Advances in Health Sciences Research, volume 37

Proceedings of the 12th Annual Scientific Meeting, Medical Faculty, Universitas Jenderal Achmad Yani, International Symposium on "Emergency Preparedness and Disaster Response during COVID 19 Pandemic" (ASMC 2021)

Temperature Optimization and Inhibition Test of *Lactobacillus acidophilus* Bacteriocin Against *Salmonella typhi* Bacteria

Siska Telly Pratiwi*, Muhammad Bagas Aditya
Department of Microbiology
Universitas Jenderal Achmad Yani
Cimahi, Indonesia
*shes_ziffa@yahoo.co.id, m.bagas.aditya.1998@gmail.com

Endah Hamidah Abbas
Department of Anatomical Pathology
Universitas Jenderal Achmad Yani
Cimahi, Indonesia
endah.hamidah123@yahoo.co.id

***Abstract*—Typhoid fever is an infection of the digestive tract caused by Salmonella typhi bacteria. One of the Lactic Acid Bacteria (LAB) in the digestive tract is Lactobacillus acidophilus which has bacteriocins to protect the digestive tract. Bacteriocin is an antimicrobial protein compound that is bactericidal or bacteriostatic. Bacteriocin protein have an optimum temperature range to provide antimicrobial effect. The aimed of this study was determine the temperature optimization of *L.acidophilus* bacteriocins on *S.typhi* growth by agar diffusion method. In this research, the extraction of *L.acidophilus* ATCC 4356 bacteriocin were treated with a temperature of 40o, 60o, 80o, 100o, and 121oC. The results of research observations were carried out by measuring the diameter of the inhibition zone in each treatment. The result temperature optimization test for *L.acidophilus* bacteriocins did not give positive inhibition zone. This is due to several reasons, namely the storage and McFarland 0.5 standard setting is too small to inhibit *S.typhi* growth. This is evidenced by the preliminary test without McFarland arrangement which can provide 3mm diameter inhibition. Therefore, further research is needed related to the use of the Mcfarland standard and its bacteriocin storage.**

Keywords—bacteriocin, antibacterial, Lactobacillus acidophilus, Salmonella typhi, inhibition

I. INTRODUCTION

Typhoid fever is an acute infection caused by *Salmonella typhi (S.typhi)* bacteria [1]. The therapy given for typhoid fever cases is antibiotics. However, long-term use of antibiotics can cause resistance. Antibiotic resistance results in inefficient treatment of these drugs so that alternative treatments are needed, namely by finding new components of antibiotic compounds, including bacteriocins. Bacteriocins can be produced by *Lactobacillus acidophilus* (*L. acidophilus*) which is a species of Lactic Acid Bacteria (LAB).

Bacteriocin is a bactericidal or bacteriostatic antimicrobial protein compound produced by the LAB species group. Bacteriocin can inhibit the growth of food spoilage or pathogenic bacteria [2]. LAB which is a microbiota in the digestive tract and vagina is *L. acidophilus* [3,4]. *L. acidophilus* can inhibit the growth of pathogenic bacteria and can be used as a safe probiotic because of its non-hemolytic characteristics, resistant to acids and resistant to bile salts so that it has the potential as a safe probiotic in the digestive tract [3-6].

Based on previous research, *L.acidophilus* ATCC 4356 bacteria can be identified and extracted but the bacteriocin has not given positive results in inhibiting *S.typhi* growth for several reasons, one of which is that the *L.acidophilus* used is thought to have no bacteriocin gene so that it does not produce bacteriocin protein. In addition, the bacteriocin extraction method is not optimal or the amount of bacteriocin produced is not optimal so that optimization is needed. Temperature has an influence on bacteriocin production so that protein denaturation does not occur [7]. Based on this, researchers are interested in knowing the optimization of the bacteriocin temperature from *L.acidophilus* as a potential probiotic or antibiotic candidate in inhibiting pathogenic *S. typhi* bacteria in vitro.

II. METHODS

This study used *L.acidophilus* and *S.typhi* microorganisms which were obtained from imported ATCC bacteria. The samples of this study were *L.acidophilus* ATCC 4356 grown on MRS Agar media and *S. typhi* ATCC 6539 bacteria grown on Tryptone Soya Agar (TSA). The bacteria used were The American Type Culture Collection (ATCC) bacteria which were used for research and certified.

L.acidophilus were cultured on MRSA incubated at 37ºC for 18-24 hours. *L.acidophilus* was taken from one colony and put into de Man Rogosa Sharp Broth (MRSB) then incubated at 37ºC for 24 hours [8,10].

