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Optimization of pH to Bacteriocin Production by Lactic Acid Bacteria Growol Isolate Against *Salmonella typhi*

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ABSTRACT
Introduction – Growol is a traditional food from Yogyakarta which made from fermentation by Lactid Acid Bacteria (LAB) of cassava. LAB growol isolate as probiotics have ability to produce bacteriocins. Bacteriocins are useful for eradicating gram-positive and negative pathogenic bacteria. Bacteriocin activity is influenced by many factors, including pH, temperature, incubation time, and others, but the research about optimization of pH to produce bacteriocins that have maximum activity against *S. typhi* bacteria has been very limited. **Purpose** – This study aims to determine the ability of the bacteriocins produced by LAB growol isolate to inhibit the growth of *S. typhi* bacteria and to optimize the pH of the culture to increase its activity. **Methodology/Approach** – This research used a quasi-experimental method using post control group design. There were 5 samples of *S. typhi* bacteria which were divided into 5 groups. Group 1 was used as normal control without pH treatment. Groups 2, 3, 4, 5 were treatment with pH 2; 4; 6; 8. Each pH treatment was carried out with 4 repetitions. After the treatment, diameter of the inhibition zone of the disc was measured. The data obtained processed were using anova as a statistical analysis to see the difference in the sample mean. **Findings** – This study showed the results that optimizing of pH bacteriocin was optimum at inhibiting the growth of *S. typhi* bacteria is 2 with average diameter of inhibition zone is 11.10 mm. **Originality/ Value/ Implication** – The results of this study prove that pH has ability to affects bacteriocin activity produced by LAB growol isolate against *S. typhi*. LAB growol isolate has potential to produce bacteriocins to kill *S. typhi*.

Keywords: LAB, growol, pH, *Salmonella typhi*

INTRODUCTION

Indonesia is a country rich in local food, one of which is fermented food from cassava such as growol. Growol is a speciality food from Kulonprogo Regency, Yogyakarta Special Region made by fermenting cassava to produce probiotics (Eni et al., 2010). This probiotic is produced from Lactic Acid Bacteria (LAB) due to the fermentation of growol by soaking it (Dwi & Putri, 2012). Changes in the microbiological and biochemical properties of cassava marinade that occur naturally or spontaneously during fermentation, resulting in several effects such as a decrease in endogenous cyanogen levels and the formation of organic acids, especially lactic acid (Hawashi et al., 2019).

The bacteria Lactobacillus lactis, Leuconostoc mesenteroides, and Lactobacillus plantarum are responsible for producing high concentrations of lactic acid (Sari & Puspaningtyas, 2019). In the fermentation of cassava to make growol, 13 strains of Lactobacillus plantarum, Lactobacillus rhamosus, and Lactobacillus pentosus were found from the cassava soak (Dwi & Putri, 2012). In addition to producing lactic acid, experimental animal studies have shown that growol has a positive effect on preventing diarrhoea (Prasetia & Kesetyaningsih, 2014).

Lactic acid bacteria can act as bacteriocins, which have great health benefits. Bacteriocins are extracellular compounds in the form of antimicrobial proteins or peptides that can show a response to certain bacteria in the opposite way (Jagadesswari S & Vidya P, 2010).

Growol which contains a lot of LABS is certainly a good source of probiotics because the short amino acids contained in probiotic sources have benefits that can lower blood pressure, boost the immune system, and inhibit the activity of cholesterol-forming enzymes, so as to reduce cholesterol levels in the body (Beltrán-Barrientos et al., 2016). Growol can serve as a functional food in diarrhoea prevention efforts because besides containing probiotics, it also contains prebiotics. The synergistic combination of probiotics and prebiotics in growol can maintain the health of the digestive tract (Eni et al., 2010).

Various factors such as pH, temperature, and bile salt concentration can affect the growth and production of lactic acid. Each species and even strain of lactic acid bacteria may have the most suitable pH and temperature values for optimal growth and lactic acid production. Studies conducted by Abdel-Rahman et al., (2013) and Aghababaie et al., (2015) showed that pH, temperature, and bile salt concentration affect the growth and production of lactic acid in three types of lactic acid bacteria isolates used in the fermentation of foodstuffs.

Several studies related to the effect of pH on bacteriocin activity have been conducted, including: bacteriocin produced by Lactobacillus casei from dried cuttlefish has active antibacterial activity in the pH range of 2-6 (Andarilla et al., 2018), bacteriocins isolated from lemea have resistance to pH 2 to pH 7 (Okfrianti et al.,

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2018), Other studies related to pure bacteriocin extracts from Lactobacillus plantarum have antimicrobial activity at pH 2 and 6 (Ohenhen et al., 2015).

