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The Effect of Temperatures and pH on Bacteriocin Activity of Lactic Acid Bacteria Strain Pr 4.3L From Peda Fish

Charis Amarantini[1,*], Vinsa Cantya Prakasita[1], and Lidia Ester Cahyani[2]

[1]*Biology Department, Faculty of Biotechnology, Universitas Kristen Duta Wacana. Jl dr Wahidin Sudirohusodo 5-25 Yogyakarta.55224*
[2]*Undergraduate Program, Biology Department, Faculty of Biotechnology, Universitas Kristen Duta Wacana. Jl dr Wahidin Sudirohusodo 5-25 Yogyakarta.55224*
[*]*Corresponding author. Email: charis@staff.ukdw.ac.id*

ABSTRACT
Lactic acid bacteria (LAB) produce bacteriocin, which is potentially administered as biopreservative. It is important to study bacteriocin continuously from an applied perspective. However, isolate LAB strain Pr 4.3L obtained from peda fish are known to produce antimicrobial compounds such as bacteriocin. Therefore, it is important to optimize the production of bacteriocin from these strains by examining the effect of incubation temperatures and pH on the activity of LAB strain Pr 4.3L. The biomass of lactic acid bacteria strain Pr 4.3L was grown in MRS broth medium with pH conditions of 4-8 which was adjusted using 1 N HCl and NaOH. Furthermore, the culture was incubated at 30 and 37°C for 36 hours, and the bacteriocin activity was tested for its inhibition against pathogenic bacteria (*S.aureus* ATCC 25923, *S.typhi* BPE 122.4 CCA and *S.typhi* NCTC 786) using agar well diffusion method. The treatment at 30°C on MRS broth medium with a pH of 7 is the best growth condition for bacteriocin production. Under these conditions, antimicrobial and bacteriocin activities are more inhibited than other treatments. This inhibition showed a broad-spectrum against indicator bacteria (*S.aureus* ATCC 25923, *S.typhi* BPE 122.4 CCA and *S.typhi* NCTC 786). Therefore, the results provided useful information on the potential of bacteriosinogenic LAB as a natural preservative.

Keywords: *Temperature, pH, Bacteriocin, Lactic Acid Bacteriocin Strain Pr 4.3L, Peda Fish.*

1. INTRODUCTION

Lactic acid bacteria (LAB) play an important role in the fermentation of food as natural microorganisms that are added as a starter [1]. It changes the aroma and texture of fermented foods and affects the durability of the final product [2]. LAB grows naturally in traditional fermented food products such as peda fish, and some obtained are *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Weisella*, and *Leuconostoc* species [3]. In addition, it has the potential of being substituted as antibiotics and biopreservatives in traditional fermented foods [4], and its inappropriate consumption can cause biological resistance [5]. Therefore, the LAB is chosen as one of the alternatives to limit the use of antibiotics and not cause side effects on human health. As a biopreservative, it produces antimicrobial agents, including organic acids, hydrogen peroxide, diacetyl, and bacteriocin which inhibit the growth of spoilage and pathogenic microorganisms [2].

Bacteriocin is a bioactive protein that is synthesized by the ribosomes of Gram-positive and negative bacteria, which possess inhibitory effects on pathogens [6]. Its production is influenced by environmental factors, such as temperature and pH. The activity is stable in various temperatures and pH settings, but the sensitivity level is different [7]. Furthermore, bacteriocin production depends on cell biomass, and the initial pH of culture media significantly influences growth [8]. However, the previous study showed that LAB strain Pr 4.3L derived from the isolation results of peda fish immersion water has the potential as a probiotic that produces antimicrobial compounds such as bacteriocin and it provides a stronger inhibitory effect on *Staphylococcus aureus* ATCC 25923

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compared to *Salmonella typhi* BPE 122.4 CCA [3]. Therefore, it is important to test the potential product of LAB strain Pr 4.3L at various temperatures and pH by analyzing the growth and bacteriocin activity against pathogenic bacteria.

2. MATERIALS AND METHOD

2.1. Sample preparation

The isolate LAB strain Pr.4.3L [3] stored with glycerol stock in cryovial tube was taken as much as 500 µl and then cultured for 36 hours at 37°C on MRS broth medium (Oxoid) containing 1% glucose. The cells were harvested through a centrifugation process with a speed of 13.500 rpm for 15 minutes, and the pellets were inoculated into the MRS agar medium. This medium was supplemented with 1% $CaCO_3$ and 1% glucose using the streak and spread plate methods, which was re-incubated at 37ºC for 36 hours. The LAB colony that grew was validated for its purity through Gram staining.

