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OPTIMIZATION OF PEDIOCIN N6 PRODUCTION BY ISOLATE *Pediococcus pentosaceus* STRAIN N6 WITH MODIFICATION OF CARBON AND NITROGEN SOURCES

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ABSTRACT

Pediocin bacteriocin was produced by isolates *Pediococcus pentosaceus* strain N6. This *Pediococcus pentosaceus* strain N6 was isolated from Hot Springs RimboPanti West Sumatra. The aim research was to optimize the production Pediocin N6 using modified of carbon and nitrogen sources. Moreover, the testing temperature and pH optimum to obtain the highest antimicrobial activity against pathogens *Escherichia coli O157:H7, Salmonella* thyphimurium and *Listeria monocytogenes* as well as optimum time in producing Pediocin N6. The growth of cells isolate and optimum production of Pediocin N6 occurs at the end phases logarithms that is between 19-20 hours until the end of the exponential phase is between 35-36 hrs. Antimicrobial Activity of isolated *Pediococcus pentosaceus* strain N6 was reached optimum 50ºC with diameter inhibition zone of 29.6 mm against the bacteria *Listeria monocytogenes,* 20.3 mm against the *E. coli O157: H7* and 18.4 mm against the *S. thyphimurium.* The optimum pH of antimicrobial activity is pH 5 with diameter inhibition zone of 29.7 mm against the bacteria *Listeria monocytogenes*, 21.4 mm forthe bacterium *E. coli O157: H7* and 19.3 mm forthe bacterium *S. thyphimurium*. Incorporation of molasses as a carbon source to produce diameter inhibition zone of 34.5 mm against the *bacterium L. monocytogenes,* 27.9 mm against *E. coli O157: H7* and 26.2 mm against *S. thyphimurium* and skim milk as nitrogen sources to produce diameter inhibition zoneof 3.7 mm against the bacterium *L. monocytogenes,* 27.6 mm against *E. coli O157: H7* and 26.8 mm against *S. thyphimurium*. Modification of molasses as a carbon source and skim milk the nitrogen source produces highest antimicrobial activity.

Key words: *Pediococcus pentosaceus* strain N6, Carbon Sources, Nitrogen Sources**,** Pediocin N6, Pathogenic bacteria

INTRODUCTION

Lactic acid bacteria (LAB) is a microorganism that can be used in controlling the growth of pathogenic bacteria in food stuffs because it can lower the pH and produce bacteriocins (Laverentz *et al.,* 2006). Bacteriocins are protein aceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain (s). They are similar to yeast and paramecium killing factors and are structurally, functionally and ecologically diverse. Applications of bacteriocins are being tested to assess their application as narrow-spectrum antibiotics *(*Cotter *et al.,* 2012). Bacteriocins antimicrobial peptides are synthesized by ribosomes of bacteria, which have the property of inhibiting other bacteria, either of the same species (narrow spectrum) or across genera (Cotter *et al.,* 2005). Some of them are inhibitory towards food spoilage and food-borne pathogenic bacteria Including *Bacillus, Clostridium, Staphylococcus* and *Listeria.* Therefore, bacteriocins of LAB are particular interest because their existing and potential applications as natural preservatives in foods. The first bacteriocin to appear on both the European food additives list and the United States FDA list was nisin, which was intended for use in the product ion of processed food (Forouhandeh *et al.,* 2010).

Bacteriocins can be produced by lactic acid bacteria such as *Pediococcus, Leuconostoc* and *Lactobacillus spp*. Bacteriocins by *L. brevis* bacteria can inhibit the growth of *E. coli, Salmonella typhimurium* and *Listeria monocytogenes.* Bacteriocins by *L. plantarum* and *L. sakei* able to inhibit the growth of *L. monocytogenes* bacteria found in cooked meat. Bacteriocins by *L. plantarum*is able to inhibit the growth of *E. coli* (Charnley, 1992). In addition, bacteriocins have advantages over antibiotics because bacteriocins can be destroyed by digestive enzymes. This shows that the consumption of this compound will not interfere with digestion and will not pose a risk to health such as the use of antibiotics in general (Todorov, 2009).

