann Rogosa Sharpe Broth (MRSB) medium then incubated at 37°C for 18 hours. The cultures were then transferred to an Eppendorf tube and centrifuged at 11.180 G force for 10 minutes to obtain cell-free supernatant. The supernatant obtained was then used for inhibition test to pathogenic bacteria using paper disc diffusion method or Kirby Bauer method.
The lawn of *E. coli* and *S. aureus* were prepared by inoculate 1 loop of pathogenic bacteria into medium Nutrient Broth (NB) incubated at 37°C for 8 hours, then 1 mL culture was poured in 20 mL of medium NA. The mixture was homogenized and then allowed to stand until the medium solidified. After the medium solidified, a 6 mm diameter disc paper which had been dropped with 20 µL supernatant was placed on the bacterial lawn. The test culture was incubated at 37°C for 24 hours and the inhibition zone formed was measured their diameters, then calculated as an average value.

Bacteriocins Production and Confirmation of Isolate LG-50 and LG-90

Inoculum of LG-50 and LG-90 was prepared by taken 1 loop and inoculated into a bottle containing 10 mL of MRSB medium then incubated at 37°C for 18 hours. As much as 1 mL inoculum was inoculated into a bottle containing 100 mL MRSB medium and incubated in shaker incubator at a speed of 150 rpm at 37°C for 24 hours. After the incubation period, the culture was centrifuged 11.180 G force at 4°C for 15 minutes. The supenatant obtained was added with ammonium sulfate as much as 50 mL slowly until the end of saturation (out salting method). After the addition of ammonium sulfate, the solution was again centrifuged at a speed of 11.180 G force at 4°C for 15 minutes. Then the supenatant was removed and the protein precipitate obtained was dissolved in 0.1 M phosphate buffer pH 5.3 2 mL. Furthermore, the bacteriocin solution was tested for confirmation.

As much as 200 µL of bacteriocin extract was mixed with 20 µL of proteolytic enzyme solution (enzyme solution was made by dissolving 1 g of proteolytic enzymes added with PBS 0.1 M pH 5.3) in an eppendorf tube then incubated at 37°C for 2 hours. The solution was tested on pathogenic bacteria using the Kirby Bauer method. One loop of pathogenic bacteria was inoculated into a test tube containing 10 ml medium NB and incubated at 37°C for 8 hours. The 1 mL pathogenic culture was then poured into medium NA, homogenized and allowed to stand until the medium solidified. After the medium solidified, the 6 mm diameter disc paper which had been dropped with 20 µL of bacteriocin extract added papain was placed on the NA medium in a dish. The incubation was carried out at 37°C for 1x24 hours. The absence of a clear zone (zone of inhibition) around the disc paper indicates that the compound was a bacteriocin which was sensitive to proteolytic enzymes.

Bacteriocin inhibition test against pathogenic bacteria *E.coli* and *S.aureus*

Determination of the inhibition of bacteriocins against pathogenic bacteria was carried out by the Kirby Bauer method. One loop of pathogenic bacteria inoculated into a test tube containing 10 ml NB medium and then incubated at 37°C for 8 hours. Then, 1 mL of 8 hours of pathogenic bacterial culture was put into a petri dish and then 20 mL of NA medium was added with the melted medium by pour plate method. The result of the mixture was homogenized and then allowed to stand until the medium solidified. After the medium solidified, the 6 mm diameter disc paper which had been dropped with 20 µL of bacteriocin compound was placed on the NA medium. Then the incubation was carried out at 37°C for 1x24 hours and the inhibition zone formed was measured their diameters, then calculated as an average value.

Characterisation of LG-50 and LG-90 isolates:

Observation of Colony Morphology

Isolate LG-50 and LG-90 was taken 1 loop and inoculated on MRSA medium incubated for 48 hours at 37°C. The macromorphological characteristics of the isolates were observed such as colony shape, colony surface, colony color, colony edge, colony size, and colony consistency

Observation of Cell Morphology

Observation of cell morphology was carried out by Gram stain, and endospore stain. The isolates of LG-50 and LG-90 was taken 1 loop and transfered to

object glass and dropped with distilled water. The smear was fixed over a bunsen fire 2-3 times. The crystal violet reagent as a basic dye was dropped on the smear and allowed to stand for 30 seconds then washed with running distilled water and dried. Lugol's iodine reagent as a mordant dye was dropped on the smear and allowed to stand for 30 seconds then washed with running distilled water and dried. The ethanol 96% reagent as a bleaching agent was dropped repeatedly over the smear until the last drop of ethanol that falls was clear, then washed with flowing distilled water and dried. The safranin reagent as a dye was dropped on the smear and allowed to stand for 30 seconds then washed with running distilled water and dried. The preparates were observed under a microscope with a total magnification of 1000x. The interpretation of Gram-positive bacteria is that cells appear purple and Gram-negative bacteria are red colored cells.