2.2. Optimization of LAB strain Pr 4.3L growth

Isolate LAB strain Pr 4.3L was cultured following existing procedures [9] with slight modifications. A complete loop of the colony was inoculated into 5 ml of MRS broth and incubated at 37°C for 36 hours. After growing, the cells were harvested using a centrifugation process at a speed of 13.500 rpm for 15 minutes. As much as 10% bacterial suspension was inoculated into 4.5 ml of MRS broth. Also, the suspension was adjusted at pH variations using 1 N of HCl and NaOH and then incubated at various temperature treatments for 36 hours. The pH range used was 4-8, while the incubation temperatures used were 30 and 37°C [10]. Cell biomass was measured using density with a spectrophotometer
(OD 600 nm) [9].

2.3. Determination of bacteriocin activity

Bacteriocin activity of LAB strain Pr 4.3L was determined using an agar well diffusion method [3] against several pathogenic bacteria with the potential of causing damage to food products and spread disease. Preparation of cell-free culture supernatant (CFCS) was conducted by harvesting LAB strain Pr 4.3L cells that had been grown at various pH and temperatures through a centrifugation process at a speed of 10.000 rpm for 10 minutes. The solution was neutralized at a pH of 6.5 using 1 N NaOH before its antimicrobial testing. Furthermore, the supernatant is collected and stored in the refrigerator until it is required [3].

Preparation of pathogenic bacteria. Three pathogenic bacteria consisting of *Salmonella typhi* BPE 122.4 CCA, was obtained from previous research collections [11] [12], *Salmonella typhi* NCTC 786, which was obtained from PT Biofarma, and *Staphylococcus aureus* ATCC 25923 was cultured in BHI broth (Merck) at 37ºC for 16- 18 hours [3]. Meanwhile, the cells are harvested through a centrifugation process at a speed of 13.500 rpm for 15 minutes. The supernatant is removed and then sterile H_2O_{dest} is added to the pellet since the suspension is equalized using the 0.5 McFarland standard [13].

In the bacteriocin activity test, sterile cotton swabs are dipped in a suspension of pathogenic bacteria. However, a sterile cotton swab containing an inoculum is rubbed on the MHA (Himedia) media. The sweeps were repeated twice until the test cultures were evenly distributed. A well of 8mm in diameter is made on MHA medium using a sterile blue tip, and a total of 150 µl of CFCS solution that had been heated at 100ºC for 10 minutes was added into the well. Meanwhile, Petri dishes are incubated at 37ºC for 24 hours, and the inhibitory zones formed around the wells were measured [3]. Therefore, the bacteriocin activity is determined based on the inhibition against pathogenic bacteria [14]. The same procedure is performed on CFCS solutions without heating treatment to test antimicrobial activity.

3. RESULTS AND DISCUSSION

3.1. Activation of isolate LAB strain Pr 4.3L

Macroscopically, the LAB strain Pr 4.3L colony that grew on MRS agar-$CaCO_3$-glucose medium had a milky white appearance with a clear zone around the colony (Figure 1a). Furthermore, $CaCO_3$ served as an early marker of the lactic acid bacteria growth that is dissolved form a clear zone around the colony [15]. Also, the MRS agar medium is given glucose as an additional carbon source. Glucose is the type of saccharide that is most easily metabolized by bacteria [16] and used for cell growth and maintenance as well as the formation of organic acids such as lactate [17]. In addition, LAB strain Pr 4.3L is known to have a fermentation type of homofermentative [3], which can directly remodel glucose into lactic acid through the Embden-Meyerhof Parnas (EMP) pathway [16].

3.2. Growth of LAB strain Pr 4.3L

The growth was determined based on cell biomass observed using a spectrophotometer (OD 600nm) [9]. At first, the LAB grew to 0.102 Å. Furthermore, LAB strain Pr 4.3L was known to grow at temperatures of 30 and 37°C under pH conditions of 4-6 (acid), 7 (neutral), and 8 (base) as indicated by an increase in cell biomass after being grown at various pH and incubation temperatures (Figure 2).

