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Characterization of Bacteriocin *Lactobacillus casei* on Histamine-Forming Bacteria

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ABSTRACT: Tuna which has undergone a process of decay will be poisoned if being consumed. It caused by the contamination of pathogenic bacteria such as *Escherichia coli, Salmonella, Vibrio cholerae, Enterobacteriacea* etc. Some types of fish contain histidine family scmbroidae high free, such as yellow tail tuna 740 mg/100g of meat, bigeye tuna 491 mg/100 g, mahi-mahi 344 mg/100 g, mackarel 600 mg/100 g, skipjack 1192 mg/100 g and albakor highest to 2 g/100 g. The formation process of histamine in fish is influenced by the activity of the enzyme *L-Histidine Decarboxylase* (HDC). Bacteriocin *Lactobacillus casei* extract is able to inhibit the activity of *Pseudomonas sp, Proteus morgani* and *Micrococcus sp.* The extract of bacteriocin *Lactobacillus casei* has a high temperature stability which has inhibitory activity against bacteria test at a temperature of 90°C. Bacteriocin *Lactobacillus casei* from bacteria *Pseudomonas sp* has the optimum activity at pH 5 with inhibition diameter of 8.25 mm, while the bacteria *Micrococcus sp* has the optimum activity at pH 4 with a inhibition diameter of 9.25 mm. Bacteriocin *Lactobacillus casei* has a molecular weight of 14.34 kDa which included in the group of class III bacteriocins, generally has a large size (> 10 kDa), and can not survive against the heat. Bacteriocins *Lactobacillus casei* extract can inhibit the activity of histamine-forming bacteria growth and have stable properties to high temperature and pH. Future research is recommended to do the production of bacteriocins *Lactobacillus casei* optimization and its application in fishery product.

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INTRODUCTION

Swordfish which belonging to the family of *scombroidae* will being easily decayed at the room temperature, and also the high water content in fish would be a suitable medium for the metabolism of spoilage bacteria that will causing the process of decay and the fish will no longer fresh anymore. The swordfish that has been decayed will be poisoned if being consumed. It caused by the contamination of pathogenic bacteria such as *Escherichiacoli, Salmonella, Vibrio cholerae, Enterobacteriacea* etc. Poisoning that often occurs by tuna were the histamine *(scombroid fish poisoning)* [1]. The process of formation of histamine in fish is influenced by the activity of the enzyme *L-Histidine Decarboxylase* (HDC). Various types of bacteria are capable to producing the enzyme HDC, including the Enterobacteriaceae, for example: *Enterobacter agglomerans, Enterobacter cloacae, Enterobacter intermedium, Hafnia alvei, Klebsiella pneumoniae,* and *Morganella morganii* [2].

To inhibit the bacterial growth, it is necessary to do the preventive measures in order to slow down the change of histidine so it will not cause the allergie such as by using bacteriocins as an antibacterial agent. Bacteriocins is one of the antimicrobial compounds that produced by lactic acid bacteria. Bacteriocins is defined as the active peptides or peptide complexes that were synthesized at the ribosomes, and also have the activity of bakteriostatic and bactericidal [3]. Bacteriocin that produced by LAB is a secondary metabolite produced by ribosomes, sensitive to proteolytic enzymes and may be inactivated by the digestive tract protease enzyme, generally has a heat resistance (60 ° C or 100 ° C for 30 minutes or more), stable at acid pH and neutral, inactivated at pH above 8.0 [4].

It is known that *Lactobacillus casei* bacteria can produce the bacteriocin that has antibacterial activity against several common pathogens and spoilage microorganisms in food production. It was explained by Chotiah [5] with the result that the crude bacteriocin *Lactobacillus casei* has antagonistic properties against pathogens *(S.typhimurium; E. coli; B. cereus* and *S. enteritidis.* Inhibitory activity against *E.coli* K99 enterotoxigenic and *S.*BCC *aureus* B2062 / ATCC 25923 is not visible. Based on the result above, this research will discuss about the chemical and physical characteristics of bacteriocins *of Lactobacillus casei* and its application on histamine-forming bacteria.

