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EVALUATION OF ANTIBACTERIAL ACTIVITY PRODUCED BY *Weisella* sp. GMP12 AND ITS POTENCY AS A STARTER TO ENHANCE FISH FERMENTED PRODUCTS QUALITY

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Abstract

Fish fermented products are popular products among the coastal community in Indonesia. Generally, the products are naturally produced with the addition of salt without any selection of the bacterial community. This situation resulted in a variety in the quality of the final products. One strategy to overcome this problem is by adding a potential lactic acid (LAB), a good bacterium, to the fermentation process. We have conducted a screening of LAB from several local Indonesian fermented fish products, including P*akasam* and *Wadi*. As many as 28 isolates were successfully obtained from the first step of screening, which was characterized as LAB by Gram stain and catalase activity. The second screening was done to screen a LAB that possessed antibacterial activity against common contaminant bacteria, namely *Staphylococcus aureus* ATCC 6538, *Salmonella* sp. 230C, *Escherichia coli* 563 B, *Citrobacter freundii* CK1, *Klebsiella* sp. CK2, and *Morganella morganii* TK7. Among those 28 isolates, we selected one isolate with the highest antibacterial activity and successfully identified molecularly as *Weisella* sp. GMP 12. Further isolation of antibacterial substances targeting bacteriocin showed a good inhibition to *Staphylococcus aureus* ATCC 6538 with 3694 AU (Activity Unit), *Salmonella* sp. 230C with 2254 AU, *Citrobacter freundii* CK1 with 3166 AU but not to *E. coli* 563B. This finding concluded that *Weisella* sp. GMP12 could be a potential candidate as a starter to be used in the fermented fish products to enhance its quality.

Keyword: Antibacterial activity, Bacteriocin, Contaminant bacteria, *Pakasam*, *Wadi*

Introduction

Fermented fish products are one of the products that are widely distributed and consumed by Indonesian people, especially in coastal area. Several Indonesian popular fermented products from fish or shrimp such as *Cincalok*, *Pakasam*, *Bekasang*, and fish sauce are often used as a complement (Irianto, 2012). Fish fermented products in Indonesia utilizes either whole fish, meat, head, or viscera as the main raw materials. Improper handling of raw materials and contamination from the environment can cause the products to cause a food-borne disease (Ly et al., 2020; Satomi, 2016). Contaminant bacteria contained in food can cause disease, like typhus (*Salmonella* sp.) and diarrhea (*E. coli*). Besides, histamine-producing bacteria (HPB) can also cause symptoms of nausea, vomiting, diarrhea, hypotension, local inflammation, and heavy breathing which is often known as scombroid fish poisoning (SFP). Histamine content in fermented fish products, particularly in the Southeast Asian region, ranges from 0.1 to 1220 mg/kg, where the histamine content limit set by the Codex Committee on Fish and Fishery Products (CCFFP) is 200 mg/kg (Anal et al., 2020). Therefore, it is necessary to have treatment to prevent those disease in fermented fish products. Prevention of food-borne disease in fermented fish products can be done by utilizing lactic acid bacteria (LAB) (Sidhu & Nehra, 2019). LAB can be used as a bio preservative agent as a starter bacterium or

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utilizing secondary metabolites, such as bacteriocins to hinder the growth of food contaminating bacteria (Calo-Mata et al., 2008; Ghanbari et al., 2013; Sidhu & Nehra, 2019).

