Characterization of Bacteriocin Produced by Lactic Acid Bacteria Isolated from Solid Waste of Soymilk production

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Abstract. Biopreservation of food using bacteriocin from lactic acid bacteria (LAB) was an innovative breakthrough. Lactic acid bacteria can protect against food spoilage and pathogen bacteria by producing bacteriocin. The purpose of this study was to characterize the bacteriocin produced by LAB isolated from solid waste of soymilk that had probiotics properties. The LAB having antibacterial activities was evaluated their growth, and identified by using 16S rRNA gene sequence analysis. Its bacteriocin activities was tested on various pHs (2, 3, 4, 5, 6, 7, 8, and 9) and temperature (60–100 °C). Its activities was evaluated againsts pathogenic bacteria (Staphylococcus aureus ATCC 25923 and Listeria. monocytogenes CFSAN004330), enzymes (trypsin, catalase and protease-K), and antibiotics (penicillin and ampicillin). The results showed that LAB A23.4 isolates, which had 16S rRNA gene sequence were L. plantarum strain TMW 1.1623. Its Bacteriocin had antimicrobial activity against S. aureus ATCC 25923 and L. monocytogenes CFSAN004330 at pH 2-7, at temperatures of 60, 70, 80, 90, 100 ° C for 60 minutes and lysed by the enzymes trypsin and protease-K. Bacteriosin activity was stronger than that of the antibiotics of penicillin and ampicillin against S. aureus and L. monocytogenes. The inhibition zone of supernatant bacteriocin was 10 and 20 mm for S. aureus and L. monocytogenes. On the other hand, penicillin and ampicillin inhibition zones were 0 and 3 mm, respectively. From these results, it can be concluded that the antimicrobial produced by L. plantarum strain TMW 1.1623 was a bacteriocin used as food preservation that its processing using relatively wide range temperature (60-100) with pH 2-7.

1. Introduction
Food is a material that is very easy to contamination by spoilage microorganisms because it contains nutrients that are needed by these microorganisms for its growth. The amount of microorganism contamination in food products can affect the quality and shelf life. One way to reduce the occurrence of contamination by microorganisms is to add preservatives.

Bacteriocin is an alternative bio preservative agent that can be used as a food preservative. Bacteriocin is a protein compound produced by lactic acid bacteria [1] that has bactericidal properties against Gram-positive and Gram-negative bacteria. It is very beneficial for the food industry because its activity can inhibit the growth of disease-carrying bacteria that are usually present in food [2]. Some
LAB species produced chemical compounds that had microbial activities such as bacteriocin and hydrogen peroxide [3].

Research on antimicrobial compounds from LAB had been carried out including, research by Ref. [4] about bacteriocin produced by Lactococcus lactis subsp. lactis strain isolated from traditional food fermentation. The antimicrobial activity of bacteriocin produced by Lactobacillus Sp. isolated from traditionally produced milk was observed by Ref. [5]. Purification and characterization of the F1 bacteriocin, a bacteriocin produced from the L. paracasei subsp. from Tibetan kefir, China [6]. The results showed that F1 bacteriocin was the first bacteriocin produced by L. paracasei subsp. and this bacteriocin is potentially used in the food industry.

Lactobacillus isolated from solid waste of soy milk processing [7] was a potential source of isolates for producing antibacterial, and they also have probiotics properties. LAB, which has probiotic properties and can also produce bacteriocin, is a characteristic of LAB that is very well used in food processing. This LAB can act as a probiotic and a food preservative that can extend the shelf life of the food. The objectives of this study were to characterize bacteriocin produce Lactobacillus spp having probiotics properties that were isolated from solid waste of soy milk production,

2. Materials and Methods

2.1. Materials

Materials used were Lactobacillus isolated from solid waste of soy milk production, indicator bacteria (Escherichia coli O167: H7, Staphylococcus aureus ATCC25923, and Listeria monocytogenes CF30004330), antibiotics (Penicillin, Ampicillin and Kanamycin) disks, EDTA, Lysozim, Rytomysis, Nuclear Solution -nase solution, Protein precipitation solution, isopropanol, ethanol, Rehydration solution, Primer R and F, TE Buffer, dH2O, Master Mix, Template, Agarose, TAE, and redsafe / gel view staining. (Merck), MRS Broth (Merck), Nutrient Agar (Merck), Nutrient broth (Merck), Nutrient broth (Merck), Buffer Pepton Water, glycerol, aquades, alcohol spritus, HCl6M, NaOH 6M.