MATHERIAL AND METHODS

The Growth character of *Lactobacillus casei*

The growth curve of bacteria for 24 hours of incubation and sampling every 1 hour to determine the existing phases. A total of 5% (v / v) of the cultivation *Lactobacillus* MRS agar were grown in MRS broth and incubated at 37ºC. Bacterial growth is followed every hour to observe the optical density value or *optical density* (OD) of the starter on MRS media with turbidimetric method with a wavelength of 620 nm [6].

Making the Cultivation

The isolates of lactic acid bacteria that used in the form of agar slant culture MRS broth were rejuvenated with *yeast* addition as much as 0.5% (w / v). Rejuvenation or activation of *Lactobacillus casei* activation were done by growing a loop isolates grown in an agar medium slant into 10 ml MRS broth, then it were incubated at 37 ˚ C for 24-48 hours. After that, 1 ml of MRS Broth cultivation were taken to be grown in 10 ml MRS broth, then it were incubated at 37 ˚ C for 24-48 hours to obtain a cultivation [7]. Rejuvenation is also were done on bacteria test using *Tryptone Soya Agar* (TSA) media.

Extraction of Bacteriocin

The active cultures *of Lactobacillus casei* as much as 10% (v / v) were propagated in the 1000 ml of MRS broth (pH 7.0; glucose 0.25% w / v; peptone 0.5% w / v) for 20 hours at a temperature of 30˚C. Culture centrifuged at a speed of 10,000 rpm for 20 min at 4 ˚ C and neutralized using 1M NaOH to pH 7.0. The solution that were obtained then being filtered using a 0.2 µm membrane filter to obtain the cell-free supernatant [8]. Cell-free supernatant obtained from the extraction process that were precipitated with ammonium sulfate saturated solution of 60% (w / v) and were homogenized with a magnetic stirrer for 24 hours at a temperature of 4 ˚ C. Then, the precipitation solution was centrifuged for 30 minutes at 12000 rpm at 4 º C. The pellets were dissolved in 25 ml of 0.05 M *potassium phosphate buffer* (pH 7.0) [9]. The pellets obtained from the bacteriocins with ammonium sulfate precipitation that were dialyzed using a dialysis membrane (1.0 kDa). The buffer that being used were *potassium phosphate buffer* (pH 7.0) for 18 hours, then buffer have to be replaced at every 6 hours at a temperature of 4˚C to obtained the bacteriocins extract [8]. Bacteriocins solution that obtained by dialysis was centrifuged at ultracentrifugation at a speed of 50,000 rpm for 30 min at 4˚C and the supernatant that obtained were the bacteriocins extract [10].

Biological Characterization (Inhibitory Activity Bacteriocin)

Media MHA which have been sterilized then were put into petri dishes 20 ml each and allowed to solidify at room temperature. The medium were inoculated with 0.1 ml of bacterial suspension test and trimmed with a hockey stick, then allowed to stand to dry for 15 minutes. Bacteriocins extract as much as 50 mL were dripped on paper discs. Then placed on a paper disk media that has been inoculated by bacteria MHA test, then it were incubated at the temperature of 37 ˚ C for 24 hours. The clear zone that being formed indicates the existence of barriers of the growth of test bacteria by supernatant. We then measured the diameter of clear zone (mm) using calipers. The diameter of each inhibition zone was measured three times in different areas and then the results are averaged [11, 12].

Stability against temperature and time

This test were done by heating the bacteriocins as 400µl at a temperatures of 45 ˚ C, 70 ˚ C and 95 ˚ C for 15, 30 and 45 minutes. Then, the bacteriocins extract were tested about its inhibitory activity by disc diffusion method.

Stability against pH

This test were performed by addition of 0.1 M NaOH or 0.1 M HCl to make a difference in the pH level of 2 to 9. The volume of bacteriocins used is 400µl. Then, a solution of NaOH or HCl bacteriocins were homogenized and allowed to stand for a few minutes before being tested to its inhibitory activity against histamine-forming bacteria.

Chemical characterization (Molecular weight Bacteriocin)

Determination of molecular weight bacteriocins were using *Deodecyl Sodium Sulfate Polyacrilamide gel electrophoresis* (SDS-PAGE) [13].

