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ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF LACTIC ACID BACTERIA PRODUCING BACTERIOCIN-LIKE FROM SMOKED GIANT CATFISH (*Arius thalassinus*)

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ABSTRACT

Bacteriocin was bacterial metabolite that have antimicrobial properties, so it had the potential to be used as food bio preservatives. Bacteriocin was produced by lactic acid bacteria (LAB), one of the sources of which was from smoked fish products. Some regions in Indonesia produce various types of smoked fish from various types of fish, which were thought to contain bacteriocin-producing lactic acid; one of them was giant cathfish *(Arius thalassinus).* This study aims to obtain LAB isolates that have strong antimicrobial activity and have the potential to produce bacteriocin-like from smoked giant catfish (*Arius thalassinus*). The research method used an experimental method that analyzed descriptively. Based on the results, there were 15 isolates LAB isolated from smoked giant catfish. Three selected isolates showed strong antimicrobial activity inhibiting *E. coli* and *S. aureus* bacteria, and the most effective inhibiting *Salmonella sp.* One selected LAB isolates identified Pediococcus *acidilactici* suspected to produce pediocin bacteriocin-like, while the other two isolates identified *Lactobacillus plantarum sp 1* and *Lactobacillus plantarum sp 2* which suspected to produce plantaricin bacteriocin. Bacteriocin from the three isolates of LAB had characteristics stable to temperatures up to 121°C, stable in pH range 2-6, and bacteriocin activity increased with the addition of SDS (Sodium Dodecyl Sulfate) and EDTA (Ethylenediaminetetraacetic acid) surfactants. The conclusion was that the bacteriocin produced was stable at high temperature, low pH, and resistence in the presence of surfactants, so it had the potential to be developed as biopreservatives material in preserving fish-based foods.

1. INTRODUCTION

Indonesia is the largest archipelago in the world that consists of 17,502 islands, and a coastline of 81,000 km with an area of fisheries at approximately 5.8 million km^2. The potential of capture fisheries in Indonesia is 6.4 million tons per year, while new marine products are utilized at 63.5% or around 4.1 million tons per year (Bappenas, 2014). The types of fish developed in Indonesia include freshwater, saltwater (sea), and brackish water (ponds) (Hidayati et al., 2012). Fish is a source of animal protein consumed by many people, in addition to highly nutritious, fish is also relatively easy to obtain and the price is quite affordable.

Fish have high nutritional content, namely protein (6-24%), fat (0.2-2.2%), water (58-80%), and minerals (2.5-4.5%) (Susanto, 2006). Fish has a water content of 76 g per 100 g of fresh fish. The high-water content makes the fish a suitable medium for the life of spoilage bacteria or other microorganisms so that the fish very easily damaged. Prevention of the damage process can be done in various ways such as salting, fermenting, or fogging. One of preserving fish product widely consumed in Indonesia is smoked fish. The smoking method was carried out to increase the endurance of the fish so that the quality of the fish can be maintained longer, and to give flavor and color to the fish.

One type of sea fish that is generally used as smoked fish is giant catfish (*Arius thalassinus*). Giant catfish are found in almost all coastal waters of Indonesia, especially on the beach that has a river mouth (estuary) at a depth of 20-100 m (Burhanuddin et al., 1987). Smoke for fish, besides being able to increase the endurance of fish, also increases the content of LAB which has the potential to produce bacteriocin (Todorov et al., 2011).

Bacteriocin is an antimicrobial compound synthesized by various species of bacteria, including the LAB group in the ribosome. Peptide compounds in bacteriocin show antibacterial activity (bactericidal and bacteriostatic) against sensitive bacterial species. This protein is active against other species that have a close kinship with the producing microorganism LAB (Gálvez et al., 2010).