The endospore test was carried out the colony of bacterial isolates transfered to object glass and dropped with distilled water. The smear was fixed over a bunsen fire 2-3 times. The preparates were covered with filter paper and dropped with malachite green, placed over boiling water for 5 minutes, then were washed carefully under running water. The preparates were dripped with safranin, allowed to stand for 60 seconds, then washed and dried. The preparates were observed under a microscope, the endospore test was positive if the vegetative cells were red and the spores were green.

Catalase Test

The colony of bacterial isolates was taken 1 loop and applied on the object glass using a loop needle, then dropped with H_2O_2 reagent. Catalase positive interpretation if gas bubbles were produced, and negative if no gas bubbles were formed.

Oxidase Test

The bacterial colony was applied to a wet paper and dropped with Tetramethyl-p-phenylene diamine dihydrochloride reagent 1%. The change in color was observed. A positive result was interpreted by changes in color to dark blue of bacterial culture.

Temperature growth observation

The effect of temperature on isolate growth was carried out by incubating bacteria in MRSB medium at 4ºC, 37ºC, and 50ºC. The interpretation was characterized by the presence of turbidity.

pH growth observation

The effect of pH on the isolates was carried out by growing the isolates in MRSB with initial pH variations of 4 and 8. Incubation at room temperature for 24 hours. The interpretation was characterized by the presence of turbidity.

RESULT AND DISCUSSION

The cell-free supernatants of isolates LG-50 and LG-90 showed antibacterial activity against *E. coli* and *S. aureus*. Those activity were characterized by the formation of an inhibition zone around the disc. The average diameter of isolates LG-50 was 10.5 mm and LG-90 was 9 mm on *E. coli* lawn and isolates LG-50 was 10 mm and LG-90 was 10.5 mm on *S. aureus* lawn (Figure 1). The inhibition zone formed was not wide enough because the antimicrobial compounds in the supernatant have not been completely purified so that they do not work optimally, as stated by Saputri *et al*., (2017). Based on Kusharyati *et al*. (2020), cell-free supernatant isolates LG-50 and LG-90 were able to inhibit the growth of *Listeria monocytogenes*, *Shigella flexneri* and *Salmonella thypi*. Accoding to Rahayu *et al*. (2007), the lactic acid bacterial supernatant was able to inhibit the test pathogenic bacteria due to the metabolite components produced such as organic acids (lactic acid and acetic acid), bacteriocin, hydrogen peroxide, and diacetyl.

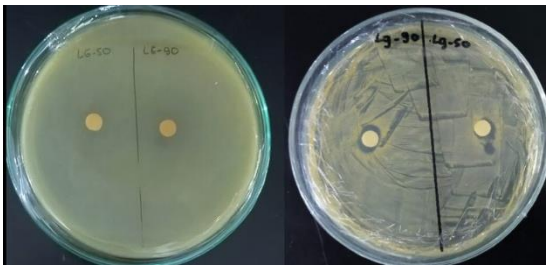

Figure 1. Test results of cell-free supernatant isolates LG-50 and LG-90 on pathogenic bacteria *E. coli* (left) and *S. aureus* (right)

The result of the bacteriocin confirmation test of LG-50 and LG-90 crude extracts showed that the crude extract that have been mixed with papain (proteolytic enzyme) did not inhibit the growth of *E. coli* and *S. aureus.* It was indicated by the absence of an inhibitory zone around the disc. Based on these results, it can be confirmed that the crude extracts of LG-50 and LG-90 isolates contained bacteriocins. These results accordance with the statement of Sari *et al*. (2018), that the bacteriocin sensitivity test can be observed from the inhibition zone that is not formed when bacteriocin compounds are added to proteolytic enzymes (such as papain or proteinase K). Bacteriocins as a natural protein can also be degraded by enzymes in the digestive tract. According to Ningsih *et al*. (2018), the purpose of antimicrobial sensitivity to proteolytic enzymes is to ensure that the antibacterial substance produced by LAB is a bacteriocin. The addition of proteolytic enzymes can eliminate bacteriocin activity by reducing or disappearing the inhibition zone around the disc. As an antibacterial, bacteriocin has properties that can be degraded by proteolytic enzymes. Because bacteriocins are proteins composed of amino acids forming peptide bonds. The peptide bond will be damaged so that the antibacterial activity of bacteriocin will be lost or unstable in the presence of proteoitic enzymes (Parada *et al*., 2007).

The results of antibacterial assay of bacteriocins contained in crude extracts of isolates LG-50 and LG-90 showed in Figure 2. The crude extract was able to

inhibit the growth of *E. coli* and *S. aureus* bacteria with an average inhibition zone diameter isolates LG-50 was 13.5 mm and LG-90 was 13 mm against *E. coli* bacteria and isolates LG-50 is 15 mm and LG-90 is 15.5 mm on *S. aureus* bacteria. Compared to Andarilla *et al*. (2018), bacteriocin *Lactobacillus casei* was able to inhibit the growth of *E. coli* and *S. aureus* with an average inhibition zone diameter of 8.45 mm and 12.225 mm.