The processing of fermented fish products is tightly related to LAB. LAB, which is a Gram-positive bacterium, can inhibit the growth of not only closely related Gram-positive bacteria but also several Gram-negative bacteria (Ahmad et al., 2017; Putra et al., 2022). LAB produce proteinogenic substance namely bacteriocins that are bacteriostatic or bactericidal and able to kinfluesnce the growth of food contaminant bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Bacillus thuringiensis*, *Salmonella* sp., *S. aureus* and histamine-producing bacteria such as *Morganella morganii* (Sasanti & Fitria, 2012; Goh & Philip, 2015). The addition of LAB as starter in fermented products are reported to give positive effect to the product. The uses of *Pediococcus* sp. B1.0 in the *Bekasang* (Indonesian fermented fish) fermentation process was reported to inhibit the histamine formation (Lawalata & Ruangkat, 2019). Moreover, the addition of *Streptococcus salivarius* in *Plasom* (traditional Thai fermented fish) was able to decrease pathogenic bacteria during fermentation (Hwanhlem et al., 2011). Our previous research succeeded in the identification of potential LAB namely *Weisella* sp. GMP12 from Pakasam. From the preliminary observation, *Weisella* sp. GMP12 cell free supernatants was capable of preventing the progression of HPB, e.g., *Morganella morganii*, *Citrobacter freundii*, and *Klebsiella* sp. (Al Hammam et al, 2023). For evaluation of the potency of *Weisella* sp. GMP12 as a starter in fish fermented products, we purified the bacteriocin and characterized it against various conditions such as temperature, proteolytic enzyme and salt concentration.

Materials and Method

Materials

Weisella sp. GMP12 was isolated from *Pakasam* (Indonesian traditional fermented fish) and identified previously (Al Hammam et al., 2023).

The materials used in this study included de-man rogosa and sharpe broth (MRSB), de man rogosa and sharpe agar (MRSA), trypticase soy broth (TSB), trypticase soy agar (TSA), nutrient agar (NA), nutrient broth (NB),(Merck KGaA, Darmstadt, Germany), 30% glycerol, 0.85% NaCl, 1% CaCO3, 70% alcohol, 70% ethanol (Merck), distilled water, 50 mM citrate buffer, 50 mM potassium phosphate buffer, 50 mM glycine phosphate buffer, kanamycin (Meiji Indonesia), Presto Mini gDNA Bacteria Kit (Geneaid), proteinase-K enzymes (Sigma, USA) and papain (Sigma, USA). Histamine-producing bacteria used were *M. morganii* TK7, *C. freundii* CK1, *Klebsiella* sp. CK2 from the collection of the Laboratory of Fishery Products Quality and Control, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada. Other tested bacteria namely *Escherichia coli* 563B, *Salmonella* sp. 230C and *Staphylococcus aureus* ATCC 6538 were provided by Yogyakarta Fish Quarantine, Quality Control and Fishery Products Safety Station.

The tools used in this study were microscopes (Olympus Model BX51TRF, Japan), vortex (Barnstead M37610-33), refrigerator -30°C (Sanyo SR-D180F), incubator (Isuzu SSJ-115), centrifuge (Kokusan H-26 F), autoclave (Hi-clave HVE-50 Hirayama), thermal cycler (Bio-Rad Laboratories Ltd), freeze drier Labadeco Corporation Cascade Console Freeze Drying System (Labconco Corporation, Kansas, MO, USA) and UV-Vis spectrophotometer (Genesys 10s UV-Vis).

Method

Bacterial Strain Preparation

Each strain was inoculated from glycerol stock in appropriate agar media (MRS-agar for lactic acid bacteria and nutrient agar or trypticase soy agar for other bacteria) and incubated at 37°C for 24 h.

Determination of Lactic Acid Bacteria Growth and Antibacterial Production Curve

The determination of the LAB growth curve adapted from Maulidayanti et al. (2019), by incubated *Weisella* sp. GMP12 in 60 mL of MRS broth and incubating at 37°C for 48 hours without shaking. Determination of the growth curve was calculated by total plate count (TPC) every 6 hours. The TPC spot test method refers to Whitmire & Merrell (2012) by dripping 10µl samples that have been diluted serially. Development of bacteriocin and antibacterial activity against *M. morganii* TK7, *C. freundii* CK1, *Klebsiella* sp. CK2, *E. coli* 563B, *Salmonella* sp. 230C and *S. aureus* ATCC 6538 were also observed every 6 hours.