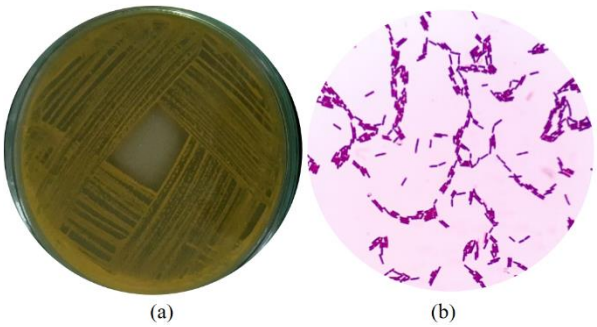

Figure 1. LAB strains Pr 4.3L colony (a) macroscopically on MRS agar-CaCO3-glucose medium using streak plate method (b) microscopically with 1000x magnification.

In treatments with incubation temperatures of 30 and 37° C, the average growth of LAB strain Pr 4.3L was not significantly different, but optimal conditions were observed at 37°C. A previous study showed that *Lactobacillus viridescence* as an optimum bacteriocin producer grows at 37°C [10]. Similarly, other results stated that the best incubation temperature for LAB growth was 37°C [8]. In the treatment with pH variations, the highest LAB growth was at pH 7. Furthermore, previous studies showed that the highest growth was also at pH 7 [10]. However, some showed that the best LAB growth was at pH 6.2 [5]. In this study, LAB which grew at pH 6 also showed quite optimal results and was not significantly different from treatment at pH 7. Regulation of LAB growth at various pH and incubation temperatures did not only affect its growth but also affected the production of antimicrobial compounds such as bacteriocin.

3.3. Determination in bacteriocin activity of isolate LAB strain Pr 4.3L

LAB bacteriocin activity was determined using the agar well diffusion method [3]. In this study, the CFCS solution was tested on 3 pathogenic bacteria, namely *S. typhi* BPE 122.4 CCA and *S. typhi* NCTC 786 which are Gram-negative bacteria and *S. aureus* ATCC 25923 which is a Gram-positive bacterium. The bacteriocin activity testing on 3 pathogenic microbes is performed because *Salmonella* sp. and *S. aureus* plays quite a role in causing food and beverage poisoning [22]. The results showed that the CFCS solution of isolate LAB strain Pr 4.3L before being heated possessed a strong and moderate inhibitory spectrum against indicator bacteria. On the contrary, its inhibitory power after being heated has moderate and weak activity against indicator bacteria (Table 1). Therefore, LAB strain Pr 4.3L can produce bacteriocin compounds even though it has a different inhibitory effect on indicator bacteria.

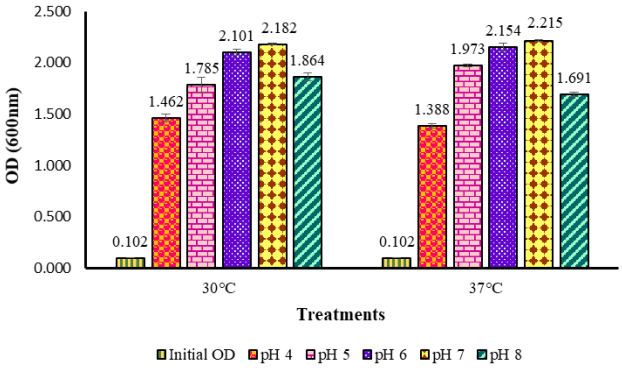

Figure 2. Growth of LAB strain Pr 4.3L at various pH and incubation temperatures

In antimicrobial activity testing, CFCS was first neutralized at pH 6.5 using 1 N NaOH. Neutralization is used to determine the presence of antimicrobial activity which is not caused by the influence of acids produced by LAB. Generally, Gram-negative bacteria are sensitive to lactic acid because they weaken cell permeability by damaging the outer membrane of the bacteria [23]. The main characteristics of bacteriocin are acid-resistant and stable to heat. Therefore, the neutralized CFCS solution is heated at 100°C for 10 minutes to determine bacteriocin activity [3]. In addition, bacteriocin which is used as preservatives in food needs to have resistance to heat because one of the food processing methods requires heating with high temperature [24]. However, it will be damaged in the absence of resistance to high temperatures since the antibacterial activity is ineffective in the food preservation process [25]. Bacteriocin is a bioactive protein that is synthesized by the ribosomes of Gram-

positive and negative bacteria and has antagonistic properties against pathogenic bacteria [6]

