Isolation and Identification of Lactic Acid Bacteria Producing Biopreservative Bacteriosin from Smoked Fish

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Abstract. Bacteriocin was a product of bacterial metabolites that have antimicrobial properties, so that it had the potential to be developed into biopreservation material in the preservation of food products. 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 bacteria. This study aims to isolate and identify BAL from several types of smoked fish, and to determine the antimicrobial activity of LAB isolate as candidate biopreservative to food product relative to fish product. The research method used was explorative which was analysed descriptively. The results showed that several LAB isolates from various types of smoked fish could potentially produce bacteriocin which can inhibit pathogen bacteria with a broad inhibitory spectrum.

1. Introduction

Fish is one food that has been used by humans because it contains good nutrition when consumed. The Planning Bureau of the Secretary General of the KKP of the Republic of Indonesia [1] stated that the potential of Indonesian marine fish resources is estimated at 7.3 million tons per year spread across Indonesian territorial waters. Fish resources in Indonesia cover 37\% of fish species in the world, where some of them have high economic value, such as tuna, shrimp, lobsters, reef fish, various types of ornamental fish, assault, and seaweed. With this potential, fish cultivation in Indonesia includes developing freshwater, saltwater (sea) and brackish water (ponds).

Fish have high nutritional content, such as protein (6-24\%), fat (0.2-2.2\%), water (58-80\%), and minerals (2.5-4.5\%). Generally fish have a water content of 76 g per 100 g of fresh fish. The high water content in fish flesh can be suitable medium for the life of decomposing bacteria or other microorganisms, so it easy to decay. Prevention of the decay process can be done by processing that can preserve, such as salting, fermentation, and fumigation. This is done to maintain the characteristics of fresh fish [2].

Smoked fish become durable because of the reduction in water content due to the heating process and the presence of chemical compounds in smoke such as phenol which can inhibit the growth of microorganisms and act as antioxidants; however fish fumigation is currently carried out with the aim to provide a distinctive color, texture and flavor. Salmon smoked fish have been identified to contain lactic acid bacteria (LAB) which produce bacteriocin [3].

Lactic Acid Bacteria (LAB) is a group of bacteria from the order Lactobacillales that have characteristics: Gram positive, rod-shaped or cocci, non-spore, non-motile, negative catalase and...
negative oxidase. LAB can produce substances that can extend shelf life such as lactic acid, hydrogen peroxide, reuterine, and active peptides called bacteriocins [4]. Bacteriocin is a ribosomal synthesized peptide which functions to inhibit the growth of other bacteria. Bacteriocins measure around 900 to 5,800 daltons [5]. Bacteriocins produced by LAB have been studied in fermented fish products and non-fermented fish products [6,7,8].

Most of the LAB are as generally recognized as safe (GRAS) by the United States Food and Drug Administration's (USFDA). They exist in most of the fermented foods either as the natural microflora or as starter cultures added under controlled conditions. Bacteriocins are of interest for prospective applications in the food industries because of their potential to increase safety and to extend shelf life of food [9]. Although several LAB strains have been reported to produce antimicrobial bacteriocin but the production of bacteriocins is dependent on multiple factors such as potent LAB strain, pH, temperature, medium compositions and bacterial growth conditions. In recent years, studies are focused on isolation of potent LAB strains and optimization of bacteriocin production conditions to achieve maximum bacteriocin production for individual strains [3].

Preserved fish products that are widely consumed in Indonesia, one of which is smoked fish. 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. This study aims to: (1) to isolate and identify the LAB producing bacteria from several smoked fish from the local market in Indonesia; (2) to determine the antimicrobial activity of LAB isolate as candidate biopreservative to food product relative to fish product. The obtained bacteriocin-producing LAB isolate is expected to be used as a starter for the production of safe processed fish products and also to produce bacteriocins which can be used to preserve processed fish products and general food products.

2. Materials and Methods

2.1. Material
Main materials were smoked fish from “Layang”, “Sero”, “Pari”, “Manyung”, and “Tongkol” fish, 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% BaCl2, Hacker crystal violet, 1% H2SO4, 3% H2O2, Iodine, NaCl, Safranine. Bacterial cultures Staphylococcus aureus and 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 x 108 CFU / ml).

The research phase consisted of analysis, pH, bacterial isolation, morphological identification with Gram staining, and catalase test. The research method used was a qualitative method which was analyzed descriptively, with replications three times.

2.2. Sample Preparation
A total of 25 g of the sample was added with 225 mL of physiological saline solution, and vortexed for 1 minute. Dilution was carried out three times and each of them was taken as much as 100 ul on sterile petridish then added with NA and MRSA medium. The cultures were incubated at 37oC for 24-48 hours. Colonies that form clear zones on the medium are taken and imprinted on the MRSA medium to obtain pure colonies.

2.3. pH Analysis
As much as 10 g of fish meat samples were crushed using mortar and mixed with 20 ml of distilled water. Then poured into a glass beaker and measured the pH using a pH meter.