Until now no specific research has been found on bacteriocins from smoked fish products. This is the basis for the need to do research on bacteriocin-producing LABs in traditional Indonesian processed fish products. In this study, the isolation and identification of bacteriocin-producing lactic acid bacteria were carried out on processed products of traditional Indonesian fish, especially smoked giant catfish. Isolates tested antimicrobial activity and performed the initial characterization of bacteriocin-producing LAB candidates

2. METHODS

2.1. Materials

Main materials were smoked fish from giant catfish (*Arius thalassinus*) which were obtained from the "Asap Indah" Smoke Fish Processing Center, Wonosari Village, Bonang District, Demak Regency, Central Java, Indonesia. The bacterial media used Man Rogosa Sharpe Agar (MRSA) media, Man Rogosa Sharpe Broth (MRSB) media, Nutrient Agar (NA) media, Nutrient Broth (NB) media, and Mueller Hinton Agar (MHA) media, which obtained from Bratachem, Bandung. Other materials used were 95% alcohol, sterile distilled water, 1% $BaCl_2$, Hacker crystal violet, 1% H_2SO_4, 3% H_2O_2, Iodine, NaCl, Safranine. Bacterial cultures *Escherichia coli*, *Staphylococcus aureus* anda *Salmonella sp.* tested a 24-hour incubation period was prepared in a physiological saline solution, then standardized with a standard McFarland solution no. 3 (equivalent to 9×10^8 CFU / ml).

The research phase consisted of bacterial isolation, morphological identification with catalase test and Gram staining, testing antimicrobial activity and characterization of

bacteriocins. The research method used was a qualitative method which was analyzed descriptively, with replications three times.

2.2. Isolation of Lactic Acid Bacteria

A total of 5 g of sample was put into 45 mL MRSB + 1% $CaCO_3$ media sterile and homogenized. The sample was incubated in a shaker incubator for 72 hours at 30 °C at a speed of 150 rpm. Dilution was carried out to 10^{-7} from 10^{-5} to 10^{-7} dilution was taken as 1 ml in a petri dish and then added with MRSA by an additional 1% $CaCO_3$. Furthermore, culture incubated at 37 °C for 72 hours. Bacterial colonies that formed a clear zone in the medium and streak have taken on MRSA to obtain pure colonies.

2.3. Identification of Lactic Acid Bacteria

Morphological Test Colony: The isolate was inoculated on MRSA then incubated at 37ºC for 24 hours. Furthermore, the shape of the colony, elevation, and edge shape were observed. Catalase and Gram Staining Test: Every isolate obtained taken one loop on object glass and then a few drops of 3% hydrogen peroxide for catalase test. Isolates showing negative catalase were stained by Gram. Gram staining was done by taking a loop that isolates the glass object that was passed over the bunsen. After drying drip with crystal violet solution, followed by strengthening colors with Potassium Iodide and rinsed with ethanol, followed by dripping paint safranin cover. Colonies that show purple are Gram-positive bacteria. LAB produces catalase-negative and Gram-positive. Bacteria Identification*:* The selected isolate producing bacteriocin was identified using the VITEK-2 Analyzer.

2.4. Antimicrobial Activity Test

LAB have been identified were grown in MRS broth at 37°C for 24-48 hours. Furthermore, MRS broth inoculum on medium speed centrifuge at 5000 rpm for 15 minutes to separate the supernatant with solids. This test uses the supernatant part. Then do the antimicrobial activity testing with the agar diffusion method. MHA sterile medium is poured into a petri dish to solidify and then applied a bacterial culture test with the swab method. MHA made pits with a diameter of ± 7 mm and put as much as 50 mL bacterial supernatant. After that the culture incubated at 37 °C for 24 hours, and observed the diameter of the clear zone produced.

2.5. Characterization of bacteriocin

Bacteriocin Production: Bacterial isolates were grown in MRSB at 37°C for 48 hours. Cultures were centrifuged at 8,000 rpm, 4°C for 15 minutes to obtain supernatant. Furthermore, the pH adjustment of the cell-free supernatant was carried out until it reached pH 6, by adding 1 M NaOH (to eliminate the effect of organic acids), then filtering it with a 0.2 µm cellulose acetate filter. The cell-free supernatant was heated at 80 ° C for 10 minutes to remove proteolytic activity and hydrogen peroxide, resulting in crude bacteriocins. Effect of pH: A total of 5 ml crude bacteriocin was put in different tubes, each arranged at a pH of 2, 4, 6, 8, and 10 using NaOH or HCl. After incubating for 4 hours at room temperature, the bacteriocin activity was tested using the agar diffusion method. Temperature resistance test: A total of 5 ml crude bacteriocin each tested for resistance to 50°C for 20 minutes, 100 °C for 20 min and 121ºC for 15 minutes. Furthermore, the bacteriocin activity was tested using diffusion agar method. Effect of surfactants: Bacteriocins stability test against some of the surfactants by adding surfactant (SDS, EDTA, urea) into each solution at a concentration crude

bacteriocin 0.1 ml or 0.1 g of surfactant/ml bacteriocin into a suspension of bacteriocin by 1%. Crude bacteriocin solution without surfactant was used as a control. Then incubation was carried out at 37 °C for 60 minutes, and the bacteriocin activity was tested using the agar diffusion method.