S.typhi were cultured on TSA media and the *S.typhi* culture was taken as much as 1 ose then diluted with 0.85% NaCl solution to have the appropriate turbidity according to the standard solution of 0.5 Mc. Farland. The bacterial suspension inoculated on MHA media and incubated at 37ºC for 24 hours to test for bacteriocin activity [9].

The identification of *S. typhi* and *L. acidophilus* bacteria in this study is by using Gram staining and biochemical tests. includes sugar fermentation tests, carbohydrate fermentation, catalase, indole, urea, motility and simon citrate.

A. Extraction of Bacteriocins

L.acidophilus suspension with a McFarland standard of 0.5 equivalent to $1.5x10^8$ CFU / mL was grown in MRSB 5 mL incubated at 37 ° C for 24 hours. After the incubation was completed, centrifugation was carried out at a speed of 10,000 rpm at 4ºC for 15 minutes to separate the cell mass that did not contain bacteriocins from the supernatant. The pH was adjusted to 6.8 then the filtrate was sterilized with a 0.45 µm diameter bacterial filter into a sterile tube to obtain a sterile cell-free supernatant. The supernatant carrying bacteriocin was then tested for bacteriocin activity [8,10].

B. Bacteriocin Sensitivity Test to Proteolytic Enzymes

Amount 750 µL of trypsin enzyme with a concentration of 1 mg / mL was dissolved in a phosphate buffer pH 7,6. The prepared enzyme was added with a bacteriocin supernatant as much as 250 µL and then incubated for 2 hours at 25oC. The filtrate is sterilized with a Millipore filter with a diameter of 0.22 µm into a sterile tube. The sterile supernatant of 20 µL was immersed in sterile disc paper with a diameter of 5 mm. MHA media containing S. typhi indicator bacteria will be placed on a disc paper on the surface. The diameter of the inhibition zone formed was measured using a caliper around the disc paper after being incubated for 24 hours at 37ºC [10].

C. Temperature Optimization of Bacteriocin Activity

Amount 5 ml of Bacteriocin supernatant each heated at a temperature of 40, 60, 80, and 100°C for 1 hour on a hot plate and 121°C with a pressure of 1 atm for 15 minutes in autoclave with the optimum pH at pH 6.8. The bacteriocin activity was tested by agar diffusion method [10].

D. Bacteriocin Activity Test

The method used to test the antibacterial activity is the agar diffusion method, 15 mL of agar (50ºC) was poured into a petri dish and allowed to solidify, then 5 µL of S.typhi indicator bacteria aged 24 hours (total 10^5-10^6 cfu / ml) was poured over it and let it at 4ºC for 1 hr [11].

Disc paper with a diameter of 5 mm was immersed in a sterile supernatant. Disc paper was placed on MHA media containing *S.typhi* bacteria then incubated for 24 hours at 37 ° C. The diameter of inhibition zone generated around the disc paper is measured using a caliper [12].

The calculation of the diameter of inhibition zone uses the following formula:

Inhibition zone diameter :

$$\frac{(Vd-Dd) + (Hd-Dd)}{2}$$

Information:

Vd : Vertical diameter

Hd : Horizontal diameter

Dd : Disc diameter

The criteria for the strength of antibacterial power based on David and Stout are categorized into a weak category with an inhibition zone diameter of 5 mm or less, a moderate category with an inhibition zone of 5-10 mm, a strong category with an inhibition zone of 10-20 mm, and a very strong category with an inhibition zone of 20 mm or more.

Positive control used chloramphenicol disc at a dose of 30 µg. Negative control used sterile disc paper (paper disc) soaked for 5 minutes with sterile distilled water [13].

III. Results and Discussion

The culture results of *L.acidophilus* in this study can be seen microscopically and macroscopically. Macroscopic examination of *L.acidophilus* was carried out by looking at the morphology of the bacteria on MRSA media. The results of macroscopic examination showed that the culture was round with pinpoint size, grayish-white in color, and impenetrable to light. Microscopic examination that is performed is an examination using Gram stain. The microscope examination results showed that Gram-positive bacteria.

The macroscopic examination of *S. typhi* was carried out by looking at the morphology of the bacteria on the TSA medium. Macroscopic examination results show a round, grayish-white, and impenetrable image. Microscopic examination is performed using Gram stain. The microscope examination results showed Gram negative bacteria.

The biochemical tests in this study could not be carried out due to the limitations of the test media, but the bacteria used for the research were certified ATCC bacteria.