Production of bacteriocins are generally performed in culture liquid substrates. Various factors may affect the production of bacteriocins in culture media. Production activity of bacteriocins by LAB are influenced the pH of media, incubation temperature, the type of carbon source, nitrogen source, the growth phase and concentration of NaCl. The type of carbon and nitrogen source used in the production of medium affects the growth rate of LAB cells, then influence the metabolism production of bacteriocins (Kim *et al.,* 2000).

Bacteriocins division based on the biochemical characteristics and genetic properties consist of three classes: class I, bacteriocins is referred to as l antibiotic. Bacteriocins peptides are small molecular weight peptides (<5 kDa) modified the phase of post-transcription and contains one or more amino acids such as lanthionine. Class II are small molecular weight peptides (<10 kDa) and were not modified and are resistant to heat. This class of bacteriocins form the structure helik (amphipathic) with variable hydrophobicity and E- sheet structure. This peptide is stable in heating 100-121ºC. Class III are large molecular weight peptides (> 30 kDa) and heat stable. Only a few bacteriocins from this class have been identified like helvetisin J, helvetisin V, acidophilusin A, laktisin A and B (Barefoot and Nettles, 1993). Class-II bacteriocins have two disulfide bonds C terminal to maintain the overall structure at the temperature increase. Changing the structure of helical regions and an increase in temperature causes a loss in the activity of the peptide are for receptor recognition and specific to a particular organism (Kaur *et al*., 2004).

The process of food preservation was combined with heating in industrial processes and this decreased the number of pathogenic microbes. Microbial resistance in industrial processes is a problem, therefore in food preservation, modern technology improves the quality of food. Preserving biological microbes involves assistance in the form of LAB due to production of lactic acid component. LAB utilization as a preservative is indispensable because it has antibacterial activity and resistance to heat (Jeevaratnam *et al.,* 2005). Pediocin N6 are potentially used as a food preservative in food processing industry which involves heating.

MATERIALSAND METHODS

Materials: Materials used isolates *Pediococcus pentosaceus* strain N6, isolated from Rimbo Panti hot springs located bordering the province of West Sumatra and North Sumatra, precisely in the District of East Pasaman regency Pasaman of West Sumatra Province Approximately 200 km from the city Padang. Pathogenic bacteria strains used are *Escherichia coli O157: H7, Salmonella* typhimurium and, *Listeria monocytegenes*. The strain was cultured in MRS broth (Merck), isolates *Pediococcus pentosaceus* strain N6 bacteriocins producing, a carbon source (glucose and molasses), a nitrogen source (yeast extract, NPK and skim milk), MHA (Merck) and Nutrient Agar (NA) (Merck)

Bacteriocin bioassay: Antimicrobial effects were tested on pathogens bacteria the agar well diffusion assay as described by Girum*et al*., (2005), a sample isolate colony was selected from MRS agar and transferred to grow in MRS broth then incubated aerobically at 50°C for 48 hours, centrifuged at 4^0C for 20mins to obtain the supernantant. 15ml of MHA was prepared and sterilized using the autoclave and it was dispensed aseptically into petri dishes. After it solidified, wells were created using the blue tip that cut edges so large diameter wells of 5 mm. Cultivated in the bottom of well is not perforated so that isolate LAB added not seeping everywhere. A sterile cotton bath was dipped in one type of pathogenic bacteria inoculum with density to inside 10^8 CFU/ml and rotated several times. Pressed on the inside of tube wall for removal of excess fluid inoculum, then inoculated to the entire surface of the medium MHA. The procedure was repeated while playing a petri for guarantee equal of inoculum. Then, it was incubated during 24 hours at 37°C.A total 50 µl the supernatant of lactic acid bacteria is dripped into the well to media MHA and during to 15-20 minutes. The petri dishes were incubated at 37°C for 48 hours. Each one petri was evaluated diameter of inhibition zone-including the diameter of agar wells the measured at six points by using a caliper.

Measurement growth: Method to Nghe and Tu (2014), the tested microorganisms were growing during 48 hours at temperature the optimum growth LAB isolates of thermophilic (50°C). Isolates BAL thermophilic grow in MRSB containing 9 ml then incubated at 50°C for 48 hours and then counted in the number cell cultures generated by measuring the growth of bacteria every 2 hours for 2 days against optical density (OD), changes in pH medium and bacterial population in the $\log_{10}$ CFU/ml in media MRS broth using the spectrophotometer at wavelength of 630 µm.