Total plate count (CFU/mL) = [(number of colonies) × (1/dilution factor) × 1,000 µL/mL]/(volume plated)

Production of Bacteriocin

The bacteriocin isolation was carried out based on Abrahams et al. (2011) and Lim (2016). *Weisella* sp. GMP12 isolate was inoculated on MRS agar and incubated at 37°C for 24 hours. The single colonies formed were then moved to MRS broth and incubated at 37°C for 24 hours or until full growth (OD_{600} = 1). Furthermore, 1% (v/v) of previously cultured was then re-inoculated into MRS broth media with a total volume of 300 mL followed by incubation at 37°C for 24 hours without shaking. After 24 hours, the supernatant was collected through 15 minutes centrifuging at 15,000 x g at 4°C. The supernatant which became cell free was then added with phosphate buffer pH 6.5 to avoid acid effects and added with catalase treatment (200 U/mL) for 30 minutes at 25°C to remove hydrogen peroxide. Then the cell-free supernatant was filtered using a filter membrane/Millipore with a pore diameter of 0.22 µm and continue to bacteriocin purification.

Bacteriocin Purification

The purification of bacteriocin is carried out partially by ammonium sulfate precipitation and dialysis (Aslam et al., 2011). The cell-free supernatant was mixed with ammonium sulfate with different saturations (40%, 60%, and 80% w/v) at 4°C with constant stirring overnight. The suspension was then centrifuged at 10,000 x g for 10 minutes at 4°C, the supernatant was removed and the precipitate (presumed to be bacteriocin) was obtained. The crude bacteriocin precipitate was then dissolved in 0.2 M phosphate buffer at a neutral pH (1:1 ratio) and followed by dialysis using a 1 kDa cut-off dialysis bag. Dialysis was carried out by inserting a dialysis membrane that had been filled with crude bacteriocin precipitate on 0.2 M phosphate buffer into 0.01 M potassium phosphate. The process was carried out on a stirrer at 4°C and the buffer was replaced twice (2nd and 4th hours), then every 6 hours. Dialysis was stopped if after dropping $BaCl_2$ no white precipitate formed. The resulting bacteriocin extract was then dried using the freeze dry method at -86°C (Yu et al., 2012).

Antibacterial Activity Test

Antibacterial activity was conducted according to Arfani et al. (2017) using the disc diffusion method. Histamine-producing bacteria were grown on 0.7% TSA soft agar medium and others were grown on 0.7% NA soft agar medium. Cell-free supernatant (CFS) or bacteriocin (final conc. 1 mg/mL in phosphate buffered saline) was dripped onto a paper disc with a diameter of 6 mm. Confirmation of bacteriocin was conducted by treating it with 4% proteinase-K. Kanamycin with a final concentration of 50 µg/mL was used as a positive control. The paper disc is placed on a medium that has been inoculated with indicator bacteria. The inhibition activity of the bacteria was measured from the cleared zone formed around the paper disc. Antibacterial activity was calculated as AU/mL (arbitrary unit) with one AU defined as area of inhibition zone per volume of mL of sample.

Bacteriocin Characterization Test

The characteristics of bacteriocins were tested for their sensitivity to temperature, pH and enzymes. Temperature sensitivity testing was carried out by heating the bacteriocins at 40°C, 60°C, 80°C, and 100°C for 60 minutes and 121°C for 20 minutes. At every temperature the residual bacteriocin activity was measured and the unheated bacteriocin was used as a control. pH sensitivity testing was carried out by adjusting the bacteriocins to pH 2, 3, 4, 5, 6, and 7 with NaOH and HCl (Wang et al., 2018), and maintaining them at the appropriate pH for 20 minutes. The activity of residual bacteriocin at each pH was measured using unadjusted pH bacteriocin as a control. The enzymatic sensitivity of bacteriocins was tested by incubating bacteriocins with proteinase-K (Sigma, USA), trypsin (Ameresco, USA), and papain (Sigma, USA) (each enzyme 1 mg/mL) at optimal temperature and pH for 1 hour. The mixture was then heated to 100°C for 5 minutes to inactivate the enzyme.