The equipment used is cool box, anaerobic jar, thermometer, pH meter, petri dish, ose needle, incubator (Infors HT-ecotron), PCR (Biometra centrifus Sartorius Sigma), Biodoc Analyze (Biometra), Electrophoresis (Mupid Exu), measuring cup, analytical scales, erlemeyers, drop pipettes, bunsen lamps, test tubes, measuring cups, hockey stics, cotton buds, beaker glass, autoclaves, and micropipettes.

2.2. Research Methods

2.2.1. Cell-free supernatant antimicrobial activity

This method referred to diffusion method 9 as much as 50 µl of the supernatant was put into the wells that had been provided. Petri dishes and their contents were placed for two hours in a refrigerator so that the supernatant seeps into the agar medium. Then incubated at 37°C for 24 hours. The clear zone formed indicates the inhibition of the growth of test bacteria by the supernatant. Clear zone diameters were measured using a caliper three times with different positions and averaged.

2.2.2. Determination of Lactic Acid Bacteria Growth

Lactic acid bacteria isolate was inoculated in 1 ose. It was incubated in 9 ml MRS-B media for 48 hours at 37 ° C. Bacterial growth was observed in optical density / OD values at 0 and 3 hours and followed every 6 hours for up to 48 hours. It used the turbidity method with 650 nm wavelengths by using a visible UV spectrometer to a constant absorbance value indicating the LAB growth has reached the stationary phase. If the value of absorbance reading is above 1, it was necessary to dilute it with sterile MRS-B10.

2.2.3. Identification Microorganism Using 16S rRNA

The species of selected LAB were identified based on 16S rRNA gene sequence analysis. Genomic DNA from the isolate was extracted using kit Presto™ Mini gDNA Bacteria. The DNA gene was amplified by PCR using the universal primers 27 F (5'-GAGTTTGATCCTGGCTAG-3'), 1525 R (5'-
AGAAAGGAGGTGATCCAGCC-3'). The conditions of PCR amplification were programmed as follows: Initial denaturation for 5 min at 95°C. 40 denaturation cycles at 94°C for 45 sec/each, 1 min annealing time at 56°C, 1 min and 30 sec for the extension at 72°C, and 7 min for a final extension at 72°C. The amplification products were analyzed by electrophoresis using 1% (w/v) agarose gel and visualized. The electrophoresis gel was stained with 5 µg/ml ethidium bromide solution by immersion, then visualized over UV light (WiseUV WUV-M20) and photographed with a digital camera (Olympus SP-500 UZ). The 16S rRNA sequence was compared with the sequences available in the nucleotide database using the BLAST (Basic Local Alignment Search Tool) at the NCBI server.

2.2.4. Antibacterial Activity of Bacteriocin against Pathogens and Antibiotics
One ml of culture was incubated for 24 hours in 9 ml of MRS Broth at 37 °C. Then, centrifuged at 14,000 rpm for 5 minutes. The supernatant was filtered with a 0.22 µl membrane filter. Cell-free supernatant is adjusted to pH 6.5 with 1N NaOH, to eliminate the inhibitory effect due to the presence of organic acids11. Pathogenic bacteria are grown aerobically at 37 °C for 24 hours. Then a 0.2% pathogenic bacterial culture was inserted into 20 ml NA at 50 °C. After solidifying, the well is 4 mm in size, using a cork borer. Fifty ml supernatant put in each well, and an antibiotic disk was placed on the surface of the media, allowed to stand for 15-20 minutes. Furthermore, it was incubated for 24 hours at 37 °C under aerobic conditions. Then the inhibition zone is measured using a caliper.

2.2.5. Antibacterial Activity of Bacteriocin at Different Degrees of Acidity
Ten ml of MRS Broth were prepared with pH 2, 3, 4, 5, 6, 7, 8, and 9 respectively using 1 N HCl or 1 N NaOH and autoclaved. After that, it was incubated for 2 hours at 37 °C, and each pH was adjusted back to pH 6.0. The inhibitory activity measured by all treatments was determined by the agar diffusion method as described above, and S. aureus and L. monocytogenes as indicators of bacteria (Chen, et al. 2014). Each was carried out three times, and repeated standard deviations were measured.

2.2.6. Antibacterial Stability Bacteriocin against Heat
Cell-free supernatant was treated at 40, 60, 80, and 100 °C for 60 minutes compared to controls (without heat treatment). Then tested by diffusion method so that it uses bacterial indicators12. The method used in this research was the descriptive method. The temperature treatments are: (a). 40 °C, (B). 60 °C, (C). 80 °C, and (D). 100 °C for 30 minutes. The activity of barriers measured by all treatments was determined by the agar diffusion method as described above and S. aureus and L. monocytogenes as indicators of bacteria. Each was done three times, and standard deviations were measured.