Effect of temperature and pH antimicrobial activity against isolates of *Pediococcus pentosaceus* strain N6*:* Methods to Ogunbanwo *et al.*, (2003b), isolate *Pediococcus pentosaceus* strain N6 of1ose was inoculated in MRSB then incubted during 48 hours at a temperature of 50, 60, 70 and 80 °C. Centrifuged and the supernatant produce is tested for antimicrobial activity using agar diffusion method. Determination the effect pH, 1ose isolates were grown on MRSB during 48 hours at pH 2-8 in aerobic conditions. Centrifuged and the supernatant produced is tested for antimicrobial activity.

Optimization of Culture Conditions to Pediocin N6 Activity:

Modification of Carbon and Nitrogen Sources on Pediocin N6 Production

Carbon sources: Methods to Ogunbanwo *et al.*, (2003b), isolates were grown during 48 hours in MRSB at 50°C with plus 2% glucose and 0.2% molasses. The thermophilic LAB isolates were centrifuged at a speed of 10.000 rpm for 15 minutes and the resulting supernatant tested for it's antimicrobial activity.

Nitrogen sources: Methods to Ogunbanwo *et al.*, (2003), modification of nitrogen source anti-microbial activity against isolates with the addit-ion various types of nitrogen sources are yeast extract, NPK and skim milk with each as 3%, 0.2 % and 3%. The addition is done at medium MRS broth. Centrifuged at a speed of 10.000 rpm for 20 minutes and the resulting supernatant was tested antimicrobial for activity.

Production Pediocin N6 liquid laboratory scale in 400 and 800 ml MRS broth and anti-microbial activity test: Method to Kim *et al.*, (2000), the production in 400 and 800 ml MRS broth, each one is inoculated as 1.0 ml culture isolates in 9.0 ml MRS broth solution have been added with 0.2% molasses and 3% skim milk (according to treatment modification the best carbon and nitrogen sources) and 1% NaCl then incubated at 50 °C for 24 hours. Production of bacteriocins on laboratory scale is performed with growing the LAB in 400 and 800 ml MRS broth and propagation as two stages that is first, inoculated 4.0 and 8.0ml culture LAB in 36 and 72 ml to MRS broth and the fermentation process in an incubator shaker speed of 150 rpm, 50 °C for 24 hours. The second, inoculated 40 and 80 ml bacterial lactic acid culture in 360 and 720 ml in MRS broth pH 5, then fermented on the incubator shaker speed of 150 rpm, 50°C for 9 hours (time of production). The next centrifuged at a speed of 10,000 rpm for 20 minutes. Super-natant was taken, neutralized pH (pH 6) with addition of NaOH. Cell-free liquids bacteriocins was heated water bath at 100°C, for 5-10 minu-tes. This process is intended for immobilize cells LAB lagging in the fluid bacteriocins. Separat-ion conducted of LAB cells using a filter size of 0.2 µm. Results of filtration are bacteriocins a liquid the free from LAB cells. Bacteriocins a liquid to remain sterile and maintained its acti-vity, bacteriocins packaging is the bottles lid that has been sterilized and then stored in a cold room.

RESULTS AND DISCUSSION

Measurement growth: The growth curve or increasing the number of cells isolates *Pediococcus pentosaceus* strain N6 during the incubat-ion period can be seen in Figure 1.