Results

Bacterial growth, antibacterial activity and bacteriocin purification

Fish fermented products are popular among coastal community in Indonesia. Generally, those products were fermented naturally without any selection of beneficial bacteria involved in fermentation process. This condition

sometimes led to the non-uniformity of final products. Thus, using a bacterial starter became one of the strategies to solve the problem. We successfully isolate *Weisella* sp. GMP12 from *Pakasam*, one of a traditional fermented food with fish as the main raw material produced in Sumatra and Kalimantan (Al Hammam et al., 2023). To evaluate the potential of *Weisella* sp. GMP12 as a starter, we first observed the growth rate and the production of antibacterial substances. As shown in Figure 1, *Weisella* sp. GMP12 was in the log phase in the first 6 hours. The growth increased from log 6 cfu/mL to log 8 cfu/mL and remained steady until 36 hours.

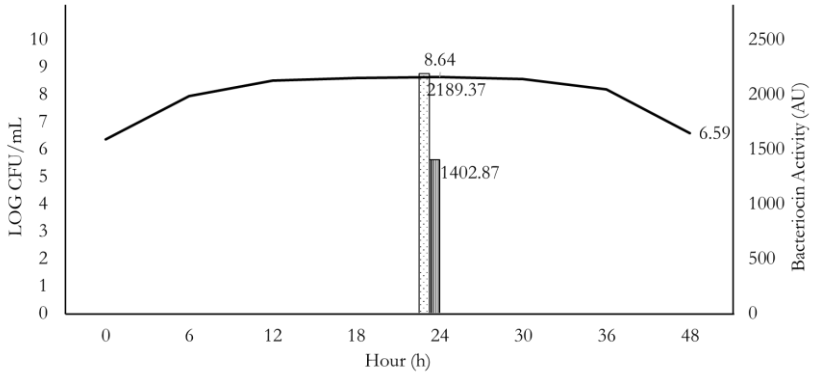

Figure 1. Growth and antibacterial substance production of *Weisella* sp. GMP12 (dot: antibacterial activity against *Salmonella* sp. 230C; strip: antibacterial activity against *E. coli* 563 B)

The antibacterial activity test suggested that antibacterial substances were only produced in a very short period of time between 18 and 30 hours of incubation. The crude cell-free supernatant only showed an inhibition to Gram-negative *Salmonella* sp. and *E. coli* but not to tested Gram-positive bacteria. This incubation time was then used for the production of bacteriocin.

Bacteriocin extraction from *Weisella* sp. GMP12 was obtained from supernatant with 24 hour incubation based on the growth curve and antibacterial activity test (Figure 1). Purification of bacteriocin was carried out by the precipitation method using ammonium sulfate salt ($(NH_4)_2SO_4$) and continued with dialysis. The precipitation of ammonium sulfate aims to separate protein from other components contained in the supernatant. Ammonium sulfate salt will bind water in protein molecules thereby increasing protein saturation and making protein in the solution settle. The bacteriocin purification results can be seen in Table 1.

Table 1. Partial purification of bacteriocin from *Weisella* sp. GMP12

Sampel	Volume (ml)	Activity (AU/mL)	Dissolved protein (mg/ml)	Total activity (AU)	Total protein (mg)	Specific Activity (AU/mg)	Yield (%)	Purification Fold
CFS	100	2184	0.081	218400	8.11	26936.94	100	1
40% saturation	20	1566	0.074	31320	1.48	21196.68	0.72	0.79
60% saturation	20	1935	0.062	38700	1.24	31264.13	0.89	1.16
80% saturation	20	2310	0.046	46200	0.92	50345.85	1.06	1.87
Dialysis 80%	10	2529.67	0.01	25296.67	0.10	242383.72	1.16	9.00

Note: Tested bacteria: Salmonella sp.

The data obtained from partial bacteriocin purification by ammonium sulfate precipitation and dialysis are presented in Table 1. The protein content obtained in each purification process has decreased along with the increase in the level of saturation of ammonium sulfate. CFS or partially purified bacteriocin has higher protein

content compared to each purification fraction. This is because CFS still contains bacterial suspension or other impurities. The value of dissolved protein that is gradually decrease indicates the separation of partially purified bacteriocin with impurities.