2.2.7. Bacteriocin Extraction and Purification
LAB isolates that have the best resistance to pathogenic bacteria based on temperature, followed by bacteriocin purification13. LAB isolates were grown in 60 ml MRSB. Then centrifuged at a speed of 13,000 rpm for 10 minutes at 4 °C. The supernatant was neutralized with 1 N NaOH to pH 6.5 and filtered using a 0.22 µm membrane filter. Ammonium sulfate (30%, 60%, and 90% gradually) is poured into 200 ml supernatant and stirred until the ammonium sulfate dissolves. The solution was stored at 4 °C for 3 × 6 hours. Then the solution was centrifuged at 4000 rpm for 30 minutes at 4 °C. The precipitate formed was dissolved in 9 ml of citrate phosphate buffer (50 mM: pH 5.0). Then bacteriocin activity was measured in two stages: Cell-free supernatant and bacteriocin extract after precipitation with Ammonium Sulfate.

2.2.8. Effect of Enzymes on the Pathogenic Bacterial Activity
Testing was done by the agar diffusion method (Djordjevic et al. 2015; Muse and Hartel 2004). The 200 µl supernatant from LAB was dissolved in 20 ml each of the enzymes dissolved protease-K (pH 7), catalase (pH 7), and trypsin (pH 7). These enzymes were dissolved in NaOH or phosphate buffer pH 7. Supernatant that has been mixed with this enzyme was incubated for 2 hours at 37 °C, and followed by heating in boiling water for 5 minutes. Supernatants that are sensitive to enzymes are not clear zones.
2.3. Data Analysis
All experiments were carried out in quadruplicate. Statistical analysis was carried out using Excel software (Windows 2010).

3. Results and Discussion

3.1. Cell-free supernatant antimicrobial activity
Six of the 24 LAB isolates isolated from solid waste from soy milk production had antimicrobial activity on all three indicator bacteria. One of these isolates (A23.4) has the highest antimicrobial activity, which is characterized by the formation of a clear zone greater than 8 mm in each bacterial pathogens. Cell-free supernatant had inhibitory activity against the growth of Escherichia coli 0157: H7, Staphylococcus aureus ATCC 25923, and Listeria monocytogenes CFSAN004330. The diameter of the cell-free supernatant inhibition against pathogenic bacteria could be seen in Figure 1.

![Fig 1. Antimicrobial activity of cell-free supernatant of A23.4 isolate against pathogens, Clear zone diameter does not include wellbore](image)

In Figure 1 the highest antimicrobial activity of cell-free supernatant was found in L. monocytogenes (13.43 ± 0.24 mm). These results illustrate that LAB isolates could inhibit the growth of pathogenic bacteria. Cell-free supernatants had different abilities to inhibit the growth of pathogenic bacteria and produce antimicrobials. Secondary metabolites of LAB were produced extracellularly so that the supernatant was the result of the separation between the cell and the liquid portion containing the secondary metabolite. The secondary metabolite products include organic acids, hydrogen peroxide, diacetyl, and bacteriocin. The LAB secondary metabolites can function as self-defense [15].

3.2. Determination of Lactic Acid Bacteria Growth
Based on the selection results to obtain LAB having the potential as a probiotic in previous studies, the A.23.4 isolate was chosen. In Figure 2. it can be seen that in the initial incubation stage from 0 to 3 hours, there was a lag phase. In this phase, LAB was in an adaptation period where growth was very slow, or at this time, there was no significant bacterial growth. The results obtained were almost the same as the results of research on determining the optimum incubation time of LAB to produce bacteriocin, where the first 2 hours of incubation period did not occur significantly LAB growth. The LAB growth could be observed through an increase in OD values, which increases OD values (Figure 2) in line with the increase in turbidity of the suspension, which indicates that there is an increase in bacterial multiplication.
The A23.4 LAB isolates entered the period of suspension at the 24 to 36 hours from the time of incubation. At this time in the medium of nutrient availability had decreased, and LABs began to produce secondary metabolic, namely bacteriocin, which was extracellular secondary metabolic.