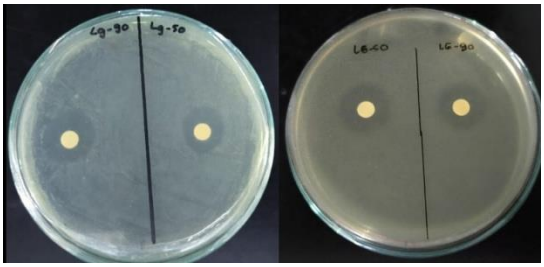

Figure 2. Test results of crude extract of bacteriocin isolates LG-50 and LG-90 on *E.coli* (left) and *S.aureus* (right)

The inhibition zone formed by bacteriocins against Gram-positive *S. aureus* bacteria was higher than that on Gram-negative *E. coli*. These results are in accordance with the statement of Andarilla *et al*. (2018), the antibacterial activity of bacteriocins produced by *Lactobacillus casei* is more sensitive to Gram-positive bacteria *S. aureus* when compared to Gram-negative bacteria *E. coli*. The difference in sensitivity could be caused by Gram negative bacteria have an outer membrane that acts as a barrier so that Gram negative bacteria are more difficult to be penetrated by bacteriocins. According to Kusharyati *et al*. (2020), bacteriocin activity tends to be more resistant (narrow) to Gram-negative bacteria than Gram-positive bacteria because Gram-positive cell walls are composed of thick peptidoglycan, while Gram-negative bacteria are composed of thin peptidoglycan but layered with outer membrane.

The results of the characterization assays LG-50 and LG-90 isolates were confirmed as lactic acid bacteria. Cell observations showed that isolates LG-50 and LG-90 were rod-shaped or curved to form the letter V, non-endospore and Gram- positive cells. This was in accordance with statement of Surono (2004), which stated that some lactic acid bacteria are rod-shaped and some are spherical.

The macroscopic observation of isolates LG-50 and LG-90 that the purified single colony had a circle colony shape, shiny colony surface, milky white colony color, entire colony edge, medium colony size (medium), and colony consistency is not slimy. This was in accordance with the statement of Putri *et al*. (2018), that was macroscopic LAB were yellowish white, round in shape with smooth edges. The colors of the colonies found were white, yellowish white, to transparent light brown. The edges of the colony are smooth, wavy, and some were rhizoid. The elevation of bacterial colonies found was convex and flat.

The results of some biochemical and physiological tests are presented in Table 1. Isolates LG-50 and LG-90 showed no gas bubbles after being dripped with H_2O_2. This is in accordance with Abun (2008), that LAB is not able to produce the catalase enzyme which is used to break down hydrogen peroxide into dihydroxy oxide (H_2O) and oxygen O_2. The result of the oxydase test was positive. The results of the physiological test of temperature and pH resistance of isolates LG-50 and LG-90 were able to survive at pH 4, pH 8 and grew at 37ºC and 50ºC. While at a temperature of 4ºC did not experience turbidity. According to Susilawati (2016), the optimum temperature for LAB growth is 35°-55°C. Based on these characteristics and referring to Bergey's Manual of Determinative Bacteriology showed that isolates LG-50 and LG-90 was thought belong to the LAB Lactobacillus.

The characterization results also accordance with Datta *et al*. (2019), isolates belonging to the LAB group of the Lactobacillus genus have general characteristics in the form of colonies that are round, milky white in color, flat-edged, small to medium, milky white or cream colored, glossy surface with raised elevation, round in shape, and entire colony edges. The cells were rods or V-shaped rods and are Gram positive. The isolates only grew on puncture marks in SIM A medium (non-motile), negative on catalase test. Lactic acid bacteria have a pH of 4.4 and grow in a temperature range of 37º-55ºC for their optimum growth. Based on Holt *et al*. (1994), Lactobacillus showed a positive response to the oxidase test, negative response in catalase test and non-spore forming cell. Further identification, through identification based on the 16SrRNA gene sequence is needed.

Table 1. Characterization of LG-50 and LG-90

No	Characterization	Results	
		LG-50	LG-90
1.	Surface colony	Shiny	Shiny
2.	Shape	Circle	Circle
3.	Color	Milky white	Milky white
4.	Elevation	Convex	Convex
5.	Edge	Flat	Flat
6.	Size	Medium	Medium
7.	Gram staining	Positive	Positive
8.	Shape cell	Rod	Rod
9.	Endospore	-	-
10.	Catalase	-	-
11.	Oxydase	+	+
12.	Temperature 4ºC	-	-
13.	Temperature 37ºC	+	+
14.	Temperature 50ºC	+	+
15.	pH 4	+	+
16.	pH 8	+	+

CONCLUSION

Based on result and discussion, it can be concluded from this research that isolates LG-50 and LG-90 were able to produce bacteriocin. Bacteriocins LG-50 and LG-90 were able to inhibit the growth of *E.coli* with inhibition zone diameters of 13.5 mm and 13 mm, in *S. aureus* with inhibition zone diameters of 15 mm and 15.5 mm. Isolates LG-50 and LG-90 from Logending beach mangrove sediment were thought to be a species of Lactobacillus.

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