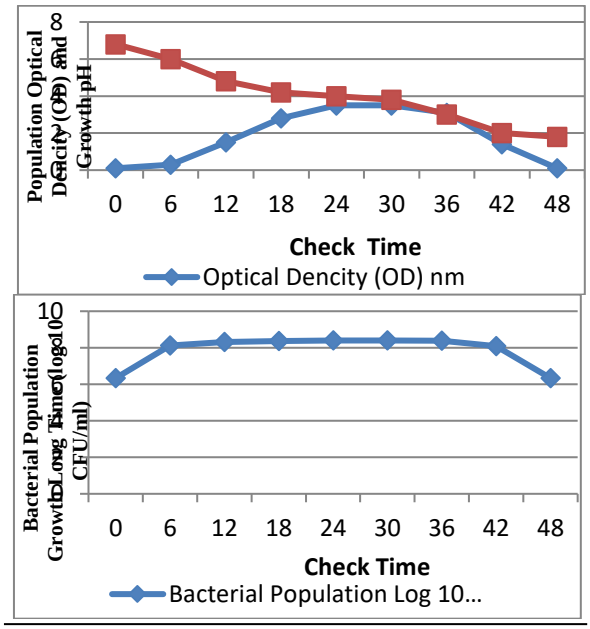

Figure 1: Growth curve, change of pH and bacterial population growth over

The cell isolates through a phase lag (adaptation) on the hour-0 until the 6th, the number of bacterial populations on the hour-0 of 6:33 $\log_{10}$ CFU/ml. Lag phase is a period adaptation or adjustment of microbes to medium conditions. The after a lag phase is completed the cells through a phase of logarithms (exponential), in this phase got under way cellular reproduction, the concentration of cellular or biomass increased so that the mass of cells to be doubled by the rate at which the cells will undergo cleavage at a constant speed 14 Log phase lasts from hours to 6th till the 20th, where the value of population density (OD) at the 6th hour as 0.1 and 2.4 at the 20th hour. Isolates experiencing the end phase of logarithma at the 20th hour. At the 6th, the bacteria have already started to experience the logarithma phase with a total population of 6.81 $\log_{10}$ CFU/ml. Bacteria experiencing the end phase of logarithm the total population of 8.53 $\log_{10}$ CFU/ml.

On entering the stationary phase to a maximum concentration of biomass, the number of cells tends stable, growth to stops and causes modification of cell biochemical structure. These undergo a stationary phase at the 20th hour to the 36th hour of incubation. Isolates began experiencing a phase of death on the 36th hour to hour 48. Bacterial populations on the 36th hour of 8.52 $\log_{10}$ CFU/ml and the value of population density OD= 2.3. Total population of bacteria on the hour to 48 together with the number of bacteria early to 6.33 $\log_{10}$ CFU/ml and OD value = 0.1. In this

phase, the availability of nutrients has begun to decrease, the energy reserves in exhausted cells, the accumulation of acid and other metabolites. The changes of pH medium and metabolites may reduce the number of cells that grow in the next phase.

Effect of temperature and pH antimicrobial activity against isolates of *Pediococcus pentoseceus* strain N6: The mean of diameter inhibition zone the resulting can be seen in Figure 2 below:

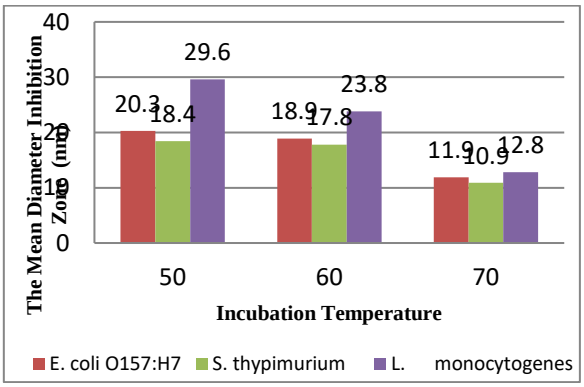

Figure 2: Effect of temperature antimicrobial activity against isolates *Pediococcus pentosaceus* strain N6 on bacterial patogens

Antimicrobial activity to isolate *Pediococcus pentosaceus* strain N6 were grow at 50°C the bacteria against *E. coli O157:H7*of 20.3 mm, *S.* thyphimurium of 18.4 mm and *L. monocy-togenes*of 29.6 mm. Isolates were grow at 60°C resulted in inhibition zone of 18.9 mm against the bacteria *E. coli O157:H7*, against *S.* thyphimurium 17.8 mm and 23.8 mm against the bacterium *L. monocytogenes*. Isolates were growing at 70°C resulted in inhibition zone of 11.9 mm against the bacteria *E. coli O157:H7*, against *S.* thyphimurium10.9 mm and 12.8 mm against *L. monocytogenes*.

The results of study show mean of diameter inhibition zone can be seen in Figure 3.