3.3. Identification of Lactic Acid Bacteria with 16S-RNA
Isolation and PCR amplification of 16S-RNA genes to the genome DNA isolate A.23.4 showed in Figure 3. These results could be seen from the visualization of electrophoresis, which was a 1444 bp DNA tape using 27F primers using 27F primers Primer R (GTATACCTT GTACGACTT) and F (AGAGTTTATCCTGCTGTCAG). The DNA sequencing results of A.23.4 isolates were analyzed using the BLAST software program on the NCBI website (http://www.ncbi.nlm.nih.gov).

The results of genome base sequence analysis with 16S-RNA showed that isolate A.23.4 had similarity (100%) with sequence nucleotide L. Plantarum strain TMW 1.1623. The phylogenetics was shown in Figure 4.

Based on the results of the BLAST analysis identified BAL species from soybean milk solid waste production in the form of soybean pulp with code A.23.4 have 100% similarity. L. Plantarum strain TMW 1.1623, and this is also from phylogenetic trees (Figure 5). L. Plantarum bacteria were mostly found by previous researchers on spontaneously fermented vegetable ingredients such as spontaneous fermented green olives [18], from Amasi, a Zimbabwean fermented milk product [19], and Nigerian fermented product [20]. Besides, L. Plantarum isolated from dairy products also has activity as a probiotic and antimicrobial as reported by Ref. [21].
3.4. **Antibacterial Activity of Bacteriocin at Different Degrees of Acidity**

The bacteriocin characterization was carried out by testing its antimicrobial activity against pathogenic bacteria. The bacteriocin antibacterial activity of *Lactobacillus plantarum* strain TMW 1.1623 was evaluated in different pH environments, i.e., 2, 3, 4, 5, 6, 7, 8, and 9. The results were shown in Figure 7, where an increase in the diameter of inhibitory zones against *L. monocytogenes* at pH 2 and 3 and a decrease in inhibition zone diameter to pH 7. However, at pH 8 and 9, no inhibition zone was formed. On the contrary, this antimicrobial activity against *S. aureus* shown that the inhibition zone was up to pH 7, but the diameter of the inhibition zone against *S. aureus* was lower than the inhibitory zone against *L. monocytogenes*. The formation of inhibitory zones at relatively high at pH 8 and 9. It was due to the bacteriocin, which was a secondary metabolic of *Lactobacillus plantarum* strain TMW 1.1623. It was a protein molecule with a relatively low molecular weight, so an extreme increase in pH resulted in protein denaturation.

![Phylogenetic tree of Lactobacillus plantarum strain TMW 1.1623](image)

**Fig 4.** Phylogenetic tree of *Lactobacillus plantarum* strain TMW 1.1623

Denaturation of this protein caused physical changes in its structure so that its biological function becomes damaged. Because these bacteriocins did not exhibit antimicrobial activity, not found a clear zone. It was a condition, due to physical damage caused by protein denaturation. This situation was expressed by Ref [22] that there was a strong intra-molecular electrostatic interaction that causes dissociation between amino acids and carboxyl groups so that protein denaturation occurs in alkaline conditions.

3.5. **Antibacterial stability of bacteriocin against heat**

The sensitivity test results of bacteriocin extract could be seen in Figure 7. The results showed that an increased in temperature from 60-100 °C decreased the bacteriocin antimicrobial activity, which was
shown to decrease the diameter of the clear zone (Figure 10) both against S. aureus or L. monositogenes. Bacteriocin was a short-chain peptide compound that was easily damaged by heat, according to Ref [23], bacteriocin would be damaged due to heat treatment which would adversely affect its bioactive activity.

![Fig 6. Heat sensitivity to the antimicrobial, antifungal bacteriocin of L. Plantarum strain TMW 1.1623](image)

In the results of this study can be seen in Figure 7, that the activity of bacteriocin extract at treatment up to 100 °C for 60 minutes against L. monocytogenes bacteria still shows antimicrobial activity. It appears that the formation of a clear zone at a temperature of 100 °C whose diameters are 6.6 and 9.5 mm respectively for S. aureus and L. monositogenes. Whereas the antimicrobial activity decreases with an increase in temperature of more than 80 °C. This is because bacteriocin was a protein compound that was denatured due to high temperatures. The protein could be easily denatured if exposed to relatively high temperatures (> 80 °C) [24].

