

The effect of heating temperature on the stability of bacteriocins produced by *Growol* isolate lactic acid bacteria

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Abstract. *Growol* is a functional food resulting from fermented cassava which is made through a process of soaking in water. During the soaking process, fermentation occurs by probiotic lactic acid bacteria. The aim of this research is to determine the effect of heating temperature on the stability of the bacteriocin produced by lactic acid bacteria isolating *Growol* against *Escherichia coli*. This research method was a laboratory experiment, with several stages, namely: 1) making *Growol* using fermentation techniques, 2) isolating lactic acid bacteria from *Growol*, 3) isolating bacteriocins, 4) testing the stability of heating temperature on bacteriocin activity. The place of research was carried out at the Microbiology Laboratory, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta. The results of the research showed that the *Growol* isolate of lactic acid bacteria had microscopic characteristics of rod-shaped cells and was gram positive. Bacteriocin was able to inhibit the growth of *E. coli* ATCC 25922 with an inhibition zone diameter of 6.5 mm. The bacteriocin produced by the *Growol* isolate of lactic acid bacteria is stable against heating at a temperature of 40-121°C. The heating temperature does not affect the stability of the bacteriocin produced by *Growol* isolate lactic acid bacteria.

1 Introduction

Growol is made from cassava which is soaked for several days. The *Growol* fermentation process that occurs during the soaking period produces lactic acid bacteria. [1] Lactic acid bacteria produce lactic acid and bacteriocins which have antimicrobial properties. [2] Bacteriocins as antimicrobials can be broken down by protease enzymes and do not cause resistance. [3] Bacteriocins prevent the colonization of pathogenic bacteria that enter the digestive tract. [4] Through competition for receptors, bacteriocin-producing bacterial colonization of the intestine can prevent pathogenic bacteria from adhering to the intestinal wall. [5]

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The highest bacteriocin activity against *Escherichia coli* and *Staphylococcus aureus* was detected at a temperature of 100°C.[6] Bacteriocins are stable against the effects of heating temperature, pH, and administration of high concentrations of NaCl.[7] Heating all types of proteins can affect protein stability and activity. [8] This effect is caused by changes in protein structure during heating at moderate temperatures. [9] Bacteriocins are proteins that are expected to be used as antimicrobial agents. Research on the impact of heating on the antibacterial activity of bacteriocins against *Escherichia coli* is required to complete the knowledge about bacteriocins produced by lactic acid bacteria isolates from *Growol*.

2 Research method

This type of research is a laboratory experiment. The research was conducted in the Microbiology Laboratory, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, from March to August 2022. The samples contained lactic acid bacteria isolated from *Growol* and *E. coli* ATCC 25922 as the test bacteria. *E. coli* ATCC 25922 was obtained from the collection of the Microbiology Laboratory, Faculty of Medicine, Gadjah Mada University.

2.1 The raw material used to make *Growol* is cassava

Cassava is fermented by soaking it in water for six days. Cassava-soaking water is replaced every 24 hours. On the eighth day, on MRS agar media, lactic acid bacteria that were isolated from *Growol* were cultured. The media was incubated in an anaerobic atmosphere for 24 hours at 37°C. Then, subcultures of lactic acid bacteria colonies growing on De Man-Rogosa-Sharpe (MRS) agar medium were carried out on MRS broth media. Incubation continued for 2 x 24 hours. Colonies of lactic acid bacteria were identified by means of gram staining, the catalase test, and the gas-producing ability test.

2.2 Bacteriocin preparation

A 24-hour-old isolate loop was inoculated with 10 milliliters of MRS broth and allowed to incubate for 48 hours. A total of 10 ml of culture was inoculated into 90 ml of MRS Broth and then incubated for 24 hours. A 50-cc culture was centrifuged for 15 minutes at 4°C and 10,000 rpm. A crude extract of bacteriocins is obtained as the resultant supernatant. 1 M NaOH was used to neutralize 50 ml of the supernatant to its pH until a pH of 7 was attained. Next, the culture was centrifuged for 15 minutes at 4°C and 10,000 rpm. Crude extract of the resulting supernatant is neutralized bacteriocins.