3. RESULTS AND DISCUSSION

3.1. Result

3.1.1. *Isolation of Lactic Acid Bacteria*

The results of sample inoculation on media MRS + $CaCO_3$ 1% produced 15 LAB isolates from smoked giant catfish with different colony sizes and showed the characteristics of a clear zone which means inhibition zone around the bacterial colony caused by the presence of antimicrobial compound produced by LAB (Figure 1).

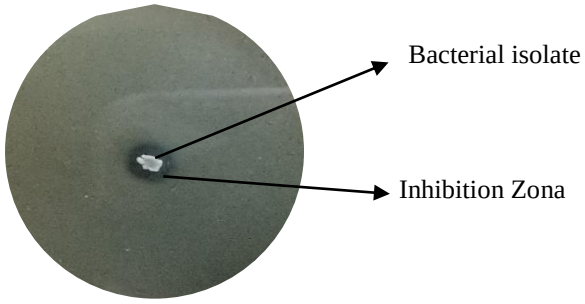

Figure 1 Inhibition zone around colony of isolated bacteria on MRSA + CaCO3 1%

3.1.2. *Identification of Lactic Acid Bacteria*

Catalase test and Gram Staining: Catalase test results of bacteria in smoked giant catfish showed that 15 isolates reacted negatively, which means no air bubbles are formed. A negative reaction was characterized by the formation of air bubbles when not dripped with H_2O_2, while a positive reaction was indicated by the formation of oxygen gas bubbles as a result of H_2O_2 degradation by the enzyme catalase (Lindawati & Suardana, 2016). Bacterial isolates tested by microscopic Gram stain to determine the shape and color of the bacterial cell so it can know the characteristics of these isolates. The results showed that all isolates were Gram-positive bacteria consisting of 12 round (coccus) bacterial isolates and 3 rod-shaped bacterial isolates (bacilli). Gram-positive bacteria are marked in purple while Gram-negative bacteria are red.

Morphological Testing Colony: The test results on the morphology of the LAB isolates giant catfish smoked fish had similar characteristics. Bacterial colonies milky white, raised elevation and convex shape, smooth edges, and form colonies on each isolate circular or round with raised edges.

3.1.3. *Antimicrobial Activity of Lactic Acid Bacteria*

Antimicrobial activity test was carried out on 15 LAB isolates from smoked giant catfish using MHA (Muller Hinton Agar). Based on the results of antimicrobial activity tests (Figure 2), all isolates showed inhibitory activity against *E. coli*, *S. aureus*, and *Salmonella sp*. It was characterized by a clear zone and turbid zones around the wells. The diameter of the inhibition zone of the isolates averaged 10-20 mm, thus including the inhibition of moderate to severe categories.

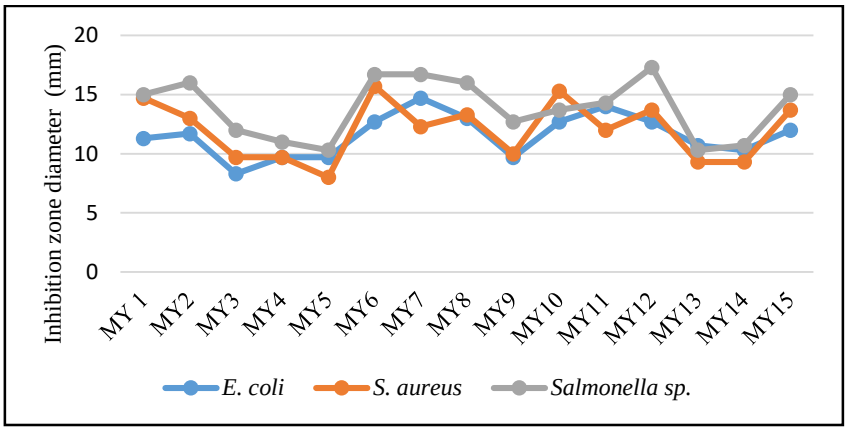

Figure 2 Antimicrobial activity of lactic acid bacteria

From the 15 isolates tested, 3 isolates showed strong antimicrobial activity, among which MY7 LAB isolates that represent the shape of cocci (round), as well as MY10 and MY12 representing the LAB isolates shaped bacilli (rod).