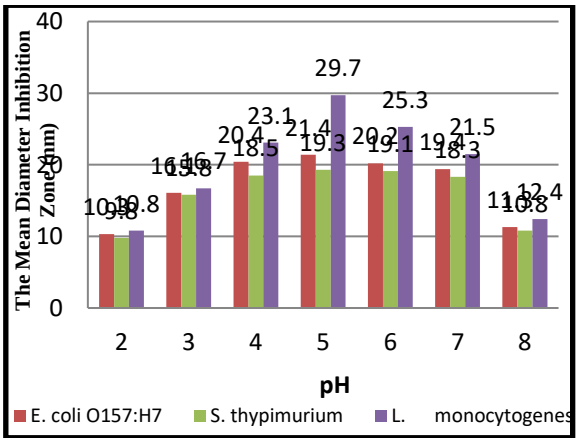

Figure 3. Effect of pH antimicrobial activity against isolates *Pediococcus pentosaceus* strain N6 on bacterial pathogens

The mean of diameter inhibition zone the most widely formed at pH 5 of 29.7 mm against *L. monocytogenes*, 21.4 mm against *E. coli O157:H7* and 19.3 mm against the bacterium *S.* thyphimurium. The diameter inhibition zone of the lowest was produced at pH 2 and 8 with the average diameter inhibition zone of 10.8 mm and 12.4 mm against *L. monocytogenes*, 10.3 mm and 11.3 mm against the bacteria *E. coli O157: H7* and 9.8 mm and 10.8 mm against *S.* thyphimurium. This indicates that the antimicrobial activity resulting from isolates *pediococcus pentosaceus* strain N6 more active at pH 5 is optimum pH isolates of growth.

The antimicrobial activity of isolates *Pediococcus pentosaceus* strain N6 was higher against *L. monocytogenes* compared to other bacterial pathogens because the isolates in addition to producing organic acids (lactic acid and acetic acid) also produce bacteriocins which Pediocin N6, where these bacteriocins have properties are more active against gram positive because it has same kinship with the producing bacteria. The according to Wu *et al.*, (2004) and Sin *et al.*, (2008), the *Pediococcus pentosaceus* is including homofermentative group. These bacteria produce lactic acid and acetic acid with fermenting pentose sugars in addition to produce bacteriocins.

According to De Vuyst and Vandamme (1994), bacteriocins are protein compounds, therefore synthesized via ribosomal protein biosynthesis mechanism involving transcription and translation. Bacteriocin was encoded by chromosome DNA and the plasmid although. Bacteriocins is produced LAB biologically active bactericidal effect, especially against Gram-positive and closely related to the producing bacterial species. According to Sin *et al.*, (2008), Pediocin K23-2 was produced by *Pediococcus pentosaceus* K23-2 includeing class IIa bacteriocins have higher antimicrobial activity against Gram-positive bacteria such as *L. monocytogenes*.

Optimization of culture conditions against Pediocin N6 activity:

Modification of carbon and nitrogen sources on Pediocin N6 production

Carbon sources: The results of study show mean diameter inhibition zone can be seen in Figure 4.

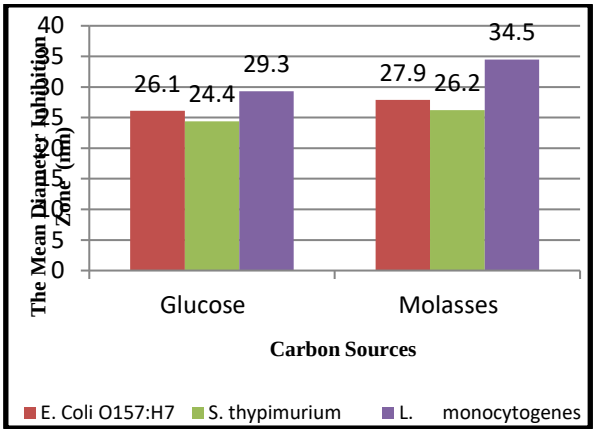

Figure 4: Effect of carbon sources of antimicrobial activity against isolates *Pediococcus pentosaceus* strain N6

The use molasses of carbon source to produce the most widely diameter inhibition zone than glucose. The resulting of diameter inhibition zone from the use molasses carbon source of 34.5 mm against *L. monocytogenes*, 27.9 mm against *E. coli O157: H7* and 26.2 mm against *S.* thyphimurium. While the carbon source glucose produces diameter inhibition zone of 29.3 mm against *L. monocytogenes*, 26.1 mm against *E. coli O157: H7* and 24.4 mm against *S.* thyphimurium.