So, from all the information that has been obtained previously, it is known that growol is able to produce good probiotics in eradicating various gram-positive and negative bacteria, but there has been no research on the optimisation of the pH factor on the ability of bacteriocins produced by LAB isolates growol in inhibiting the growth of *Salmonella typhi* bacteria. In this context, this study aims to determine the ability of the bacteriocins produced by LAB growol isolate to inhibit the growth of *S. typhi* bacteria and to optimize the pH of the culture to increase its activity. Accordingly, the sub-questions of the study are as follows:

(1) How the ability of bacteriocins produced by LAB growol isolate to killing *S. typhi* bacteria?
(2) How does the optimization of pH to bacteriocin production by LAB growol isolate against *S. typhi*?

LITERATURE REVIEW

(1) Growol

Growol is one of the functional foods obtained from fermented cassava, which is the third largest source of carbohydrates in Indonesia after rice and corn. This speciality food from Kulonprogo contains many lactic acid bacteria that act as probiotics (Eni et al., 2010). Lactobacillus plantarum and Lactobacillus rhamnosus are the main microorganisms in the growol production process (Puspaningtyas et al., 2019). The ability of probiotic bacteria derived from growol isolate lactic acid bacteria, namely Lactobacillus casei subsp. rhamnosus TGR2, has potential antimicrobial activity (Rahayu et al., 1996).

The process of converting cassava into growol through fermentation is thought to convert carbohydrate compounds such as starch, cellulose, and pectin into organic acids. Simple carbohydrates in the basic ingredients help the growth of natural microbes which then produce acidic compounds. The acidity of growol is usually less than pH 4. This kind of acidity makes it difficult for most harmful microorganisms in food to survive. Thus, lactic acid fermentation of food can reduce the potential growth of harmful microorganisms in it (Ohenhen et al., 2015).

(2) *Salmonella typhi*

Salmonella typhi bacteria are gram-negative bacteria, rod-shaped, with a width of 0.7 - 1.5 m with a length of 2.0 - 5.0 m, moving with the presence of flagellum. *Salmonella typhi* is generally pathogenic, causing many infections in both humans and animals (Parama Cita, 2011). *Salmonella typhi* is one of the main causes of diarrhoea after Escherichia coli (Kaur et al., 2018). Diarrhoea caused by *Salmonella typhi* is invasive and characterised by prolonged fever, abdominal pain, and other systemic symptoms such as confusion or headache (Kasper et al., 2009).

Diseases caused by Salmonella bacteria, called salmonellosis, can vary in severity. The illness can range from mild diarrhoea, abdominal cramps and fever to a serious life-threatening fever known as enteric fever, which requires immediate antibiotic treatment. Salmonellosis can also cause localised infections and be an asymptomatic carrier of the disease. The most common form of salmonellosis is gastroenteritis which is self-limiting and uncomplicated. If a person suddenly develops diarrhoea or fever with no apparent cause, it is important to consider salmonellosis as the cause. Diagnosis involves detecting the presence of Salmonella bacteria in clinical samples such as faeces or blood (Teshome et al., 2019).

(3) Lactic Acid Bacteria

Lactic acid bacteria produce lactic acid as their main metabolite (Agustine et al., 2018). In addition to lactic acid, lactic acid bacteria also produce various antibacterial compounds including hydrogen peroxide, antimicrobials, and other metabolic products that are beneficial to the body. These bacteria are isolated with the aim of producing antibacterial compounds that have potential as probiotics (Kormin et al., 2001).

The effectiveness of an antimicrobial agent in suppressing the growth of microorganisms is influenced by various factors, including the pH of the substrate, age of the bacteria, temperature, and duration of incubation (Lambuk et al., 2022). In general, LAB are able to thrive in the temperature range of 5 - 45°C and are resistant to acidic environments, where many strains can grow at pH 4.4. However, optimal growth of LAB occurs in the pH range of 5.5 to 6.5 and 8 (Elyass et al., 2017).

The health benefits of lactic acid bacteria include improving the ability to digest lactose, controlling harmful bacteria in the digestive system, lowering serum cholesterol levels, tumour prevention, antimutagenic and anticarcinogenic properties, stimulating the immune system, preventing constipation, vitamin B production, bacteriocin formation, and neutralising toxic compounds (Kormin et al., 2001). In addition to boosting the immune system and stopping the activity of enzymes that produce cholesterol, bacteriocins also have the potential to prevent cancer development (Agustine et al., 2018).