RESULTS AND DISCUSSION

The growth phase of *Lactobacillus casei*

During the growth of lactic acid bacteria, the maximum production occurs at the end of the exponential phase or early stationary phase. The incubation period were used at the 19th hour which this phase occurs production of bacteriocins. The best production of bacteriocins was when it reaches the end of the exponential phase or early stationary phase. The growth curve *of Lactobacillus casei* [14] can be seen in Figure 1.

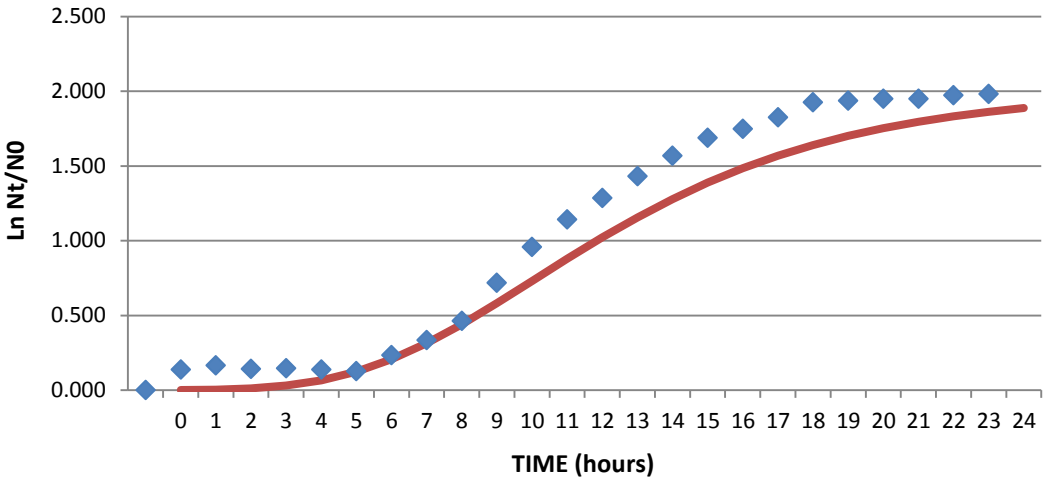


Figure 1. The growth curve *of Lactobacillus casei*

Biological Characterization

The diameter of inhibition zone was looked like diameter of the clear zone around the well which exhibits bactericidal (killing bacteria) or pseudo-diameter zone that showed bacteriostatic properties (inhibit microbial growth). The Clear zone formed by the secondary metabolites or another antimicrobial active compound that were produced. The test results inhibitory activity can be seen in Figure 2.

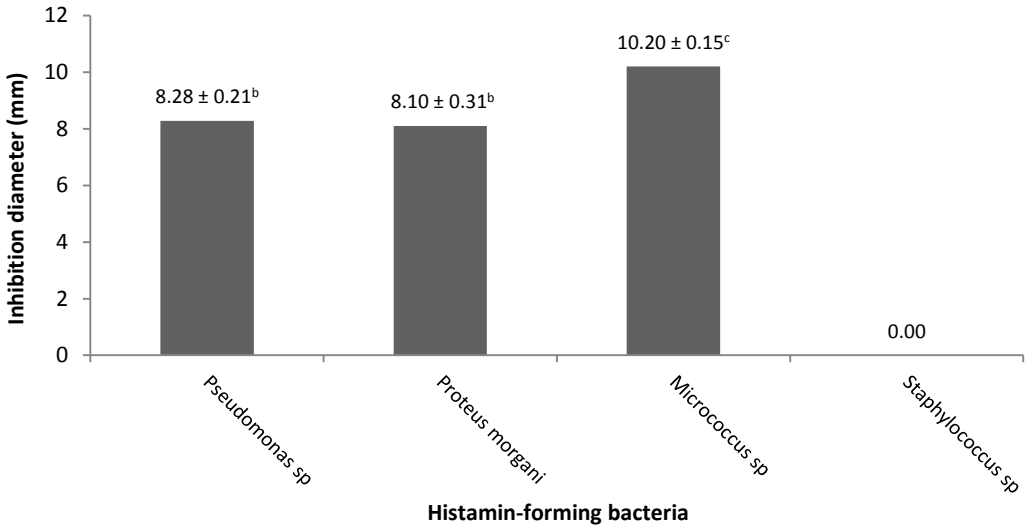
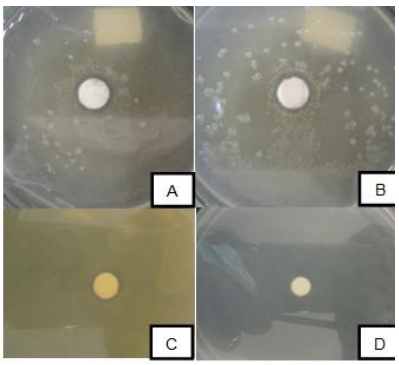