The 80% fraction showed the highest inhibitory activity compared to the 40% and 60% fraction with inhibitory activity of 2310 AU/ml with purification fold of 1.87 times. These results indicate that the salting out process of bacteriocin mostly occurs at 80% saturation. Based on this, the 80% fraction continued with the dialysis process. The dialysis fraction has a dissolved protein content of 0.01 mg/ml, the content is lower than that of 80% fraction which is 0.046 mg/ml. The dialysis fraction inhibitory activity increased 90% with purification fold of 9.00 times compared to CFS. The 80% dialysis was then further dried by freeze-dried process (-86°C) and successfully obtained ± 120 mg of bacteriocin from 20 ml of dialysis extract. We again conducted antibacterial activity test on partially purified bacteriocin and the data can be seen in Table 2.

Table 2. Antibacterial activity test on partially purified bacteriocin

Tested bacteria	Antibacterial activity (AU)
Staphylococcus aureus ATCC 6538	3694
Salmonella sp. 230C	2254
E. coli 563 B	1608
Citrobacter freundii CK1	3166

Note: final concentration 1 mg/mL

Table 2 showed the inhibitory activity of bacteriocin after freeze dried. Bacteriocin from *Weisella* sp. GMP12 interestingly able to inhibit the growth of either Gram-positive and Gram-negative bacteria.

Bacteriocin Characterization

Temperature is one of the parameters related to the process of heating and cooking in food processing. The use of heat in the cooking process generally ranges between 40°C-100°C. However, some food products need to be sterilized at 121°C. Temperature stability is needed to determine the extent to which bacteriocin produced by *Weisella* sp. GMP12 can be applied in food as an alternative antimicrobial compound to inhibit the growth of food contaminant bacteria. The stability in various temperature is depicted in Figure 2.

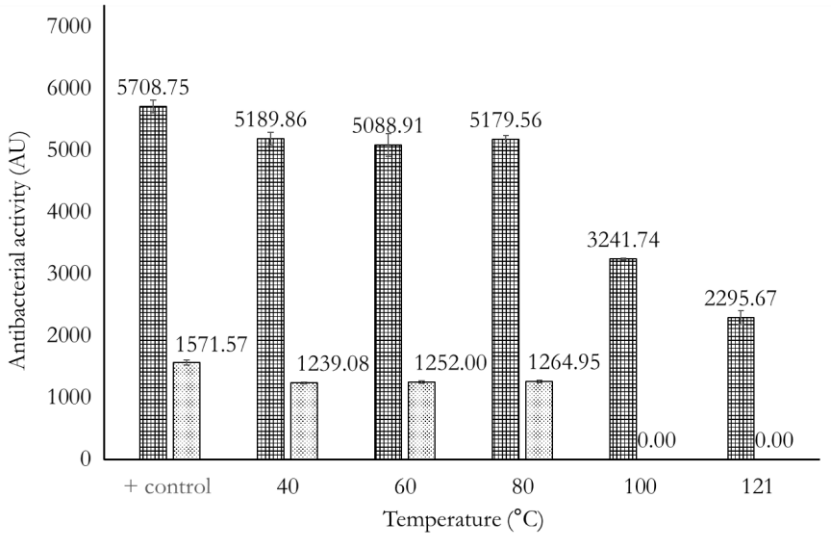

Figure 2. Bacteriocin stability in various temperature (dot: *Salmonella* sp.; square: *Staphylococcus aureus*)

Bacteriocin activity of *Weisella* sp. GMP12 against *Staphylococcus aureus* ATCC 6538 remains steady until 80°C and starts to decrease until 121°C. Compared to *Staphylococcus aureus* ATCC 6538, antibacterial activity against *Salmonella* sp. 230C is lower, and it loses its activity at 100°C and 121°C.

