RESEARCH ARTICLE | MAY 17 2017

Bacteriocin production of *Lactobacillus* sp. from intestines of ducks (*Anas domesticus* L.) incubated at room temperature and antibacterial effectivity against pathogen

Nurfitri Arfani; Fatmawati Nur; Hafsan; Rodiyati Azrianingsih

AIP Conf. Proc. 1844, 030004 (2017)
https://doi.org/10.1063/1.4983431

BACTERIOCIN PRODUCTION OF *Lactobacillus* sp. FROM INTESTINES OF DUCKS (*Anas domesticus* L.) INCUBATED AT ROOM TEMPERATURE AND ANTIBACTERIAL EFFECTIVITY AGAINST PATHOGEN

Nurfitri Arfani[1,a], Fatmawati Nur[2], Hafsan[2], Rodiyati Azrianingsih[1]

[1] *Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Indonesia*
2 Biology Department, Faculty of Science and Technology, UIN Alauddin Makasar, Indonesia

[a)]Corresponding author: nurfitri.arfani@gmail.com

Abstract. Bacteriocin is a peptide that is easily degraded by proteolytic enzymes in the digestive systems of animals, including humans. It has antimicrobial activity against pathogenic bacteria. *Lactobacillus* sp. is one type of lactic acid bacteria (LAB) that occupies the intestines of ducks (*Anas domesticus* L.). The purpose of this research was to determine the optimum time of the highest protein production by *Lactobacillus* sp. and to determine inhibitory activity of bacteriocin against pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*). Using the Bradford method, the results showed that the optimum time of highest bacteriocin production was after 36 hours of incubation, with a protein content of 0.93 mg/ml. The bacteriocin inhibitory activity against *Escherichia coli* showed that a protein concentration of 30% gave a maximum inhibition index of 1.1 mm, while for *Staphylococcus aureus,* a concentration of 70% gave a maximum inhibition index of 0.3 mm. Further research is required to determine the stationary state of bacteriocin production in this circumstance.

INTRODUCTION

The microbiological quality of food products is determined by the number and types of microorganisms that are contained in foodstuffs. This determines the durability of food products in terms of damage by microorganisms. Product safety is determined by the number of pathogenic microorganisms that are contained within food.[1] Moreover, the incidence of decay that occurs during processing, transportation, and storage of food, among others, is due to microbial activity. This can result in losses for the food industry. To overcome the problem of decay, preservatives are used for food safety and to extend durability.[2]

Currently, there is a public use of chemical preservatives such as formaldehyde, monosodium glutamate (MSG), saccharin, benzoic acid, and ascorbic acid. However, the nature of chemical preservatives has some deficiencies; for example, formaldehyde is a known carcinogen that can induce cancer in animals, including humans. If swallowed, it does not cause intoxication in the short term, but if consumed above the threshold (maximum 0.1 mg/L), it can disrupt health by interfering with digestive organ functions, causing hypotension (low blood pressure), heart disease and damage to the nervous system.

This issue has sparked a search for natural preservatives that are safe to use; for example, chemical compounds that are derived from plants, animals or produced by microbes, better known by the term biopreservation. One biopreservation, which is currently being developed is bacteriocin, produced by lactic acid bacteria. The use of bacteriocin in the food industry requires attention in terms of production, because it involves efficiency and

The 7th International Conference on Global Resource Conservation
AIP Conf. Proc. 1844, 030004-1–030004-5; doi: 10.1063/1.4983431
Published by AIP Publishing. 978-0-7354-1516-4/$30.00

effectiveness in its application. The production process is efficient in its use of materials and is effective in inhibiting pathogenic bacteria, which can be obtained by optimizing the factors of production.[2]

Bacteriocin is an antimicrobial compound that is produced by lactic acid bacteria (LAB) from the genus *Lactobacillus* sp. This can be found in the colon of duck *Anas domesticus* with the result that ducks have relatively good health levels. Indeed, the duck has its own advantages, namely that it has a higher vitamin content than chicken.[3] Therefore, I conducted research to produce bacteriocin from *Lactobacillus* sp. isolated from the intestines of ducks (*Anas domesticus*).

MATERIALS AND METHODS

Purification of Isolates

Purification was performed by growing isolates of MRSA bacteria on media and incubated at 37ºC for 2 days.