Table 1. Antimicrobial and bacteriocin activity tests of isolate LAB strain Pr 4.3L against pathogenic bacteria

Treatment		Antimicrobial activity (Inhibitory zone in mm)			Bacteriocin activity (Inhibitory zone in mm)		
Temperature (°C)	pH	*Staphylococcus aureus* ATCC 25923	*Salmonella typhi* NCTC 786	*Salmonella typhi* BPE 122.4 CCA	*Staphylococcus aureus* ATCC 25923	*Salmonella typhi* NCTC 786	*Salmonella typhi* BPE 122.4 CCA
30	4	12.3 ± 1.2*	12.3 ± 1.5*	11.7 ± 2.1	10.7 ± 1.2	10.0 ±1.0	11.0 ± 1.0
	5	12.7 ± 0.6*	9.7 ± 0.6	13.3 ± 1.5*	11.0 ± 1.0	10.0 ± 1.0	12.0 ± 0*
	6	16.3 ± 1.2**	14.3 ± 1.5*	12.0 ± 0*	12.0 ± 0*	12.3 ± 0.6*	12.0 ± 0*
	7	17.7 ± 0.6**	14.3 ± 1.2*	17.7 ± 0.6**	12.3 ± 1.2*	12.0 ± 1.0*	12.7 ± 1.2*
	8	12.3 ± 0.6*	12.2 ± 0.6*	12.7 ± 1.2*	11.0 ± 1.0	9.7 ± 0.6	10.3 ± 0.6
37	4	13.0 ± 1.0*	11.0 ± 1.0	13.0 ± 1.0*	10.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0
	5	15.0 ± 1.0*	11.3 ± 0.6	15.0 ± 1.0*	11.0 ± 1.0	10.7 ± 0.6	11.0 ± 0
	6	15.0 ± 1.0*	13.0 ± 1.0*	15.0 ± 1.0*	11.0 ± 0	11.0 ± 1.0	12.0 ± 0*
	7	15.7 ± 1.2*	15.0 ± 1.0*	15.7 ± 1.2*	11.0 ± 1.0	11.3 ± 0.6	11.0 ± 0
	8	14.0 ± 1.0*	11.3 ± 0.6	14.0 ± 1.0*	10.7 ± 0.6	10.3 ± 0.6	10.0 ± 0

Note: *moderate inhibition and **strong inhibition against indicator bacteria; well diameter: 8 mm

The inhibitions of LAB against pathogens are grouped into 4 categories, including weak (8-12 mm), moderate (12-16 mm), strong (16-20 mm) and very strong (> 20 mm) antimicrobial activities [14]. The results showed that the compound produced by LAB strain Pr 4.3L had stronger inhibition against *S. aureus* ATCC 25923 compared to *S. typhi* BPE 122.4 CCA and *S. typhi* NCTC 786 on all temperature and pH treatments (Table 1). Also, bacteriocin attaches with the cell wall of Gram-positive bacteria and forms complexes with lipoteichoic acid to cause destabilization. This acid is a specific receptor and is associated with the binding of bacteriocin compounds [26]. The same has happened with the bacteriocin activity produced by *L. brevis, L. casei,* and *L. plantarum* against *B. cereus, B. subtilis,* and *S. epidermidis* respectively [27].

The antimicrobial activity of LAB strain Pr 4.3L has stronger inhibition against all indicator bacteria at a temperature of 30°C compared to 37°C with a pH range of 6-7. The largest inhibitory zone was obtained in the treatment with a temperature of 30°C and a pH of 7. The lowest inhibitory zone with a weak category is in the treatments with a pH of 4 at temperatures of 30 and 37°C. The same profile is shown in the inhibitory zone of bacteriocin activity, even though it belongs to the medium category (Table 1). However, Figure 3 showed the differences produced based on antimicrobial and bacteriocin activity tests. CFCS produced by LAB strain Pr 4.3L before being heated at 100°C for 10 minutes possessed a strong antimicrobial activity (Figure 3: a-c), while BLS (bacteriocin like substance / CFCS after heating treatment) showed moderate bacteriocin activity (Figure 3: d-f). Likewise, the inhibitory zone shown by the CFCS solution of LAB strain Pr 4.3L, which has been treated by heating showed that the bacteriocin activity possessed high-temperature resistant characteristics.