![Fig 7. The sensitivity of bacteriocin extract at various temperatures (A = 60 °C, B = 70 °C, C = 90 °C, D = 90, and E = 100 °C)](image)

In this study, it was seen that the antimicrobial activity of bacteriocin extract was higher in L. monocytogenes more than in S. aureus pathogen. The bacteriocin activity indicated by forming a clear zone at 100 C was 10.20 mm. The bacterial activity of the bacteriocin extract produced by L. plantarum had the potential to be used as a bio preservative for food processing. This result was in line with the conclusions of Ref. [25] which produces bacteriocin from L. plantarum 2C12 which had an antimicrobial activity that can inhibit the growth of pathogenic bacteria such as E. coli, S. aureus, and Salmonella typhimurium

3.6. Bacteriocin supernatant extraction and purification
The bacteriocin supernatant activity of L. Plantarum strain TMW 1.1623 at the ammonium sulfate concentration stage for precipitation was 30, 60 and 90% compared to the control antimicrobial activity (supernatant free cell). Pellets in the form of bacteriocin were evaluated for their antimicrobial activity against pathogens (L. Plantarum strain TMW 1.1623). Figure 8 show the antimicrobial activity of
bacteriocin against S. aureus and L. monocytogenes, where the higher the fraction of the ammonium sulfate usage, the better the purity level. This bacteriocin had better antimicrobial activity, which was indicated by existing a clear zone.

![Fig 8. Bacteriocin antimicrobial activity after precipitation with ammonium sulfate (P = bacteriocin pellet, S = supernatant)](image)

### 3.7. Effect of Enzymes on the Pathogenic Bacterial Activity

The bacteriocin was evaluated using several enzymes (protease K, trypsin, and catalase), and pathogenic bacteria. The results of these three enzymes can be seen in Table 1, where the bacteriocin extract added by the protease K enzyme and trypsin loses its antimicrobial activity against S. aureus and L. monocytogenes to various enzymes. This was indicated by the absence of clear zones in the two pathogens. The absence of this clear zone was caused by bacteriocin, which was a peptide compound [26] whose low molecular weight protein was hydrolyzed by the enzyme protease K and trypsin. These data obtained were similar to the results of study of Ref. [20], where evaluated the bacteriocin produced by L. plantarum F1. The results showed that antimicrobial activity was lost or unstable after bacteriocin treated with proteolytic enzymes. The same thing was also obtained from the results of a study of the bacteriocin activity of L. plantarum derived from cow's milk [10], apparently, the bacteriocin was very sensitive to proteolytic enzymes, such as trypsin, pepsin, and proteinase K. Bacteriocin in food was be degraded by proteolytic enzymes in the digestive tract [27].

Based on the results, the enzyme could inactivate bacteriocidal activity in inhibiting bacterial pathogens. Conversely, without the addition of enzymes to the bacteriocin extract the anti-microbe activity remained active, this was shown in control (Table 1) there were inhibitory zones in S. aureus and L. monocytogenes, respectively 6.27 and 11.33 mm.

| Treatments   | Clear Zone (mm) |
|--------------|-----------------|
|              | **Staphylococcus aureus** | **Listeria monocytogenes** |
| Control      | 6.27±0.12       | 11.33±0.74               |
| Protease K   | -               | -                        |
| Trypsin      | -               | -                        |
| Catalase     | 5.33±0.35       | 10.10±0.46               |

The effect of enzyme catalase on bacteriocin extract from L. plantarum strain TMW 1.1623 continued to provide an anti-microbial activity against S. aureus ATCC 25923 and L. monocytogenes CFSAN004330. Inhibitory zones formed on the two pathogens were approximately 5.33 and 10.10 mm respectively against S. aureus and L. monocytogenes. The addition of the enzyme catalase to bacteriocin extract from L. plantarum strain TMW 1.1623 did not affect the anti-microbial activity against pathogens S. aureus and L. monocytogenes. Inhibition zones formed on two pathogens were respectively 5.33 and 10.10 mm against S. aureus and L. monocytogenes. The addition of the enzyme catalase to bacteriocin extract from L. plantarum strain TMW 1.1623 did not affect the anti-microbial activity against pathogens.
S. aureus and L. monocytogenes. Inhibition zones formed on two pathogens were respectively 5.33 and 10.10 mm against S. aureus and L. monocytogenes. The bacteriocin still had antimicrobial activity, even though the enzyme catalase was added to the media. This also shows that the bacteriocin activity is not influenced by hydrogen peroxide12. Bacteriocin from LAB was sensitive to proteases, but resistant to catalase [28].

4. Conclusion
The bacteriocin supernatant produced by Lactobacillus plantarum strain TMW 1.1623 has antimicrobial activity in the range of pH 2-7 and a temperature of 60-100°C against Staphylococcus aureus ATCC 25923 and Listeria monocytogenes CFSAN004330. This bacteriocin is sensitive to protease K, trypsin, but not sensitive to the enzyme catalase.

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