This is showing the use molasses of carbon source to produce the most widely inhibition zone because molasses composition consists two simple sugars that is glucose and fructose which can be directly used bacteria as a carbon source. According to Curtin, (1983), molasses containing about 46% total sugar, 3.0% crude protein, 0.0% fat dengan79,5 ° brix. Molasses is widely used for production of alcohol through fermentation. The next according to Mariam *et al.*, (2009), the molasses contains several components sucrose, glucose and fructose with a total carbohydrate concentration of 45-60%. According to Mintian *et al.*, (2005); Richter and Berthold (1998), reported that the 80.7, 71 and 89% total sugar from medium used to produce lactic acid during the fermentation process. Utilization of sugar from molasses on the fermentation process is very important. According to John *et al.*, (2009), sucrose in the molasses that can be used directly as a carbon source and converted into energy source for the growth of lactic acid bacteria without solving process is previously.

Glucose is a monosaccharide, or simple sugar that consists only one component simple sugars. According to Armstrong (1995), Glucose is also called dextrose constitute organic compounds most relevant in nature and constitute aldohexoses containing four asymmetric carbon. The main function of glucose is source energy in living cells. According to Ray (2004), hexose sugars (glucose) will be metabolized by LAB is homofermentative through the glycolytic pathway or pathways Emden Meyerhoff Parnas (EMP) using 2 molecules of ATP and enzyme fructose diphosphate-aldolase for convert glucose to fructose 1,6-diphosphate. Hydrolysis these molecules produce two molecules that three carbon compounds. As a result of reaction, the dehydrogenation (to produce NADH + H + from NAD), the phosphorylation reaction and produces two molecules of ATP are formed pyruvate the next converted to pyruvate. Pyruvic acid will then be converted into lactic acid through the activity of lactic acid dehydrogenase. Based on the glycolysis process glucose carbon sources will be overhauled first into fructose component so that can used by the microbial cells as an energy source for growth. According to research Khay *et al*. (2013), the optimization of production bacteriocin by *Enterococcus durans* E204 isolated from Camel Milk of Morocco the used carbon source to glucose 3% the result bacteriocin activity at hight such as of 640 AU/ml against Listeria inocua CECT 4030 in medium MRS broth the pH 5.5 and 6.0. Meera *et al*., (2012) optimize production bacteriocin by probiotik BAL the used carbon source to result bacteriocin activity and the highest such as 12800 AU/ml at pH 6 and 1300 AU/ml at 30^0C by the gastro intestinal entero pathogens.

Nitrogen sources: The results of study after isolates *Pediococcus pentosaceus* strain N6 were grow with a modified nitrogen source can be seen in Figure 5.

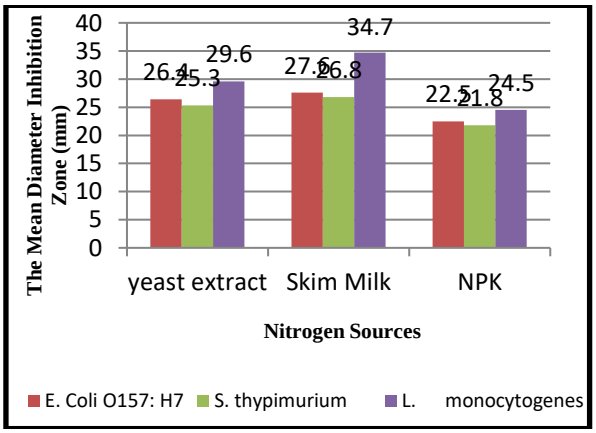

Figure 5: Effect of nitrogen sources antimicrobial activity against isolates *Pediococcus pentosaceus* strain N6

The resulting supernatant was tested antimicrobial activity against pathogenic bacteria. The results were showed that the use nitrogen source of skim milk to produce the most diameter inhibition zone compared to the use of yeast extr-

act and NPK. Diameter inhibition zone was produced nitrogen source skim milk of 34.7 mm against the bacterium *L. monocytogenes*, 27.6 mm against *E. coli O157: H7* and 26.8 mm against *S.* thyphimurium. While the nitrogen source yeast extract resulted diameter inhibition zone of 29.6 mm against the *bacterium L. monocytogenes*, 26.4 mm against *E. coli O157: H7* and 25.3 mm against *S.* thyphimurium. Nitrogen Sources of NPK produce diameter inhibition zone of 24.5 mm against the bacterium *L. monocytogenes*, 22.5 mm against *E. coli O157: H7* and 21.8 mm against *S.* thyphimum.