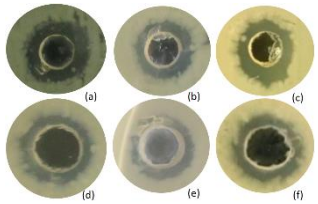

Figure 3. Inhibitory zones of LAB strain Pr 4.3L in the treatment with an incubation temperature of 30°C based on antimicrobial (a-c) and bacteriocin (d-f) activity test. Note: (a & d) in the treatment with a pH of 7 against *S. typhi* BPE 122.4 CCA; (b & e) in the treatment with a pH of 6 against *S. typhi* NCTC 786; (c & f) in the treatment with a pH of 7 against *S. aureus* ATCC 25923.

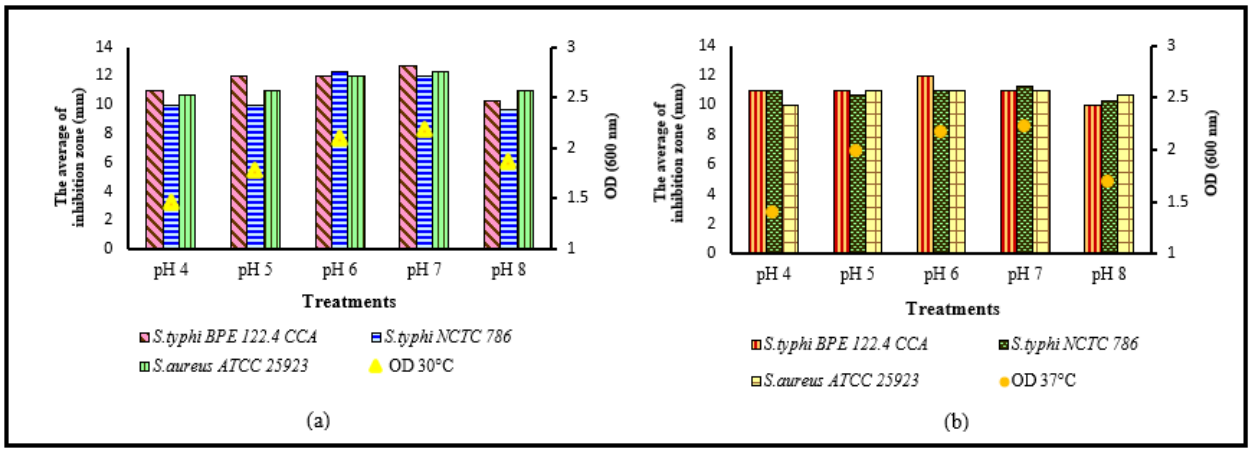

Figure 4. The relationship between growth and bacteriocin activity of LAB strain Pr 4.3L

The results showed that LAB growth is quite influential on bacteriocin activity. This activity is stronger against pathogens at incubation temperature of 30°C compared to 37°C (Figure 4). However, the growth of LAB strain Pr 4.3L for 36 hours of incubation at 37°C is faster (OD_{600nm} value = 2,215 ± 0.009) since it entered the stationary phase first compared to the growth at 30°C (OD_{600nm} value = 2.101 ± 0.031) (Figure 1). Therefore, the bacteriocin activity is slightly decreased since it is more productive in the log phase. On the contrary to the stationary phase, there is a decrease in bacteriocin activity in the stationary phase. This was caused by the release of the protease enzyme from the cell before entering the death phase [28]. Secondary metabolites such as bacteriocin are produced during the logarithmic phase, antibacterial compounds are present in maximum amounts and each LAB species has a different growth pattern at the end [29].

4. CONCLUSION

LAB strain Pr 4.3L has the potential to produce bacteriocin at a temperature of 30°C on MRS broth medium with a pH of 7. In these conditions, the CFCS antimicrobial activity has a stronger inhibition than that of BLS bacteriocin. The inhibition of antimicrobial compounds produced by LAB strain Pr 4.3L has a broad spectrum against indicator bacteria (*S. aureus* ATCC 25923, *S. typhi* BPE 122.4 CCA and S. *typhi* NCTC 786). The results provide useful information about the application of bacteriosinogenic LAB potential in the food fermentation process.

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