Changes in pH in food processing often occur with the addition of acidic substances. Moreover, the fermentation process of fishery products, which utilizes lactic acid bacteria, also produces acids that lower the pH of the products. Bacteriocin pH stability is an important parameter which is crucial to observe to govern the ability of bacteriocin in various pH ranges (Figure 3).

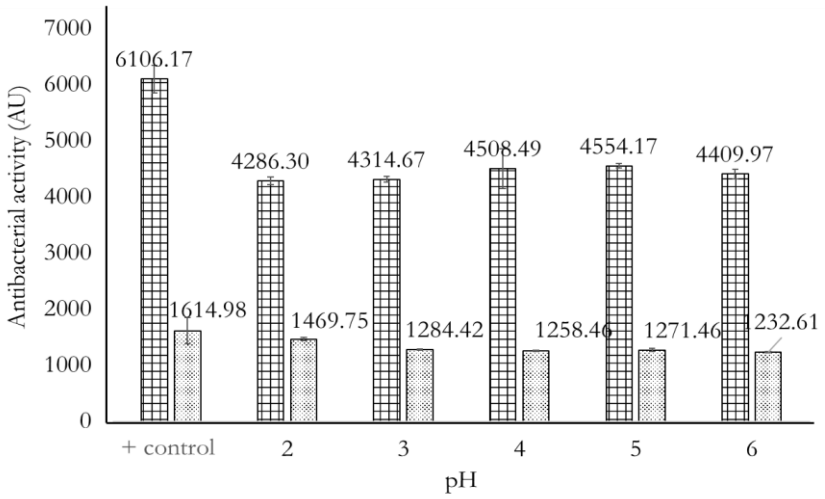

Figure 3. Bacteriocin stability in various pH (dot: *Salmonella* sp.; square: *Staphylococcus aureus*)

Data obtained from Figure 3 shows that bacteriocin produced by *Weisella* sp. GMP12 has stability in a fairly wide acid pH range. Antibacterial activity showed higher inhibition in *Staphylococcus aureus* ATCC 6538 than in *Salmonella* sp. 230C.

As a proteinogenic antibacterial substance, bacteriocin tends to lose its activity due to the presence of protease, so it is necessary to know the stability of the bacteriocin before applying it in the process of food processing. In this study, papain and proteinase-K enzymes are used as protease and lysozyme as non-protease with final concentration of 1 mg/ml (Figure 4).

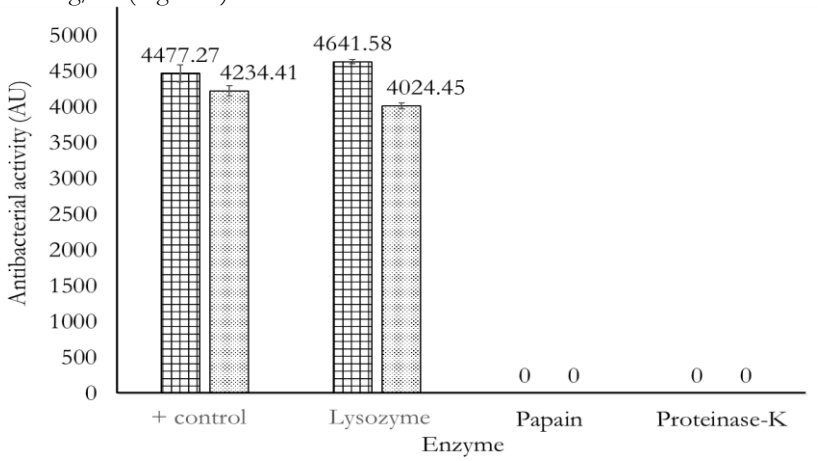

Figure 4. Bacteriocin stability in various enzyme (dot: *Salmonella* sp.; square: *Staphylococcus aureus*)

The results of the addition of enzymes showed that bacteriocin produced by *Weisella* sp. GMP12 lost its inhibitory activity after treatment with papain and proteinase-K. On the other hand, the addition of the lysozyme shows no effect on bacteriocin activity against *Staphylococcus aureus* ATCC 6538 and *Salmonella* sp. 230C. Protease enzymes are able to degrade bacteriocin, so bacteriocin loses its antibacterial activity (Huang et al., 2009). Protease can also be used as a confirmation that antibacterial activity that arises is caused by bacteriocin itself, not other metabolites (Pringsulaka et al., 2012). The addition of proteases such as papain and proteinase-K was reported to be able to eliminate the antibacterial activity of bacteriocin (Lei et al., 2020; Wang et al., 2018).