Production and Precipitation Bacteriocin of LAB Isolates

LAB isolates were inoculated into 50 mL of MRSB medium as a starter culture and were incubated at 37ºC for 24 h in a shaker incubator. The cell suspension was then inoculated in starter medium and LAB isolates, as many as 15 mL to 700 mL, were transferred into MRSB medium then incubated again at a temperature of 37ºC for 72 h. Every 12 h 10 mL were taken and centrifuged at 4500 rpm for 15 min at 4°C. A crude extract was obtained in the supernatant to be used at a later stage. Bacteriocin (concentrated) was precipitated from the crude extract by adding 50% methanol. The addition was done gradually while stirring gently using a magnetic stirrer at slow speed at a temperature of 4ºC. The mixture was then centrifuged at 4500 rpm for 30 min at 4°C. The precipitate was dissolved in 0.1 M phosphate buffer pH 7 in 2 mL volume, then the protein content was measured.

Measurement of Crude Extract Bacteriocin

The protein content was measured using the Bradford method. A total of 400 mL precipitated bacteriocin was reacted with 4 mL Bradford reagent, vortexed, then allowed to stand for 15 min. As the control, 400 mL distilled water was used, which was reacted with the same reagents, while bovine serum albumin (BSA) was used as a standard protein with a series of concentrations from 0.2 to 0.1 mg/mL of stock BSA (mg/ml). Protein content measurement was performed using a spectrophotometer at a wavelength of 595 nm. Bradford's composition was prepared by dissolving 0.033 g of Coomassie Brilliant Blue added to 100 mL of 95% ethanol. This was then added to 34 mL of 85% phosphoric acid. The mixture was homogenized by vigorous shaking, filtered with filter paper and stored in dark bottles at low temperatures. Stock Bradford reagent was diluted five times before use to reduce oxidation.

Inhibition Activity Test of Bacteriocin from LAB isolates

By using the disc diffusion method, 200 mL of indicator bacteria, with optimum incubation age, were spread on NA medium. Then, paper discs with a diameter of 6 mm were placed on the surface of the agar medium already containing the indicator bacteria. A total of 20 mL of bacteriocin, which have been deposited by dripping on a paper disc in accordance with the determined concentrations (5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) and then incubated at 37ºC every 24 h for 72 h. Inhibitory concentration was shown by the formation of a clear zone around the paper disc. The inhibition index was calculated using the equation:

$$IP = \frac{\phi clear\ zone\ (mm) - \phi paper\ disc\ (mm)}{\phi\ paper\ disc\ (mm)} \qquad (1)$$

where IP = inhibition index and Φ = diameter (mm).

RESULTS AND DISCUSSION

Optimum Time of Highest Protein Production by *Lactobacillus* sp.

The optimum time production of bacteriocin was determined by the incubation period required to produce bacteriocin antimicrobial compounds. The optimum time was based on the protein content of each 12-h sampling point using the Bradford method with a wavelength of 595 nm. The optimum time production of bacteriocin of *Lactobacillus* sp. can be seen in Figure 1.

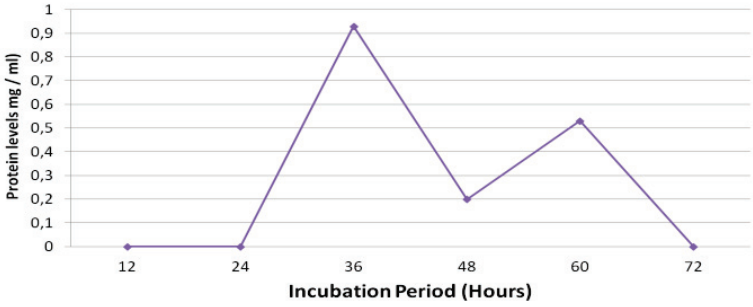

FIGURE 1. Optimum time production of bacteriocinat various incubation

As Figure 3.1 shows, the optimum time for the production of bacteriocin *Lactobacillus* sp. was 36 h. This phase is called the early stationary phase. This is similar to previous findings[4] showed that the growth curve of *Lactobacillus casei* strain Shirota at the beginning of the stationary phase showed the highest increase in the number of bacterial cells at 36 and 48 h. The results obtained in the present study indicate the optimum time to ferment bacteria in the substrate, and the possible resulting fermented metabolites quickest. The maximum production of bacteriocin is at the end of the exponential phase and the early stationary phase.[5]

In this study, the isolate used was one type of lactic acid bacteria isolated from the intestine of ducks (*Anas domesticus*). It is a microbe that strongly maintains healthy gastrointestinal function, such that the genus is the most widely used in the development of probiotic products.