2.3 Test of bacteriocin inhibition against *E. coli* ATCC 25922 bacteria using the Kirby-Bauer method

An antimicrobial activity test of bacteriocin was carried out on Muller Hinton Agar (MHA) media. *E. coli* ATCC 25922 bacteria colonies aged 24 hours were taken in several oses and suspended in five milliliters of sterile physiological NaCl solution in a test tube. The turbidity of the suspension was compared with the standard Mc-Farland turbidity of 0.5%, which is equivalent to 1.5×10^8 CFU/mL on the Mc-Farland densitometer. A sterile cotton swab is prepared and dipped in a suspension of *E. coli* ATCC 25922 bacteria, then rubbed evenly on the surface of the MHA media and left for 5–15 minutes so that the suspension can seep into the media. A total of 20µL of the antibacterial supernatant was dripped on a 5 mm-diameter sterile disc of paper. The paper discs were placed on MHA media containing *E. coli* ATCC

25922 bacteria and incubated for 24 hours at 37°C. Using a caliper, the diameter of the resulting inhibition zone surrounding the disc paper was determined. [10]

2.4 Effect of heating temperature on bacteriocin activity

Five milliliters of bacteriocin were heated for thirty minutes in a water bath at 40, 60, 80, and 100 degrees Celsius, and for fifteen minutes in an autoclave at 121 degrees Celsius. Next, employing *E. coli* ATCC 25922 bacterium, the bacteriocin activity was measured using the Kirby-Bauer method. [11]

2.5 Effect of pH on bacteriocin activity

A total of 5 ml of bacteriocin was put into different test tubes and then adjusted pH 2, 4, 6, 8, and 10 using HCl and NaOH and allowed to sit at room temperature for four hours. Next, employing *E. coli* ATCC 25922 bacterium, the Kirby-Bauer method was used to measure the bacteriocin activity. [11]

3 Results and discussion

The lactic acid bacteria isolate *Growol* has characteristic features of round colonies, white in color, mucoid, rod-shaped cells, Gram-positive, does not produce catalase enzyme and fermented lactose but do not produce gas (Fig. 1).

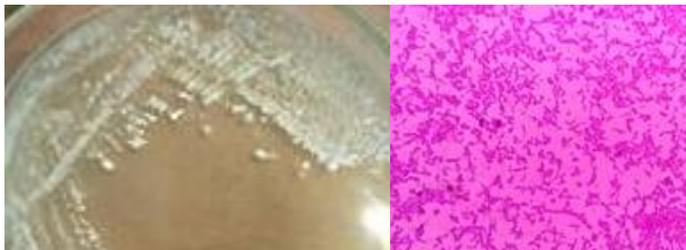


Fig.1. Morphology of lactic acid bacteria isolates *Growol*

Table 1 shows the results of the bacteriocin sensitivity test against *E. coli* produced by lactic acid bacteria isolated from *Growol*. Bacteriocins were subjected to heating treatment with temperature variations from 40 to 121°C. The bacteriocin inhibition zone with the widest diameter of 6.8 mm was found in bacteriocins treated with heating 40°C. Next, the data is analyzed using the Friedman test and shows the Asymp value. Sig.>0.05, which is 0.979. This shows that heating treatment at different temperatures does not affect the activity of bacteriocins in inhibiting *E. coli*.

Table 1. The results of bacteriocin sensitivity tests against *E. coli* with various heating temperature treatments

No.	The heating temperature (°C)	Diameter of the bacteriocin inhibition zone (mm)
1	40	6.8
2	60	6.3
3	80	6.4
4	100	6.2
5	121	6.6

Fig. 2. shows the results of the bacteriocin sensitivity test against *E. coli* produced by lactic acid bacteria isolated from *Growol*. Bacteriocins were subjected to pH treatment with

pH variations from 2 to 10. The bacteriocin inhibition zone with the widest diameter of 12.5 mm was found in bacteriocins treated with pH 2. Bacteriocins lose their antibacterial activity at pH 10 treatment. pH treatment affected the activity of bacteriocins in inhibiting *E. coli* ($p < 0.05$).