(4) Bacteriocins

Bacteriocins are proteins produced by ribosomes with antimicrobial capabilities at certain concentrations. Bacteriocins are protein toxins produced by a number of bacteria and certain members of archaea, aimed at blocking the growth of similar or closely related bacterial strains. It has antimicrobial effects against pathogenic and harmful bacteria (Negash & Tsehai, 2020).

Bacteriocins have various advantages that can be utilised. They are able to withstand extreme heat stress and are active over a diverse pH range. In addition, these antimicrobial proteins are colourless, odourless and tasteless. Their nature as proteins makes them easily broken down by proteolytic enzymes, so bacteriocin fragments do not survive long in the human body or in nature. This reduces the risk of interaction between the target strain and the degraded bacteriocin fragments. Because of these advantages, bacteriocins have become a

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popular ingredient in the food, medicine, and agriculture industries (Negash & Tsehai, 2020).

(5) Bacterial Sensitivity Test

a. Diffusion method

This method is based on the principle of antibacterial diffusion into a medium that has been inoculated with microbes. Observation of the results of this method can be seen from the presence or absence of a transparent area that shows the zone of inhibition of bacterial growth around the disc paper (Balouiri et al., 2016).

1) Disc method

The disc method, also known as the Kirby Bauer test, is a technique that uses paper discs to absorb antimicrobial compounds that have been previously soaked. This disc paper is then placed on agar media that has been inoculated with bacteria. After incubating for 18-24 hours at 35°C, a transparent zone will appear around the disc indicating the presence or absence of microorganism growth. The extent of the transparent zone correlates with the number of microorganisms present (Balouiri et al., 2016).

2) Pitting method

The well method is a technique in antibacterial testing that is done by creating vertical holes in agar that has been previously planted with bacteria. Next, the sample to be tested is inserted into the hole. After the incubation process, bacterial growth is observed based on the presence or absence of an inhibition zone around the hole (Balouiri et al., 2016).

From the questions that have been asked before, as well as the literature used, the hypotheses in this research:

(1) The bacteriocin produced by LAB growol isolate can kill *S. typhi* bacteria.
(2) pH affects the optimization of LAB in killing *S. typhi*.

METHOD

This research used a quantitative approach. This type of research is a laboratory quasi-experimental research with a post-test only controlled group design. The population studied was bacteriocin produced by lactic acid bacteria isolate growol with cassava (*Manihot esculanta*) purchased at Gamping Market, Godean, Sleman, Yogyakarta Special Region, a total of 6 pieces from four different traders.

Inclusion criteria for sample selection were:

1. Cassava was not too old.
2. Cassava is brightly coloured.
3. Fresh cassava.
4. Medium-sized cassava.

The exclusion criteria for sample selection are:

1. Cassava that is old.
2. Cassava that has been rotten.

The research was conducted at the Microbiology Laboratory of FKIK Universitas Muhammadiyah Yogyakarta in August 2022. The independent variables of this study were lactic acid bacteria isolate growol, pH, bacteriocin. The dependent variable of this study is the diameter of the bacteriocin inhibition zone against *Salmonella typhi*. The controlled variables of this study are anaerobic atmosphere during incubation, incubation time, bacterial concentration, test and test bacterial strain. The uncontrollable variables of this study are active compounds and contaminants in bacteriocin.

There were 5 samples of *S. typhi* bacteria which were divided into 5 groups. Group 1 was used as normal control without pH treatment. Groups 2, 3, 4, 5 were treatment with pH 2; 4; 6; 8. Each pH treatment was carried out with 4 repetitions. After the treatment, diameter of the inhibition zone of the disc was measured. To analyze this research, need some work steps and the following are the work steps in making growol up to the sample testing stage.

(1) Make growol from cassava, peel the cassava and cut it into small pieces and then soak it in an open container for seven days and change the water every 24 hours.
(2) Isolate lactic acid bacteria from growol on MRS Agar Media and then grow them in an anaerobic atmosphere for 2x24 hours.
(3) Then, gram staining is carried out on the growing colonies.
(4) Subculture of colonies that grow into MRS Broth Media under anaerobic conditions for 2x24 hours.
(5) *S. typhi* bacteria are planted on TSA Agar Media for 1x24 hours.
(6) Take a bacteriocin sample and put it in the effendof tube.
(7) Isolate bacteriocin by centrifuging at 8000 Rpm.
(8) Adjust the pH of each solution of 5 ml of crude bacteriocin in different tubes.
(9) pH adjustment was carried out at pH 2, 4, 6, 8 using NaOH and HCl solutions, then incubated for 4 hours at room temperature.
(10) Take the bacteriocin enzyme to test the bacteriocin antibacterial activity against *S. typhi* using the agar diffusion method.