Discussion

Weisella sp. GMP12 grew well in MRS broth media with a relatively short lag phase and directly continued to log phase from 6 hours to 24 hours. Antibacterial substances in this study were optimally produced at the beginning of the stationary phase. This result is in accordance with Kusmarwati et al. (2014), who revealed production of antibacterial substances by LAB starts at 12 hours and is optimal at the 24 hours of incubation. Bioactive compounds or secondary metabolites specific to bacteria can be produced throughout the growth phase, but production is higher when the final exponential phase is reached (Seyedsayamdost, 2019). Many metabolites including hydrogen peroxide, acetoin, reuterin, bacteriocins, diacetyl, and reutericyclin are produced by LAB (Ibrahim et al., 2021). In this study, we targeted bacteriocin as the antibacterial substance and successfully isolate partially purified bacteriocin. Antibacterial activity test of bacteriocin on Gram-positive and Gram-negative bacteria showed that bacteriocin tend to have higher inhibition to Gram positive bacteria (Table 2). Bacteriocin is more effective in inhibiting bacteria that have close kinship (Parada et al., 2007). This difference in activity is due to the structure of Gram-negative bacteria, which are more difficult to penetrate by bacteriocin compared to Gram-positive bacteria (Yoneyama et al., 2011). The outer membrane of gram-negative bacteria serves as a barrier to cell permeability so as to prevent bacteriocin from penetrating to the cytoplasm or passing target cells (Kumar et al., 2009). Gram-negative bacteria are generally composed of lipopolysaccharides (LPS) consisting of O antigens, core oligosaccharides, and lipid A. The presence of LPS, especially Lipid A, prevents bactericide activity from cationic antimicrobial peptides, so that bacteriocin is not efficient in inhibiting gram-negative growth (Paczosa & Mecsas, 2016).

In order to evaluate the potency of bacteriocin produced by *Weisella* sp. GMP12 to be used in the food system, we conducted stability test at various temperatures, pH and enzymes. Based on the results, bacteriocin produced by *Weisella* sp. GMP12 was stable after heating at 40°C-100°C and stable at various acid pH, however, it's not stable to protease. Similar results were also reported that bacteriocin produced by LAB has a high range of temperature stability (Lei et al., 2020; Zhang et al., 2018). Research by Zhang et al., (2018) succeeded in isolating the bacteriocin namely Lac-B23 produced by *Lactobacillus plantarum* J23 with a molecular weight of 6.73 KDA. Lac-B23 still has an inhibitory activity to *Listeria monocytogenes* after heating 121°C for 30 minutes. Lei et al., (2020) isolating crude bacteriocin from the *Lactobacillus plantarum* ZRX03 which has stability after treated at 121°C. From this result, we presume that bacteriocin produced by *Weisella* sp. GMP12 was belong to bacteriocin Class I (<5 KDA) or Class II (<10 KDA) which have a simple molecular shape and compact bonds so that it is stable at high temperatures (Abrams et al., 2011b; Ahmad et al., 2017). The stability in various pH also reported by Lei et al. (2020) and Zhang et al. (2018) which mention that bacteriocin produced by LAB has good pH stability in the range 2-12. The stability on various low pH shows that bacteriocin can be used as an alternative antimicrobial in food.

Conclusion

In this research we successfully purified bacteriocin from newly isolated lactic acid bacteria namely *Weisella* sp. GMP12. The evaluation of the stability of the bacteriocin in various temperature and pH conclude that *Weisella* sp. GMP12 have a high potency for the production of fermented fish products.

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Conflict of Interest

The authors declare no conflict of interest.

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