To determine the optimum production of bacteriocin, the growth curve of *Lactobacillus* sp. was used, which was produced by sampling every 12 h for 72 h. In the log phase, 0–24 h, *Lactobacillus* sp. does not experience significant growth, due to adjusting to the new medium. This means that cells cannot reproduce or cleave, but only adapt to the medium or new environment.[6] The logarithmic phase (exponential phase) is the next phase, which is characterized by increased levels of protein in the incubation period of 24–36 h. This phase was when bacteriocin was produced: at an incubation period of 36 h with a protein content of 0.93 mg/mL. The optimum production of bacteriocin *Lactobacillus brevis* OG1 shows that antimicrobial activity was originally detected in the exponential growth phase and reached a maximum at the beginning of the stationary phase, depending on the species.[7] The next phase is the stationary phase. In this study, it is not known when the stationary phase occurred due to the 12 h sampling periods, which meant that the stationary phase of bacterial incubation occurred between 36 h and 48 h. Moreover, at the incubation period of 48 h, decreased levels of protein (0.2 mg/mL) were detected, and these were caused by the bacteria *Lactobacillus* sp. producing a protease, which causes a decrease in protein content. At the incubation period of 60 h, there were increased levels of protein (0.52 mg/mL). This is due to these bacteria producing another protein, in addition to protease, which causes an increase in protein content. However, although the levels of protein increased at 60 h, the protein content at 36 h was the highest, and was thus used as the criterion for the optimum time for the production of bacteriocin, which also indicates that this incubation period is the exponential phase. The death phase is characterized by an incubation period of 72 h with a protein content of about 0 mg/mL, which indicates the bacteria *Lactobacillus* sp. no longer produces bacteriocin. Extension of the incubation period after the stationary phase caused bacteriocin activity to decrease due to the release of protease from the cell when the cell enters the death phase.

A previous study[8] showed that the levels of protein required to isolate SR21 (*Lactococcus lactis* spp. Lactis 1) is approximately 0.0033 mg/mL and to isolate SR54 (*Lactobacillus brevis*), it is 0.0034 mg/mL. These values are different when compared to the levels of protein produced by *Lactobacillus* sp., which has a protein content of 0.93 mg/mL with an incubation period of 36 h, which is higher than previous study[8]. However, another research[9] showed that Amylosin 121 can be obtained by using ammonium sulfate precipitation storage followed by the dialysis process.

Increased activity was highest when precipitating 0–20%, which is 180 times, and when precipitating 20–40%, which is 133.5 times the protein content, at 0.6 mg protein/mL and 1.4 mg protein/mL, respectively. Production of bacteriocin, which varies depending on the pH increase in growth medium to achieve optimum acidity conditions, further decreased. In addition, the temperature can affect increased production of bacteriocin, but can also kill the bacteria.

Bacteriocin Inhibitory Activity against Pathogenic Bacteria (*Escherichia coli* and *Staphylococcus aureus*)

Antibacterial activity tests can be carried out by diffusion and dilution methods. The disc diffusion method is performed by measuring the diameter of a clear zone, which is indicative of the response of bacterial growth inhibition by antibacterial compounds in the extract. In this study, the inhibition zones of a bacteriocin produced by *Lactobacillus* sp. occurred at different concentrations for the two indicator bacteria *Escherichia coli* and *Staphylococcus aureus*, which can be seen in Figure 2.

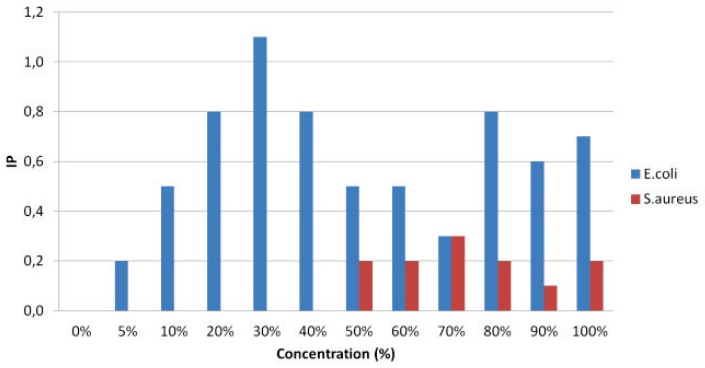

FIGURE 2. Bacteriocin inhibitory activity against pathogen bacteria (*Escherichia coli* and *Staphylococcus aureus*)

Based on the results in Figure 3.2, a bacteriocin produced by *Lactobacillus* sp. has an inhibitory effect on the pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*, which is characterized by the clear zone. Bacteriocin inhibition zones were highest in the indicator bacteria *Escherichia coli* at a concentration of 30%, with an index of inhibition (IP) of 1.1 mm, while for *Staphylococcus aureus* the inhibition zone was highest at a concentration of 70% with an IP of 0.3 mm.