3.1.4. *Identification of Lactic Acid Bacteria Using VITEK-2 Analyzer*

The bacteria isolate that will be further identified were selected based on the greatest antimicrobial activity. The result of identification of LAB showed that isolates by code isolates MY10 and MY12 identified as *Lactobacillus plantarum* with a value of probability in a row is equal to 87% and 97%, while the isolates of LAB with code MY7 showed that these isolates are identified as *Pediococcus acidilactici* with the level of similarity or probability by 89%.

3.1.5. *Characterization of bacteriocin*

Effect of pH: The test results bacteriocins sensitivity to pH indicates that the bacteriocins of LAB isolate the third still have inhibitory to bacteria test. The resulting antimicrobial activity ranged from 7.75 to 24.75 mm indicates the diameter of the inhibition zone of moderate to strong category. Bacteriocin from *P. acidilactici* still stable in the pH range 2-4, while bacteriocin from *L. plantarum sp 1* and *L. plantarum sp 2* still stable after incubation in the pH range 2-6. The three types of bacteriocins showed the greatest antimicrobial activity in inhibiting the growth of *Salmonella sp* (Figure 3).

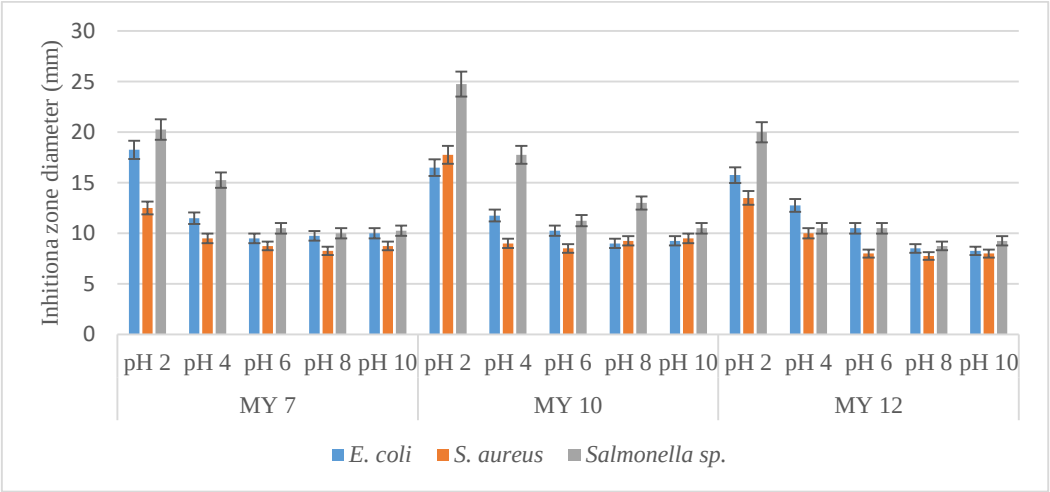

Figure 3 Bacteriocin antimicrobial activity against various pH

Temperature resistance test: Bacteriocins produced by *P. acidilactici*, *L. plantarum sp1*, and *L. plantarum sp2* still show antimicrobial activity when heat-treated at a temperature of 50, 100, and 121 °C in 15-20 minutes (Figure 4).