Figure 2. Graph bacteriocins extract inhibitory activity *of Lactobacillus casei*

On inhibition test bacteria *Staphylococcus sp* (Figure 3) there is no clear zone so that it can be stated that the bacteriocins *of Lactobacillus casei* can not inhibit the test bacteria *Staphylococcus sp* due to *Staphylococcus sp* has resistance to acids and has a strong cell wall (covalently bonded) so that this bacteria is more resistant to acids and other substances that were produced by antagonic *Lactobacillus casei* [15]. Bacteriocins extract of the bacterium *Lactobacillus casei* test has inhibitory activity which has inhibition diameter between 6-11 mm.



Description:

(A) *Pseudomonas sp*

(B) *Proteus morgani*

(C) *Micrococcus sp*

(D) *Staphylococcus sp*

Figure 3. Results of bacteriocins inhibition diameter *Lactobacillus casei* against histamine-forming bacteria

Based on the results of bacteriocin inhibitory activity against histamine-forming bacteria, bacteriocins can be concluded that the extract can inhibit the growth of *Lactobacillus casei* histamine-forming bacteria. Inhibition of the enzyme *L-histidine Decarboxylase* will affect in delayed or no product formation so that leads to the reduction or even the histamine can not be produced [16]. Bacteriocins will affect the membranes, DNA synthesis and protein synthesis. In general, bacteriocins showed bactericidal or bacteriostatic activity against other bacteria that are closely related to the producing strain. The main mechanism of bacteriocin was varied, they are the formation of pores in the cytoplasmic membrane or cell wall biosynthesis and inhibition of enzyme activity (RNAse or DNAse) in target cells [5].

Stability against the temperature

From the test results of the temperature characteristics of these bacteriocins, bacteriocins obtained inhibitory activity in bacteria *Pseudomonas sp* results can be seen in Figure 4 and the bacteria *Micrococcus sp* that the results can be seen in Figure 5.