A. Extraction of L.acidophillus Bacteriocins

The preliminary test of *L.acidophilus* bacteriocins against *S.typhi* was carried out on MHA media using the Kirby-Bauer method. Bacteriocin supernatant was dripped onto disc paper by testing three times against the growth of S.typhi bacteria. The preliminary test was carried out without adjusting the temperature, pH, and number of bacterial colonies.

Inhibition zone can be found in the preliminary test. The area of the inhibition zone is 3mm, so a bacteriocin sensitivity test to proteolytic enzymes is needed to determine whether the inhibition zone is caused by bacteriocin.

B. Bacteriocin Sensitivity Test to Proteolytic Enzymes

Bacteriocin sensitivity test to proteolytic enzymes was carried out by preparing 750 µL of trypsin enzymes with a concentration of 1 mg / mL dissolved in a phosphate buffer pH 7.6. The prepared enzyme was added with a bacteriocin supernatant as much as 250 µL and incubated for 2 hours at 25oC.

The filtrate is sterilized with a Millipore filter with a diameter of 0.22 µm into a sterile tube. A total of 20 µL of sterile supernatant was immersed in sterile disc paper with a diameter of 5 mm. Disc paper is placed on MHA media which contains S.typhi bacteria.

Bacteriocin sensitivity test to trypsin enzyme gave negative results, so it proved in the preliminary test that the inhibition was caused by *L.acidophilus* bacteriocins. Bacteriocin is a protein compound while the trypsin enzyme is a proteolytic enzyme that can destroy protein compounds so that the inhibition of bacteriocins will not be formed in the bacteriocin sensitivity test to the trypsin enzyme. This eliminates the inhibition formed by other antimicrobials such as lactic acid, hydrogen peroxide, and diacetyl because these compounds are not protein compounds. If the inhibitory power is formed in the bacteriocin sensitivity test to proteolytic enzymes, the inhibition power is formed not by bacteriocin but by other antimicrobials.

C. Optimization of L.acidophilus Bacteriocin Temperature

Testing the inhibition of *L.acidophilus* bacteriocins against *S.typhi* was carried out by the Kirbybauer method on MHA media. The bacteriocin temperatures tested were 40,60,80,100, and 121ºC, while the chloramphenicol disk as a positive control and distilled water as a negative control on MHA media.

The diameter of inhibition zone can be calculated by measuring the diameter of the clear zone formed minus the diameter of the disc paper. The inhibition zone of each treatment was compared with the control inhibition zone. The results of *L.acidophilus* bacteriocin inhibition against *S.typhi* can be seen in table 1.

TABLE I. TESTING THE INHIBITION OF *L.ACIDOPHILUS* BACTERIOCINS ON *S.TYPHI* GROWTH

Group	Inhibition Zone Diameter Test		
	Test 1	***Test 2***	***Test 3***
1	22,74	23,02	23,18
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0

Information:
Group 1: Positive control with chloramphenicol
Group 2: Negative control with distilled water
Group 3: Test the bacteriocin temperature at 40ºC
Group 4: Test the bacteriocin temperature at 60ºC
Group 5: Test the bacteriocin temperature at 80ºC
Group 6: Test the bacteriocin temperature at 100ºC
Group 7: Test the bacteriocin temperature at 121ºC

Optimization of *L.acidophilus* bacteriocin temperature did not give positive results on *S.typhi* growth in this research test but gave positive results in the preliminary test. This shows that *L.acidophilus* bacteriocin can inhibit the growth of *S.typhi* but it needs further investigation because the temperature optimization test did not give a positive result. Temperature optimization is carried out to determine the optimum temperature of bacteriocin inhibitory activity. Oh, Kim, and Worobo's research showed that the temperature optimization of *L.acidophilus* bacteriocins with a temperature of 65º and 95ºC still gave 100% inhibition but at 121ºC the inhibition power was reduced to 50% [14]. Hoda Mahrous research showed the results of temperature optimization of *L.acidophilus* bacteriocins by providing inhibition of 30.15% at 30ºC, 87.34% at 60ºC, and 11.12% at 90ºC [15]. Based on these data *L.acidophilus* bacteriocins with temperature test range 30-121ºC can still have inhibitory power against indicator bacteria but have different optimum inhibition zones.

This research test did not get the results of bacteriocin temperature optimization because it did not give results in the inhibition power of the temperature test group ranging from 40º, 60º, 80º, 100º and 121ºC. This is thought to be due to several reasons, namely the presence of storing and controlling the number of bacteria with McFarland is too small so that the level of bacteriocin extraction is too little as a result it cannot provide an inhibitory power on S.typhi bacterial growth.