After the study was completed, the data will be presented in tables, the study was conducted analytically using one-way anova test to determine the difference between treatments. This research has been declared ethically feasible by the Ethics Commission of the Faculty of Medicine and Health Sciences UMY with No. 076/EC-EXEM-KEPK FKIK/UMY/VIII/2022.

RESULT AND DISCUSSION

(1) Result

The samples used in this study are LAB (lactic acid bacteria) isolates obtained through the cassava fermentation process as the basic ingredient for making growol. The media used to grow bacteria is MRS agar media. Macroscopically, single colonies of lactic acid bacteria were round, white and dense. The shape of lactic

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acid bacteria colonies on MRS agar media can be seen in Figure 1.

Figure 1. Colony Shape of LAB on MRS AGAR Medium

The results of staining lactic acid bacteria isolate growol with gram technique showed that lactic acid bacteria have microscopic characteristics of rod-shaped cells, are gram-positive, have a single or grouped arrangement like a short chain (Figure 2).

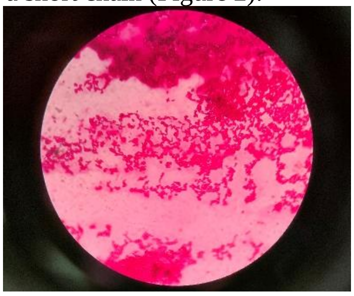

Figure 2. Gram Stain Results Lactic Acid Bacteria

After treatment, the results of the treatment obtained the diameter of the inhibition zone in millimeters (mm). The results of the treatment can be seen in Figure 3. Based on the observations, it can be seen that the diameter of the bacteriocin inhibition zone against *Salmonella typhi* is the highest at 13.00 mm at pH 2 (Figure 3).

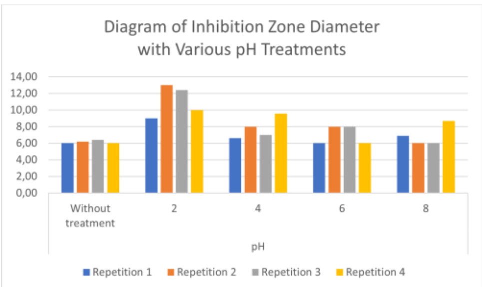

Figure 3. Diagram of Inhibition Zone Diameter with Various pH Treatments

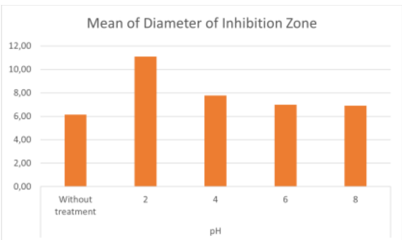

Figure 4. Mean of Diameter of Inhibition Zone

From the observations of Figure 4, the average inhibition zone of bacteriocin without pH treatment is 6.15 mm, the inhibition zone of bacteriocin pH 2 is 11.10. Bacteriocin inhibition zone pH 4 was 7.78 mm, bacteriocin inhibition zone pH 6 was 7.0 mm, bacteriocin inhibition zone pH 8 was 6.90 mm.

The data obtained were tested using one-way anova because the data used more than two treatments (>2), more than one repetitions, and data measured only post treatment. Statistical test results to determine the effect of pH on the diameter of the bacteriocin inhibition zone against *Salmonella typhi* bacteria using one-way anova test due to data distribution is normal.

Table 1. Results of Anova

Source	Sum of Squares	df	Mean Square	F	p-value
Perlakuan	98.960	4	24.740	18.227	0.00
Error	20.359	15	1.357		
Total	1371.472	20			

Based on the research results obtained, by using a significance level of 5%, it was found that there was a significant difference in the diameter of the bacteriocin inhibition zone of LAB from growol isolate after being treated with pH (p-value < 0.05). That is, this significant difference indicates that pH greatly influences the optimization of bacteriocins in killing *S. typhi*, the data obtained failed to reject H0, which means it can be concluded that the pH variation affects the average inhibition zone.

Then, Tukey Duncan test was conducted to see the most effective and significant pH in killing *Salmonella typhi*.

Table 2. Results of Tukey Duncan Test

	Perlakuan	N	Subset 1	Subset 2
Tukey HSD[a,b]	Tanpa Perlakuan	4	6.1500	
	pH 6	4	6.5000	
	pH 8	4	6.9000	
	pH 4	4	7.7875	
	pH 2	4		12.2250
	Sig.		.318	1.000
Duncan[a,b]	Tanpa Perlakuan	4	6.1500	
	pH 6	4	6.5000	
	pH 8	4	6.9000	
	pH 4	4	7.7875	
	pH 2	4		12.2250
	Sig.		.086	1.000

From Tukey Duncan Test, it was found that pH 2 is the position of the most effective pH in killing *S. typhi* with score of Tukey Duncan Test is 12.225.