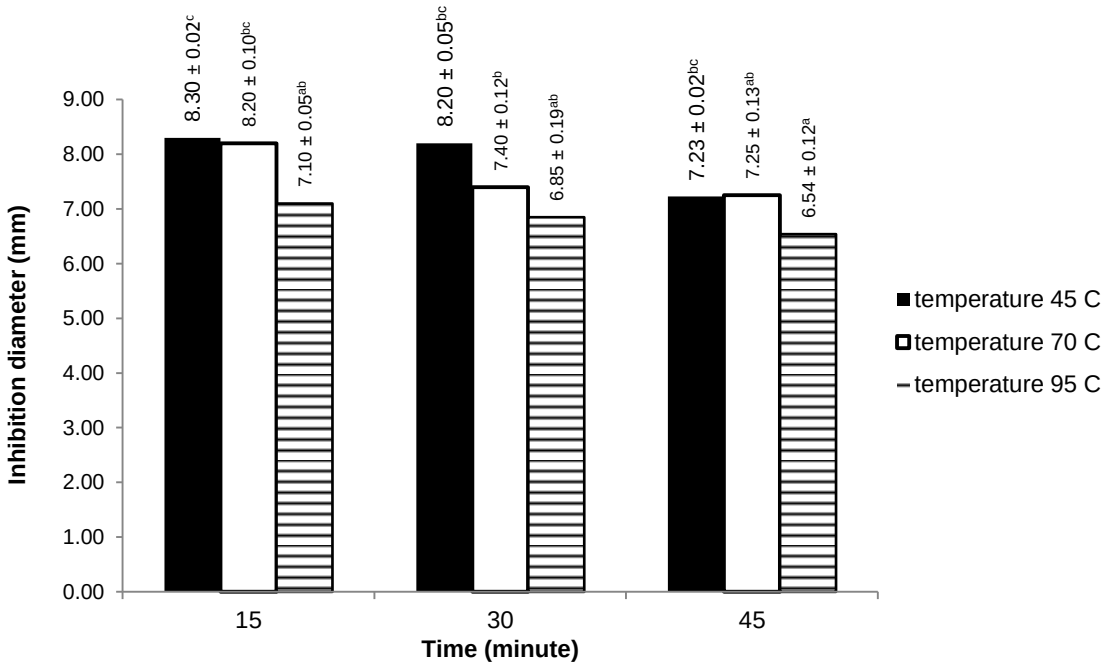


Figure 4. Graph bacteriocin inhibitory activity against the treatment temperature and heating time in bacteria *Pseudomonas sp*

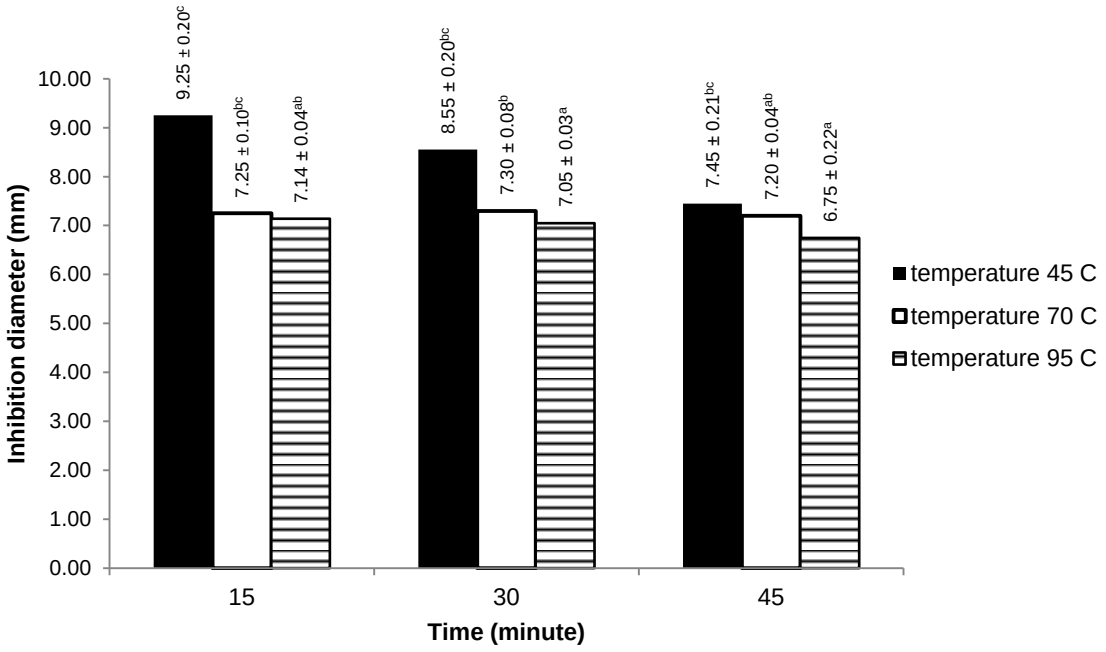


Figure 5. Graph bacteriocin inhibitory activity against the treatment temperature and heating time in bacteria *Micrococcus sp*

Bacteriocin *Lactobacillus casei* did not lose the inhibitory activity in the temperature range of 45 ° C, 70 ° C and 90 ° C, however, these bacteriocins decreased inhibitory activity against both the histamine-forming bacteria, the *Pseudomonas sp* and *Micrococcus sp.* Bacteriocin inhibitory activity decline continued with the increasing of heating temperature and the longer it has been used. It also can be seen in Figures 4 and 5 that the higher temperature was used, the smaller of diameter of the inhibition of bacteriocins against histamine-forming bacteria, as well as time. The longer it is used, the smaller the diameter of the inhibition of bacteriocins against histamine-forming bacteria. Bacteriocins of lactic acid bacteria resistant to the temperature of 100 ° C for 30 minutes even until the temperature of the autoclave. This is because in these bacteriocins may exist compounds - small globular compounds and their strong hydrophobic regions. It can be distinguished that from bakteriophage that is not heat resistant to autoclaving [17].