This is showing the use skim milk of nitrogen source to produce the most widely diameter inhibition zone the most extensive because the composition of skim milk contains milk protein is casein and lactose. Protein is a source nitrogen for build body's cells, while lactose is a source energy and carbon that can boost the growth of bacteria. The process of metabolism to growth, first isolate *Pediococcus pentosaceus* strain N6 use lactose as a source energy to grow because a simple sugar that directly can be used followed using casein from skim milk protein for build up the body's cells. According to Buckle (1987), skim milk is part of milk that remains after the cream to taken partially or entirely. Skim milk contains nutrients from milk except fat and fat-soluble vitamins. According to Pop *et al*., (2014), growth kefir in the medium skim milk increased in the first 24 hours, and then decreased with increasing that acid content and decrease the nutrient content in medium. The next according to Wang and Bi (2008), nitrogen source is not too increase growth, but the growth kefir increases when casein was added to the medium followed with the addition of peptone, Tryptone, yeast extract and yeast powder. According to Wang *et al.,* (2010), the modification culture component production bacteriocin by isolated from gut of poultry that used nitrogen source lactose combination with beef extract and soy peptone decrease bacteriocin activity of 36% (16.8 mm) against bacteria gram negative and positive such as *Micrococcus plavus* and *Candida mycoderma*.

Production Pediocin N6 liquid laboratory scale in 400 and 800 ml MRS broth with a combination of carbon and nitrogen sources

Production of liquid Pediocin N6 and antimicrobial activities

The results of study presented in Table 1, indicates that antimicrobial activity Pediocin N6 liquid produced in laboratory scale 400 ml and 800 ml MRS broth with the addition of molasses and skim milk to produce diameter inhibition zone of 35.2 mm against *L. monocytogenes*, 28.5 mm against *E. coli O157: H7* and 26.7 mm against *S.* thyphimurium. The antimicrobial activity Pediocin N6 liquid produced in 400 and 800 ml MRS broth medium can be seen in Table 1.

Table 1: The Step Pediocin N6 Liquid Production and Antimicrobial Activities

Pediocin N6 Production in (medium MRS broth)	Production Medium	The mean diameter inhibition zone (mm)		
		E. coli O157: H7	*S. thyphimurium*.	*L. monocytogenes*
400 ml	Erlenmeyer 500 ml	28.5	26.7	35.2
800 ml	Erlenmeyer 1 Liter	28.5	26.7	35.2

These results indicate the production liquid Pediocin N6 both in 400 ml and 800 ml MRS has the same antimicrobial activity or do not decreesed, meaning Pediocin N6 has stabilized antimicrobial activity. This is caused the conditions in producing liquid Pediocin N6 comparison culture isolates *Pediococcus pentosaceus* strain N6 with MRS broth medium along temperature and pH are used same, so it does not affect the antimicrobial activity Pediocin N6 liquid at each stage of production.

Conclusions

Isolates cell growth and production of bacteriocins optimum occurs on later phases logarithms that hour of 20th until the end exponential phase is the 36th hour. Antimicrobial Activity isolate *Pediococcus pentosaceus* strain N6 optimum is achieved at 50^{o}C with a diameter inhibition zone of 29.6 mm against *Listeria monocytogenes*, *E. coli O157: H7* by 20.3 mm and 18.4 mm *S. thyphimurium*. pH optimum antimicrobial activity isolate *Pediococcus pentosaceus* strain N6 is pH 5 with a diameter inhibition zone of 29.7 mm against *Listeria monocytogenes*, 21.4 mm against the *E. coli O157: H7* and 19.3 mm against the *S. thyphimurium*. Modification molasses of carbon source to produce diameter inhibition zone of 34.5 mm against the *L. monocytogenes*, 27.9 mm against *E. coli O157: H7* and 26.2 mm against *S. thyphimurium* and skim milk of nitrogen sources to produce diameter inhibition zone of 34,7 mm against the *L. monocytogenes*, 27.6 mm against *E. coli O157: H7* and 26.8 mm against *S. thyphimurium*. Pediocin N6 production in 400 and 800 ml MRS broth was resulted diameter inhibition zone of 35.2 mm against *L. monocytogenes*, 28.5

mm against *E. coli O157: H7* and 26.7 mm against *S.thyphimurium*.

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