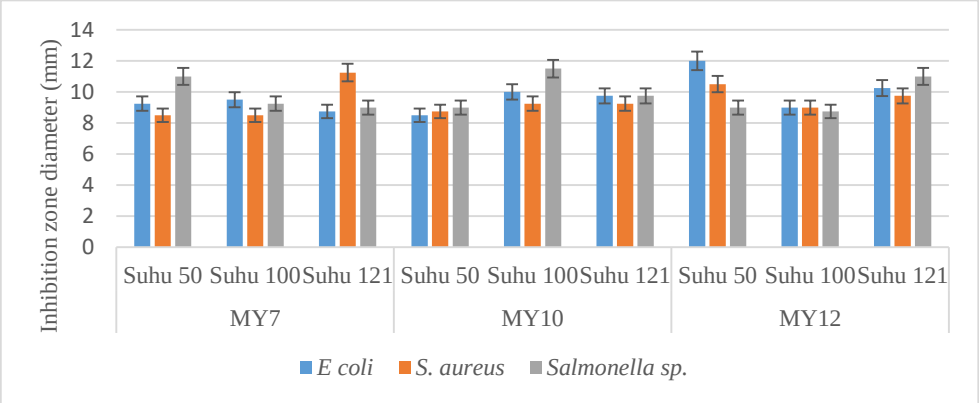

Figure 4 Bacteriocin antimicrobial activity against various temperatures

Effect of Surfactants: Bacteriocins with an added surfactant SDS and EDTA showed an increase in inhibitory activity against all three test bacteria is *E. coli*, *Salmonella sp*, and *S. aureus*. Meanwhile, the addition of urea surfactant does not affect bioactivity in inhibiting bacterial bacteriocins test (Figure 5).

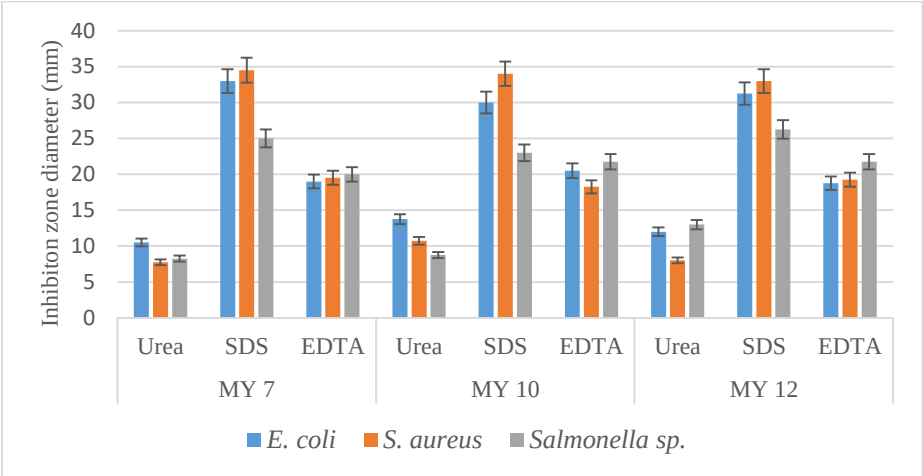

Figure 5 Bacteriocin antimicrobial activity against surfactants

3.2. Discussion

Lactic acid bacterial isolates can be observed from the presence of clear zones around the colony. Lactic acid produced by lactic acid bacteria will bind $CaCO_3$ to dissolve Ca-lactate, giving rise to clear zones. The existence of the clear zone can be used as a marker for the presence of LAB colonies (Nudyanto & Zubaidah, 2014). The colony has the characteristics of white, beige, and yellow (Irwansyah, 2018). The wider the clear zone indicates the greater the ability of isolates to produce lactic acid. LAB is a group of bacteria that do not have a catalase enzyme but have a peroxide enzyme that can convert H_2O_2 to H_2O, thus showing a negative reaction on catalase testing (Raharjo, 2012).

LAB isolates belong to the Gram-positive bacterial purple spherical or rod-shaped, spore-forming, capable of fermenting carbohydrates, catalase-negative, and is a microaerophilic group. These bacteria also possess facultative anaerobes, can dissolve the gelatin, fast-digesting protein, do not reduce nitrate, acid-tolerant, and capable of producing

lactic acid (Yousef & Carlstrom, 2003). Gram-positive bacteria have a thick cell wall in the form of peptidoglycan. When decaying with alcohol, the cell wall pores narrow due to decolorization so that the cell wall still holds violet crystals (Waluyo, 2008). Varying forms of bacteria can be influenced by the environment, both biotic and abiotic factors, growth nutrition factors (growing medium), and temperature (minimum, optimum, and maximum) (Safrida et al., 2012).

From the 15 isolates tested antimicrobial activity, only three isolates had the highest activity. These three isolates were also selected based on the ability of inhibition against the three test bacteria, as well as the resistance of bacteria to survive when grown on new media. Inhibitory zone activities are grouped into four categories: weak (<5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (>20 mm) (Ismail et al., 2017). The greater the inhibition zone was formed, the greater the inhibitory activity against bacterial isolates pathogen (Pelczar and Chan, 1986). All three isolates showed a strong inhibition against *Salmonella sp*. The compound has a bactericidal effect at a pH below 5, especially in Gram-negative bacteria than Gram-positive bacteria. The difference inhibitory activity due to bacteriocin has inhibitory activity against specific bacteria and has a close kinship with the bacteriocin-producing bacteria (USMIATI & Maheswari, 2009).