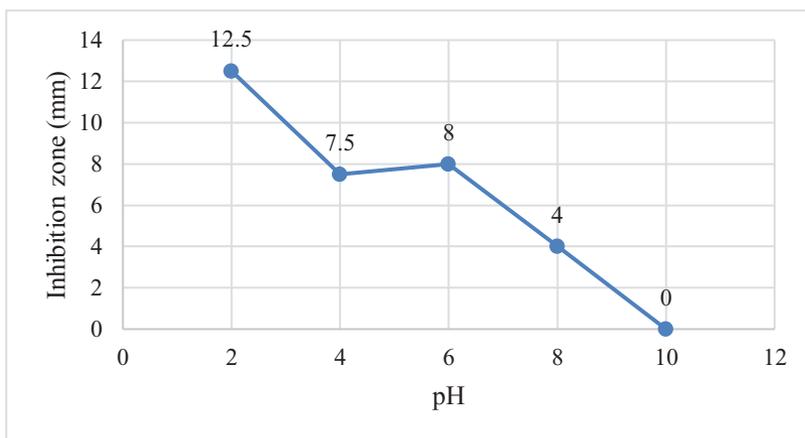


Fig. 2. The effect of pH on bacteriocin activity

The lactic acid bacteria that were recovered from *Growol* in this investigation were characterized by spherical, bacillus-shaped, yellowish-white colonies that were Gram-positive. The same study also said that all lactic acid bacteria identified from *Growol* belonged to the genus *Lactobacillus*. This study demonstrates that the diameter of the inhibition zone that bacteriocins create on *Escherichia coli* growth is not significantly affected by changes in temperature. With a diameter range of 5–10 mm, the average diameter of the inhibitory zone created by bacteriocins at all temperature treatments was 6.48 mm, classified as having moderate antimicrobial activity. There is an additional categorization of antibacterial characteristics that depends on the inhibitory zone diameter: weak (less than 5 mm), strong (10–20 mm), and extremely strong (more than 20 mm). [12] The findings of this investigation are consistent with the other study that bacteriocin activity is resistant to changes in treatment temperature. [7] The activity of bacteriocins in inhibiting *Escherichia coli* is stable over a wide range of temperature treatments. [13, 14] However, the results of this study contradict the research of Widayati et al., showed that the highest activity of bacteriocins against *Escherichia coli* and *Staphylococcus aureus* was produced by bacteriocins heated at 100°C. [6].

Bacteriocins generated by bacteria that are Gram-positive work by disrupting the integrity of the bacterial cell membrane. Some types of bacteriocins form pores in the bacterial cell membrane, resulting in increased cell permeability which leads to bacterial death. Other types of bacteriocins produce antimicrobial properties through inhibiting enzymes in the peptidoglycan biosynthesis process. Inhibition of this enzyme ultimately causes the accumulation of peptidoglycan in the cytoplasm and results in disruption of the bacterial cell membrane. [15] From this research it was found that bacteriocins are stable against temperature treatment. This stable property is because bacteriocins have a simple peptide structure without tertiary structure, a very hydrophobic area, and a high content of the amino acid glycine. [16] This research also shows that bacteriocins produce moderate inhibitory power on *Escherichia coli*. This is because the outer membrane of Gram-negative bacteria functions as a barrier, making it difficult for substances like detergents, digestive enzymes, and antibiotics to pass through the cell membrane. [17]

The four general phases of bacterial growth are the lag phase, log phase, stationary phase, and death phase. [18] Bacteriocin is produced at the beginning of the log phase then reaches

a maximum at the beginning of the stationary phase and decreases at the end of the stationary phase due to the production of more proteolytic enzymes. [7] Bacteriocins kill other pathogens such as *Bacillus*, *Staphylococcus*, *Listeria*, and *Morganella* by forming pores and modulating enzyme activity. [19] The pH, temperature, nutrients, aeration, and duration of incubation are among the external elements that impact the bacteriocins that lactic acid bacteria create. [20]

At low pH, the concentration of H⁺ ions and redox potential outside the bacterial cell will be high, thus inhibiting the work of protons, while at high pH, the concentration of H⁺ ions and redox potential outside the bacterial cell will be low. If the proton potential in the environment is lower than in the cell, it will follow the concentration gradient. As a result, there are differences in the biosynthesis process of bacteriocins produced by lactic acid bacteria. Generally, bacteriocin production is optimal at neutral pH with temperatures ranging from 25 – 37°C. [21] At low temperatures, enzymatic reactions take place slowly, an increase in temperature will accelerate the reaction, until the optimum temperature is reached, and the enzymatic reaction reaches its maximum. An increase in temperature past the optimum temperature will cause the enzyme to denature and reduce the speed of the enzymatic reaction. The temperature factor has two conflicting effects, namely increasing bacteriocin production but can kill bacteriocin-producing lactic acid bacteria. An increase in temperature before reaching the optimum temperature will increase bacterial growth and bacteriocin. High temperatures will cause denaturation of polypeptides and cause the biosynthesis process to be disrupted. So that the formation of bacteriocin will change and as a result there is a decrease in its activity.

4 Conclusion and recommendation

This research proves that temperature does not affect the activity of bacteriocins produced by *Growol* isolate lactic acid bacteria on the growth of *Escherichia coli*. The stability of bacteriocin to temperature is important to know if bacteriocin will be used as a food preservative. This is because food production usually involves a heating process. If bacteriocin is not resistant to heating, it cannot be used as a preservative in products that require heating in the manufacturing process. Bacteriocin produced by lactic acid bacteria isolate *Growol* is stable at various heating temperatures. Its antibacterial activity remains stable until a temperature of 121°C. This study also proves that bacteriocins produced by lactic acid bacteria isolate *Growol* have activity at acidic pH and are unstable at alkaline pH. If it is applied as a food preservative, it is only suitable for food products that have an acidic to neutral pH. Further, research on the characteristics of bacteriocins produced by *Growol* isolates of lactic acid bacteria is needed to provide more complete information.

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