2.4. Morphological Test (Gram Staining and Catalase Test)
Each isolate colony was taken one by one and then placed on a sterile glass object and dropped 3% hydrogen peroxide solution to test catalase. Isolates that show negative catalase are followed by Gram staining, observation of endospores, and motility [5]. LAB which showed negative catalase and Gram positive was cultured on MRS broth media for 24 hours then stored in tilted MRSA.

2.5. Testing of Antimicrobial Activity with Well Diffusion Agar Method
Media MHA (Mueller Hinton Agar) sterile is poured into a sterile petri dish sufficiently then let it solidify. The testing bacteria (S.aureus and Salmonella sp) was placed onto the well of the medium MHA, and then incubated at 37°C for 24 hours. The diameter of the inhibitory zone (mm) formed around the well was then measured using a caliper.

3. Results and Discussion

3.1. pH and Water Content of Smoked Fish
The types of smoked fish observed consisted of fish, Layang (Decapersus sp.), Sero (Sardinella lemura), Manyung (Arius thalassinus), Pari (Dasyatis sp.), and Tongkol (Euthynnus affinis) which can be seen in Fig.1. pH measurements on the five types of smoked fish tested showed an average pH value of 6, which means it was still in the neutral pH range to acid. According to the research of Oguzhan et al. [11], the water content of smoked rainbow trout (Onchorhyncus mykiss) fillet for 3 hours with a temperature of 80-90°C reduced in moisture content of 11.1% (70.3% in fresh fillets and 59.5% in smoke fillets). With the pH of smoked fish that tend to be acidic with a moisture content of approximately 60%, it was one of the factors that spread smoked fish to be more durable than fresh fish.

![Type of Smoked Fish](image)

**Figure 1.** Type of Smoked Fish

3.2. Isolates of Lactic Acid Bacteria
There were 50 isolates obtained from five types of smoked fish which showed different morphological appearances, where the highest number of isolates were obtained from “Layang” smoke fish (14 isolates), while the least isolates were obtained from “Tuna” smoke fish (5 isolates).

| No | Type of Smoked Fish | Number of Isolates (n) | Number of Lactic Acid Bacteria (LAB) |
|----|---------------------|------------------------|-------------------------------------|
| 1  | Layang              | 14                     | -                                   |
| 2  | Sero                | 11                     | -                                   |
| 3  | Manyung             | 10                     | 1                                   |
| 4  | Pari                | 10                     | 2                                   |
| 5  | Tongkol             | 5                      | 1                                   |
|    | Total               | 50                     | 4                                   |

The bacterial isolates that were grown then screened for lactic acid bacteria by catalase test. The results of the observations obtained 4 bacterial isolates that showed negative catalase test results and
included in the LAB group. There were no LAB from “Sero” smoked fish, whereas from type of “Manyung” smoked fish obtain 1 isolate (M1), 2 species of isolates (P1 and P2) were obtained from “Pari” smoked fish, and 1 isolate (T1) obtained from “Tuna” (mackarel) smoked fish.

All LAB isolates showed small round white colonies on the surface of MRSA media. All isolates showed the characteristics of Gram positive, non-motile, not spore-forming, with short stem cell forms (cocoids). In further research biochemical testing will be carried out on a series of sugar media, and further identification using the API-test kit and molecular analyses with 16s RNA.

3.3. Antimicrobial Activity Test
Antimicrobial activity tests on bacterial isolates from “Pari”, “Manyung”, and “Tuna” smoked fishes were carried out by well diffusion agar method on test bacteria representing pathogenic bacteria groups namely *Staphylococcus aureus* and *Salmonella* sp. Antimicrobial strength was measured from the diameter of the clear zone which showed an area of inhibition of the growth of test bacteria. P1 isolates were only tested against *Salmonella* sp. bacteria, while P2 isolates were only tested against *S. aureus*. The results of testing of antimicrobial activity from isolates of each type of smoked fish could be seen in Table 2.

| No | Isolates | Diameter of the Bacterial Inhibitory Zone Test (mm) |
|----|----------|---------------------------------------------------|
|    |          | *S. aureus* | *Salmonella* sp |
| 1  | M1       | 6.3        | 3.5           |
| 2  | P1       | 0          | 8.3           |
| 3  | P2       | 8.1        | 0             |
| 4  | T1       | 7.8        | 10.5          |

Note: M1 = isolate from “Manyung”; P1-P2 = isolate from “Pari”; T1 = isolate from “Tongkol” smoked fish.

Based on observations, M1 isolates could inhibit *S. aureus* and *Salmonella* sp bacteria with less strong antibacterial strength (<8 mm). P1, P2 and T1 isolates showed quite strong antimicrobial activity (≥ 8 mm) so that it was thought to produce bacteriocin. In the next stage of this research, the three isolates will be purified, characterized and grown to get bacteriocins. This was in line with the research of Barman et al. [10] in which from buttermilk products obtained 3 bacterial isolates with inhibitory zones ≥ 8 mm, and were identified as *Lactococcus lactis* spp. producing bacteriocins. The three isolates could actively inhibit the growth of *Salmonella* sp and *Pseudomonas* sp when grown on MRSB media pH 5.7, with an incubation temperature of 28°C.