Identification of isolates carried out by using the VITEK-2 Analyzer tool for selected LAB isolates that have strong antimicrobial activity in inhibiting bacterial test which code isolates MY7, MY10, and MY12. VITEK-2 is a Highly Automated System (HAS) for antimicrobial recognition and sensitivity tests based on the principle of Advance Colorimetry and Turbidimetry, thus enabling the results of identification and sensitivity of antimicrobials to be carried out quickly, which is around 5-8 hour.

The VITEK-2 tool can identify bacteria automatically using a card that contains a miniature tube or well with dried liquid and involves 30 kinds of sugar fermentation. The tool has a model like an API test kit, but smaller because it adapts to any place card slot on the device. Of the three isolates to be identified, the cards used are cards that have ANC and GP codes. Cards with ANC codes are used for isolates MY10 and MY12 which are rod-shaped and Gram-positive, while the GP card code is used for isolates MY7 which are round and Gram-positive. Generally, fish contain LAB such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* (Yuliana, 2007).

The result showed that isolates of LAB by code isolates MY10 and MY12 identified as *Lactobacillus plantarum*, while the isolates of LAB with code MY7 showed that these isolates are identified as *Pediococcus acidilactici*. *L. plantarum* was abundant in fermented foods because it was generally more resistant to acidic conditions so that there are many at the last stage of fermentation and can produce bacteriocin which can be used as food biopreservatives (Ray & Bhunia, 2001). *P. acidilactici* is a species of Gram-positive bacteria in the form of cocci which is often found in pairs or tetrads with an optimum pH of growth which is pH 4. *P. acidilactici* is an important BAL that is commonly found in meat, vegetables, and fermented products (Anriana, 2015). *Pediococcus* exert antagonism against other microorganisms, including enteric pathogens, especially through the production of lactic acid and secretion of bacteriocins called pediocin, whereas bacteriocins produced by *L. plantarum* called plantaricin.

Several studies have examined the bacteriocins produced by lactic acid bacteria to characterize bacteriocins to determine the stability of temperature, pH, and surfactant. The three types of bacteriocins produced by LAB isolated from smoked giant catfish show characterization as a bacteriocins candidate indicated by resistance to the effect of pH 2-6, can survive temperatures up to 121^{o}C, as well as an increased activity when added surfactant SDS and EDTA.

Production of bacteriocin depends on the condition of the growth pH, so the pH plays a big role in determining the quantity of bacteriocin produced by a producing bacterium

(Kusmarwati et al., 2014). Bacteriocin is a short peptide that is stable to heat. Besides, the presence of certain amino acids in the bacteriocin could maintain the structure of the bacteriocin from the influence of heat. The stability of antimicrobial activity against heating is very important for bacteriocin when used as a food preservative, this is because various procedures in food processing use the heating process. Surfactants affect the membrane cell permeability system in cell fluid. Adsorption of bacteriocin to the target cell is very important because as an intermediary the occurrence of bacteriocin insertion into the cell membrane and in forming pores is the cause of cell death so that the addition of surfactants can increase bacteriocin activity[20].

4. CONCLUSION

There were 15 isolates of LAB isolated from smoked giant catfish in MRS + $CaCO_3$ 1% media. Selected bacteria producing highest antimicrobial activity was MY7 isolate identified as *Pediococcus acidilactici*, MY10 and MY12 were identified as *Lactobacillus plantarum*. The three isolates producing bacteriocins were able to inhibit the growth of *E. coli* and *S. aureus*, as well as the most effective in inhibiting the growth of *Salmonella sp*. Bacteriocins of *L. plantarum sp1*, *L. plantarum sp2* suspected plantaricin and *P. acidilactici* were allegedly pediocin are stable until the temperature reaches 121 ^{o}C, stable in the pH range 2-6, as well as increased activity by the addition of surfactant SDS and EDTA. The resulting bacteriocin was expected to be a candidate for biopreservative material in preserving fish-based foods.

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