These results indicate that antibacterial properties of bacteriocin are stronger against Gram-positive bacteria (*Staphylococcus aureus* and *Listeria monocytes*) than Gram-negative bacteria (*Salmonellatyphi* and *Escherichia coli*). This is due to environmental factors that may allow less optimal growth, leading to low bacteriocin activity, meaning less inhibition of indicator bacterial growth. Normally, strains of Gram-positive bacteria are sensitive to bacteriocin with a very varied spectrum, whereas Gram-negative bacterial strains are resistant to bacteriocin. However, *Staphylococcus aureus* becomes insensitive to some of the bacteriocin in a normal environment, but in conditions below normal; for example, 13% salt and acidic conditions, bacteriocin is sensitive to *Staphylococcus aureus*.[10] This research used medium without the addition of acid or salt. In addition, Gram-negative bacteria may become sensitive following the destruction of the structure of lipopolysaccharide on the cell surface due to physical or chemical stress. Bacteriocin of lactic acid bacterial origin is not efficient in inhibiting Gram-negative bacteria because their outer membrane is hydrophilic and can block the action of bacteriocin. Bacteriocin inhibition activity is the ability of a bacteriocin molecule to inhibit indicator bacterial cells, measured by the extent of the clear zone formed.[11] Mechanisms of bactericidal activity of bacteriocin are as follows: (1) molecular bacteriocin in direct contact with the cell membrane, (2) the contact process can disrupt the membrane potential to destabilize the cytoplasmic membrane so that the cells become stronger, and (3) the instability of the membrane is able to affect the formation of holes or pores in the cell membrane through the process of PMF (proton motive force) disruption.[12] The effect causes inhibited cell growth and produce cell death process in cells sensitive to bacteriocin.

SUMMARY

The optimum time needed by the bacteria *Lactobacillus* sp. to produce bacteriocin is the incubation time of 36 h, where a protein content of 0.93 mg/mL is produced. The bacteriocin produced can inhibit the bacteria *Escherichia coli* at a concentration of 30% at an IP of 1.1 mm, and for *Staphylococcus aureus* at a concentration of 70% at an IP of 0.3 mm.

ACKNOWLEDGMENT

Authors thanks to Mrs. Eka Sukmawaty for helping, supporting and funding this research.

REFERENCES

1. Buckle, K. A., R. A. Edwards, G. H. Fleet, And M. Wooton, "*Food Science"*, Translate: H. Purnomo dan Adiono. *Ilmu Pangan*, (Ui Press, Jakarta, 1987).
2. Prabowo, Antara Koco, "Bacteriocin Production Of Lactic Acid Bacteria Strains From SCG in 1223 in The Medium Molasses", Bogor Agricultural University, Bogor, 2010).
3. Ditjennak, "Directorate General of Livestock of The Republic Of Indonesia", (Jakarta, 2006).
4. Pranayanti, Ida Ayu Pratiharavia and Aji Sutrisno, "Making Drinks Probiotic Coconut Water (*Cocos nucifera* L.) with Starter *Lactobacillus casei* Strain Shirota", Journal of Food and Agro-Industry vol. 3 no. 2, 765 (2015).
5. Januarsyah, T, "Study of Inhibitory Activity Bacteriocin of Lactic Acid Bacteria Strains SCG 1223", (Bogor Agricultural University, Bogor, 2007).
6. Madigan Mt, Martinko Jm., Brock: Biology of Microorganism, Pearson Education International. *ISBN 0-13196893-9*, 375-377 (2006).
7. Jimenez-Diaz, R., "Plantaricin S And Two New Bacteriocin Produced by *Lactobacillus plantarum* LPC 010 Isolated From a Green Olive Fermentation", Appl. Environ. Microbiologi, 59, 1416-1429 (1993).
8. Suardana, W.I.;Suarsana, N.I .; Sujaya, N.I, and Wiryawan, K.G, "Isolation and Identification of Lactic Acid Bacteria from Cow Rumen Fluid Biopreservatif Bali as a candidate", Veterinary Journal, 8 (4): 155 159 (2007).
9. Sudirman, Idwan, *et al*. "Utilization of Lactic Acid Bacteria Producing Bacteriocin as Seed Starter Excellence and Biopreservatif on Food", IPB, LPPM, 1999).
10. Springer-Verlag, "*Lactic Acid Bacteria: Current AvancesIn Metabolism"*, (Genetics And Aplication, Germany, 1996).
11. Bhunia, A.K., M.C. Johnson, B. Ray And N. Kalchayanand, "Mode of Action of Pediocin Ach From *Pediococcus acidilactis* H on Sensitive Bacterial Strains", J. Applied Bacteriol 70, 25-33 (1991).
12. Gonzalez, B., E. Glaasker, E.R.S. Kunji, A.J.M. Driessen, J.E. Suarez, And W.N. Konings, "Bactericidal Mode of Action of Plantaricin C", Appl. Environ. Microbiol 62, 2701-2709 (1996).