Effect Of *Bifidobacterium bifidum* Addition To Protease Activity Of Psychrophile Bacteria In Yoghurt During Storage

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ABSTRACT

Yoghurt is a fermented milk product using lactid acid bacteria (LAB), therefore it will produce milk product that has aroma, flavor, and distinctive texture. Yoghurt often damaged due to protease activity produced by psychrophile bacteria. One preventive way to restrain the damage is by adding *Bifidobacterium bifidum* to yoghurt. The aims of the research were to know the effect of *B. bifidum* addition with different concentration to psychrophile bacteria protease activity during storage and to know the best concentration of *B. bifidum* addition in reducing protease activity of psychrophile bacteria in yoghurt. The main parameter observed was the number of tyrosine production in U/ml. Protease activity was measured with modification of Lowry method, measurement was done every 5 days interval for 30 days storage. The result showed the average of protease activity controlling of psychrophile bacteria in yoghurt with starter formula 1:1:0, 1:1:0.5, 1:1:1, 1:1:1.5 on 5th day storage were 7.02 U/ml; 6.23 U/ml; 4.48 U/ml and 4.07 U/ml. The protease activity in yoghurt was decreased on 10th day storage, and the datas with starter formula 1:10.5, 1:1:1 and 1:1:1.5 were 5.80 U/ml; 3.91 U/ml and 2.10 U/ml, meanwhile the control treatment (1:1:0) was decreased on 15th day with 4.64 U/ml. Based on the result of analysis of variance (ANOVA), starter concentration and length of storage treatment showed non-significant result.

Keywords: *B. bifidum,* psychrophile bacteria, yoghurt, protease activity.

Introduction

Yoghurt is a fermented milk product using lactid acid bacteria, therefore it will produce milk product that has aroma, flavor, and distinctive texture. The main materials used in making of yoghurt is fresh milk pasteurized. Raw yoghurt despite having undergone fist processing, but in reality it is easy damaged so it can't be consumed for a long time.

Damage of yoghurt can be caused by several factors, including is lack of heating process or homogenization of milk, to low a temperature of incubation, absence of materials stabilizer and contaminated by microorganisms. According to Uyar et al., (2011) during storage in under low temperature, milk was undergoes damage because proteinase and lipase produced by psychrophile bacteria. One of the most dominant psychrophile bacteria that damage milk pasteurization is *Pseudomonas flourescens.* Damage of milk product by *P. flourescens* caused by protease activity produced, resulting in changes in biochemistry and microbiology on volatile compounds and milk protein (Reinheimer et al., 1993 in Khusniati and Nurmalia, 2010). One species of lactid acid bacteria that have the effect bactericidal and was able to suppress growth of psychrophile bacteria is *Bifidobacterium bifidum.*

The aims of the research were to know the effect of increasing *B. bifidum* culture with concentration ratio on protease activity psychrophile bacteria during storage and to know concentration of *B. bifidum* culture which has highest capability for reducing protease activity of psychrophile bacteria in yoghurt. The results of the research expected can be benefit to provide scientific information about the ability of *B. bifidum* and concentration culture of *B. bifidum* in inhibits protease activity psychrophile bacteria in yoghurt.

Method

Making of inoculum *S. thermophillus, L. bulgaricus and B. bifidum* (Whardani, 2004) Breeding of pure cultures of *S.thermophillus, L. bulgaricus* and *B. bifidum* in MRS agar slant medium inoculated into 9 ml of 1% pepton solution, then incubated for 24 hours. After that, do the OD measurement using spectrophotometer with

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wavelength of 600 nm to its absorbance value of 0.5 is obtained.

Making a Starter Culture (Koroleva, 1991 in Akmar, 2006). Making a starter culture of *S. thermophillus*, *L. bulgaricus*, and *B. bifidum* comprises 3 stages, namely making induk culture, feeds and bulk starter.