Stability against pH

The pH factor is often a consideration for preservatives to be used in food, especially food for livestock with low pH conditions such as beef, *ham,* meatballs, milk, butter, cheese etc. [18]. From the results of testing the characteristics of bacteriocins against this pH value, obtained bacteriocin inhibitory activity on histamine-forming bacteria *(Pseudomonas sp* and *Micrococcus sp),* the result can be seen in Figure 6.

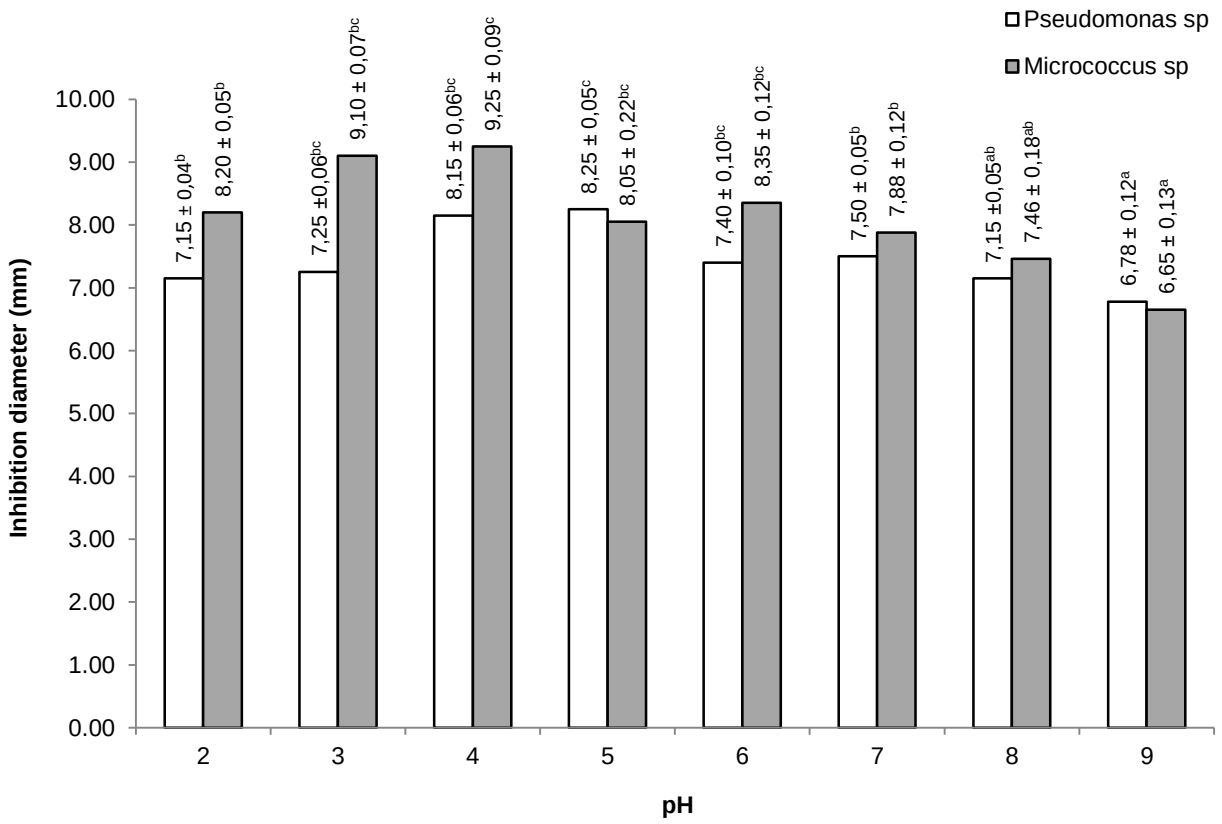


Figure 6. Graph bacteriocins extract inhibitory activity against pH

Bacteriocin *Lactobacillus casei* bacteria *Pseudomonas sp* test has optimum activity at pH 5 with inhibition diameter of 8.25 mm, while the test bacteria *Micrococcus sp* has optimum activity at pH 4 with a diameter of 9.25 mm inhibition. Bacteriocins produced by *Lactobacillus acidophilus* has optimum activity in the range of pH values of 4-5. Bacteriocin inhibitory activity decreased with increasing pH values (pH approaching the base) and active at acidic pH. The higher of the pH so the bacteriocin activity will be reduced, as seen in bacteriocins lost piscicolin activity at high pH near the pH 8 [19].