(2) Discussion

This study proves that pH affects the activity of bacteriocins produced by lactic acid bacteria isolate growol against *Salmonella typhi*. This is indicated by the difference in the average inhibition zone against pH control with variations in pH 2, 4, 6, 8. Morales (2003) classify the inhibition zone or clear zone in four categories based on antimicrobial activity, namely: weak (less than 5 mm), moderate (between 5-10 mm), strong (between 10-

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20 mm), and very strong (more than 20 mm). The diameter of the bacterial growth inhibition zone is measured in mm (Kusumawati et al., 2008). The larger the clear zone, the higher the level of antimicrobial activity against pathogenic bacteria.

So, it can be concluded that the antibacterial activity of bacteriocins from lactic acid bacteria against *Salmonella typhi* indicator bacteria at pH 2 is in the strong category, and the activity without treatment, pH 4, 6, 8 treatment is in the medium category. Bacteriocins obtained from growol isolates showed very high antimicrobial activity at pH 2 compared to the antimicrobial activity shown at pH 4, 6 and 8. Almost no antimicrobial activity was shown by serially diluted extracts in the control variable. This result may be due to the high tolerance of LAB to low pH (Chartier et al., 2014). This also shows that a high pH value has a negative effect on the antibacterial activity produced by the bacteriocin of LAB isolate growol.

This observation is similar to the research conducted by Ohenhen et al., (2015) which showed that bacteriocins obtained from LAB were more active at pH 2 and 6, compared to pH 10 and 12. The increased sensitivity of *Salmonella typhi* to a decrease in bacteriocin pH can be caused by the physiology of cell walls and bacterial membranes that experience leakage at low pH. This observation is also in accordance with the research of Malini M and Savitha J (2012) who observed that the antibacterial activity of pure bacteriocin extracted from LAB was optimal against Staphylococcus aureus at low pH, namely pH 2 and 4. While the bacteriocin extract diluted at pH 10 showed almost no antimicrobial activity against LAB. Similar research results were also reported by (Kurniawan, 2012).

Bacteriocins produced by various Lactobacillus strains show increased activity at low pH. Low pH affects the structure of all macromolecules. The components in cells that are most sensitive to pH are proteins. Low pH alters the ionisation of amino acid functional groups disrupting hydrogen bonding, promoting protein denaturation. Optimum growth pH is the pH that is most favourable for the growth of an organism. Minimum growth pH is the lowest pH that an organism can tolerate. The highest pH is the maximum growth pH. Salmonella grows in the pH range of 4 to 9 with an optimum between 6.5 and 7.5. Growth will be inhibited at pH <3.8 (Bhunia, 2007). Thus, the results of this study are also in line with the theory.

S. typhi cannot grow at pH 2 because at this pH the high acidity cannot be compensated for by *S. typhi*. The acidic atmosphere in the human stomach ranges from pH 1.5 – 2 (Pasaribu et al., 2018). That is, this also supports the fact that the bacteriocin produced by LAB from growol isolate will become a good probiotic when it passes through the human digestive tract in the stomach. When in the stomach, this bacteriocin will work very optimally to kill *S. typhi*. The optimal ability of probiotics to kill germs will reduce the risk of diarrhea. As in previous studies that explicitly showed that growol containing good bacteria of the genus Lactobacillus was very capable of overcoming diarrheal diseases caused by bad bacteria (Eni et al., 2010).

This is in accordance with the theory of Messens and De Vuyst (2002) which explains that more bacteriocin molecules are produced at low pH. Low pH conditions cause an increase in bacteriocin solubility, a decrease in hydrophobic properties, peptide aggregation thus causing bacteriocin binding to the cell surface. So it can be concluded that bacteriocin shows greater anti-bacterial activity at low pH (pH 5 and below) than its physiological pH While the higher the pH causes bacteriocin activity to decrease.

CONCLUSION AND RECOMMENDATION

The results of this study prove that pH have ability to affects bacteriocin activity produced by LAB growol isolate against *S. typhi*. LAB growol isolate has potential to produce bacteriocins to kill *S. typhi*. The pH bacteriocins from LAB growol isolate most effectively to killing *S. typhi* in 2.

Researchers propose conducting additional investigations into the optimal optimization factors of bacteriocins derived from the growol isolate for the purpose of eliminating *S. typhi*.

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