The McFarland used in the study of the temperature optimization test for bacteriocin inhibition using the McFarland 0.5 standard was equivalent to $1.5x10^8$ CFU/mL. The results of Sawitri Pertami's research, namely the inhibition power of *L.acidophilus* probiotics against the growth of *Candida albicans* showed that probiotics containing acidocin, lactacin B, lactacin F with McFarland 6 standards equivalent to $18x10^8$ CFU/mL and McFarland 8 equivalent to $21x10^8$ CFU/mL did not provide inhibition of growth. *C.albicans*, but the standard McFarland 10 equivalent to $30x10^8$ can provide inhibition against the growth of *C. albicans* with an average clear zone diameter of 8.24 mm [16]. Based on these data it is concluded that this bacteriocin temperature optimization test study did not provide inhibition results due to use The small McFarland standard is 0.5 McFarland which is equivalent to $1.5x10^8$ CFU/mL so that the amount of bacteriocin produced is small as a result it cannot provide inhibition against *S.typhi*. Standard McFarland owned by the FK UNJANI microbiology laboratory, only has a standard of 0.5 so it cannot be done to improve the McFarland standard.

Another reason for the temperature optimization test for bacteriocin inhibition does not give positive results is the storage time. The storage time occurs because there is a closed laboratory hour, but the extraction time takes a long time so that after incubation it is continued with storage at 4ºC for 16 hours to be continued. The preliminary test does not carry out storage after incubation because the preliminary test does not require temperature regulation, pH and McFarland standards so that the processing time is faster than the bacteriocin temperature optimization test.

Storage time has an influence on the bacteriocin inhibition zone. Based on the research of Ohenhen, *Lactobacillus plantarum* provides bacteriocin inhibition against Staphylococcus aureus bacteria at a temperature of 28 ± 2ºC gave 5mm of inhibitory zone. Bacteriocin stored at -4ºC for 7 days gave inhibition to 3mm. This shows that storage time can reduce the activity of bacteriocin inhibition [17].

Hassanejad Bibalad's research showed that the inhibitory effect was only 40% of all Lactobacillus isolates against one or more microorganisms. The study found only 6% of all Lactobacillus isolates had the bacteriocin gene [18]. The bacteriocin gene is required in transcripts to produce bacteriocins.

The preliminary test of this study provides the inhibitory power with a diameter of the inhibition zone of 3 mm. The inhibition zone formed is an inhibitory effect of *L.acidophilus* bacteriocins because the sensitivity test to proteolytic enzymes gave negative results.

The bacteriocin gene *L.acidophilus* ATCC 4356 has not yet been identified for the bacteriocin gene classification. Several *L.acidophilus* genes have been found for bacteriocin, including Acidocin A which belongs to the subclass IIa classification of *L.acidophilus* TK9201 found in fermented milk, Acidocin B subclass IIb classification on *L.acidophilus* M46 found in food, and Lactacin B which has not been identified as a subclass on *L .acidophilus* N2 is found in food [19]. Further research is needed to determine the classification of the bacteriocin gene of *L.acidophilus* ATCC 4356. Identification of the bacteriocin gene *L.acidophilus* ATCC 4356 can provide an overview of the mechanism of its inhibitory power. Class IIa bacteriocins such as Acidocin A are listeria-active peptides which are bacteriocins that are active against the genus Listeria / Listerisid (pediocin-like bacteriocins) having amino acid sequences in the N-terminal region which are similar to YGNGVXC and are heat stable. Class IIb *L.acidophilus* bacteriocins such as Acidocin B consist of two peptides which differ from each other by equal numbers forming pores on the target cell membrane and disrupting the proton gradient of the target cell [20-22].

IV. Conclusion

Based on the research that has been done, it can be concluded that *Lactobacillus acidophilus* can be identified and extracted bacteriocins. *L.acidophilus* bacteriocin can inhibit the growth of *S. typhi* seen in the preliminary test which gave an inhibitory power of 3 mm and the proteolytic enzyme sensitivity test gave negative results. Optimization of temperature inhibition of *L.acidophilus* bacteriocins did not provide inhibitory results for several reasons, namely the storage time after incubation due to laboratory closing hours and the use of small McFarland standards, namely the standard of 0.5 McFarland, so that the amount of bacteriocin to be produced was small, consequently cannot form the inhibition zone in the test.

Acknowledgment

The authors would like to thank all the professionals who have helped research and the staff of the Microbiology Laboratory Faculty of Medicine Universitas Jenderal Achmad Yani.

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