Chemical characterization (Molecular Weight)

Bacteriocins *of Lactobacillus casei* has a molecular weight of 14.34 kDa which can be seen in Figure 3. The molecular weight of bacteriocins were different by *Lactobacillus* species such as have a broad molecular weight range. Based on the size, morphology and physical, bacteriocins [20] produced by the bacterium *Lactobacillus casei* are grouped in Class III bacteriocins, which were generally large (> 10 kDa), and are not heat resistant consisting of two types. Type IIIa is bakteriolisin which is an enzyme of bakteriolitic. Examples are studied in this type is lisostaphin. Type IIIb is non-lytic type bacteriocins, one of which is helvetisin J (37 kDa) that were produced by *Lactobacillus helveticus.*

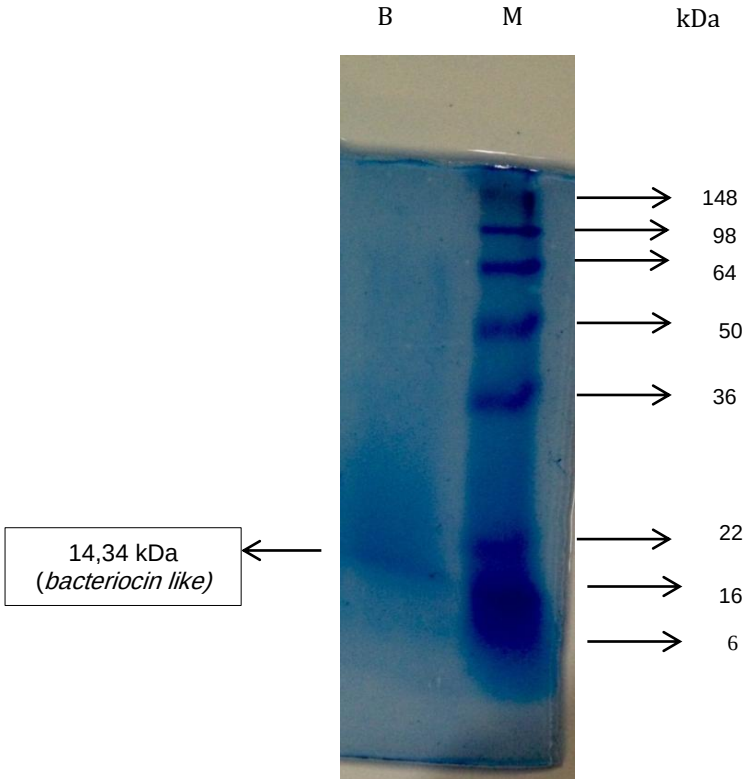


Figure 7. Result of elektroforesis SDS-PAGE

CONCLUSION

Bacteriocin *Lactobacillus casei* extract is able to inhibit the bacteria *Pseudomonas sp, Proteus morgani* and *Micrococcus sp.* Extract of bacteriocin *Lactobacillus casei* has high temperature stability which at a temperature of 95 ° C still has inhibitory activity against bacteria test. Bacteriocin *Lactobacillus casei* bacteria *Pseudomonas sp* test has optimum activity at pH 5 with obstacles measuring 8.25 mm, whereas the test bacteria *Micrococcus sp* has optimum activity at pH 4 with obstacles measuring 9.25 mm. Bacteriocin *Lactobacillus casei* active at acidic pH. Bacteriocin *Lactobacillus casei* has a molecular weight of 14.34 kDa were included in the group of class III bacteriocins, generally large (> 10 kDa), and are not heat resistant.

Competing interests

The authors declare that they have no competing interests

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