In “Roe” fish fermentation, it was found that antimicrobial producing bacteria was *Enterococcus faecium* CN-1 with microbes inhibited was *Listeria monocytogenes*. The fermented seawater fish was obtained by *Lactobacillus plantarum* as an antimicrobial producing microbe against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* [8].

In the research conducted by Todorov et al. [3] about the characterization of bacteriocins from smoked salmon, it obtained isolates of L. sakei lactic acid bacteria which have the ability to produce bacteriocin. The bacteriocin obtained was one of the bacteriocins of class Ila namely sakacin G, which has resistance characteristics at a temperature of 30 °C and pH 5.5. The addition of surfactant affects bacteriocin activity by 1.2 times compared to without the addition of surfactants. Increased bacteriocin activity was related to the interaction of hydrophilic groups and bacteriocin lipophils with pathogenic microbial cells. With the presence of surfactants, contact between bacteriocin and microbial pathogens becomes more effective. The obtained bacteriocin has a size of 3811 kDa.

4. Conclusion
There were 4 isolates of lactic acid bacteria (LAB) from 5 types of smoked fish studied (“Layang”, “Sero”, “Manyung”, “Pari” and “Tongkol”). From 4 LAB isolates 3 of them showed quite strong antimicrobial activity against pathogenic bacteria *S. aureus* and *Salmonella sp*, and were thought to
potentially produce antimicrobial peptides in the form of bacteriocins which would be developed as natural biopreservation material in food products.

References

[1] Biro Perencanaan, Sekretariat Jenderal Kementerian Kelautan dan Perikanan RI. 2016. Kementerian Kelautan dan Perikanan . Jakarta

[2] Hidayati, L., Chisbiyah, L.A., Kiranawati, T.M. 2012. Evaluasi Mutu Organoleptik Bekasam Ikan Wader. Jurnal TIBBS 3 (1): 44-51

[3] Todorov, Svetslav D. Todorov, Cinta Rachman , Ange´ lique Fourrier, Leon M.T. Dicks, Carol A. van Reenen, Herve Pre´vost, Xavier Doussset. 2011. Characterization of a bacteriocin produced by Lactobacillus sakei R1333 isolated from smoked salmon. Anaerobe 17 (2010) 23–31. doi: 10.1016/j.anaerobe.2010.01.004.

[4] Sidhu, P.K., K. Nehra. Bacteriocin-nanoconjugates as emerging compounds for enhancing antimicrobial activity of bacteriocins. Journal of King Saud University – Science xxx (2017) xxx–xxx. Review

[5] Willey, J.M., Sherwood, L.M., & Woolverton, C.J., 2009, Prescott’s Principles of Microbiology. McGraw-Hill Higher Education. Boston

[6] Matamoros, S., Pilet, M.F., Gigout, F., Prevost, H., & Leroi, F., 2009. Selection and evaluation of seafood-borne psychrotrophic lactic acid bacteria as inhibitors of pathogenic and spoilage bacteria. J. Food Microbiol., Vol. 26, hal. 638-644

[7] Montiel, I. Martin-Cabrejas, S. Langa, N. El Aouad, J.L. Arqu es, F. Reyes, M. Medina. 2014. Antimicrobial activity of reuterin produced by Lactobacillus reuteri on Listeria monocytogenes in cold-smoked salmon. Food Microbiology 44 (2014) 1-5

[8] Sonsa, N., A.R. Rodtong, M.L. Chikindas, J. Yongsawatdigul. Characterization of bacteriocin produced by Enterococcus faecium CN-25 isolated from traditionally Thai fermented fish roe. Food Control. Volume 54, August 2015, Pages 308-316. https://doi.org/10.1016/j.foodcont.2015.02.010.

[9] Barbosa MS1, Todorov SD, Belguesmia Y, Choiset Y, Rabesona H, Ivanova IV, Chobert JM, Haertlé T, Franco BD. Purification and characterization of the bacteriocin produced by Lactobacillus sakei MBSa1 isolated from Brazilian salami. J Appl Microbiol. 2014 May;116(5):1195-208. doi: 10.1111/jam.12438. Epub 2014 Feb 8.

[10] Barman, S., R. Ghosh, N.C. Mandal. Production optimization of broad spectrum bacteriocin of three strains of Lactococcus lactis isolated from homemade buttermilk Annals of Agrarian Science 16 (2018) 286–296.

[11] Oguzhan, P. 2013. Effect of salting and packaging on liquid-smoked rainbow trout fillets during refrigerated storage. Afr. J. Microbiol. Res. Vol. 7 (50), pp. 5719-5725.

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