Making Yogurt (modification of Kosokowski, 1985 in Ramadzanti, 2006). The making of yoghurt made with taking pure milk 500 ml into bottle. Then added 50 gr (10%) sugar and heated up to 90 °C temperature for 30 minutes. After that, refrigerant to 45 °C temperature. Starter with a variety of concentration added into milk has been pasteurized with comparison of different starter concentration that 1:1:0, 1:1:0.5, 1:1:1 and 1:1:1.5 as much as 3%., then shuffled several times. Milk has been given starter incubated at 45 °C temperature for 6 hours until formed yoghurt. Yoghurt stored at 4 °C temperature in refrigerator for 30 days.

Measurement of The Protease Activity Yoghurt with Lowry Method (Akmar, 2006)

Protease activity measurement is done every 5 days for 30 days of storage. Protease activity assay done with taking 10 ml yoghurt has been given various treatment concentrations, put in eppendorf, then centrifuged samples with a speed of 10.000 rpm for 10 minutes at 4°C temperature. Supernatant (Crude extract protease enzyme) is taken and put in test tube, stored at 20 °C temperature.

Supernatant taken 0.5 ml into test tubes, added 0.5 ml buffer tris-HCL 0.05 M pH 7 and vortex then incubation at 37°C temperature for 5 minutes. 0.5 ml casein substrate 2% in 0.05 M phosphate buffer pH 7 was added to supernatant and incubated for 10 minutes at 37°C, then added TCA 0.4 M 1 ml. vortex and centrifuged, then from 0.5 ml supernatant then added 2.5 ml 0.5 M Na_2CO_3, homogenize and incubated at 37 °C for 5 minutes. Added 0,5 ml folin ciocalteu reagent, homogenize and incubated at 37°C for 30 minutes, then its absorbance was measured by using spectrophotometry at wavelength of 660 nm. The use of blanko of any testing using the same procedure, but addition of TCA 0.4 M done before addition of substrate casein.

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Calculation of enzyme activity as follows:

$$\text{Protease Activity (U/ml)} = \frac{(X \times 1000 \times V)}{(p \times q) \times fp}$$

Description:
X = concentration of samples from regression equation with absorban as the value of Y
Fp = Dilution factor
V = Total volume of sample in each tube
1000 = conversion factor (mM to M)
q = Incubation time
p = Enzyme volume (ml)

Measuring content of lactd acid (AOAC, 1995). Measuring lactic acid levels were measured every 5 days for 30 days of storage. Yogurt from each sample was taken 10 ml into 250 ml Erlenmeyer flask, then added 90 ml aquades for titrated using NaOH 1 N. color indicator that use is fenolptalein 1% with color change to pink. According to the requirement of quality yoghurt on SNI 01-2981-1992, The amount of lactid acid is 0.5-2 %. Total cconcentration of lactid acid titration calculated as percent lactid acid. Lactid acid content are calculated use following formula:

$$\%\ \text{lactid acid} = \frac{\text{Vol NaOH} \times \text{N NaOH} \times \text{Mr}}{\text{Sample volume (ml)} \times 1000}$$

Description:

Vol	: Volume of NaOH NaOH used (mL)
N NaOH	: NaOH Concentration: (N)
Mr. lactid acid	: 90 g/equivalent
V sample	: sample Volume (mL) were analyzed

The calculation of the amount lactic acid bacteria and psychrophile bacteria (Hidayat et al., 2013), The calculation of amount lactic acid bacteria begins with yoghurt sample was diluted into sterile aquades with in comparison 1: 9. Yogurt samples taken 1 ml and diluted into 9 ml sterile aquades until dilution of 10^{-6}. On the dilution of 10-5 and 10-6 platting duplo in pour plate (PP) to MRSA and platting mono in PP to the King's B medium, next incubated at temperatures 37°C for 48 hours. To calculate the number of BAL and psychrophile bacteria that grow used method of Total Plate Count (TPC) with the formula:

$$\text{CFU's/ml} = \text{total colony} \times 1/\text{Fp} \times \text{PP}$$

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Results

Results of examination protease activity psychrophile bacteria average crude yoghurt extract with variety of starter concentration measured every 5 days storage time lapse is follows: yoghurt with starter formula 1:1:1.5 (A3) protease activity of equivalent value is the lowest, i.e. amounting to 0.08 U/ml on day 15; The average activity of protease produced by yoghurt tallest with formula 1: 1: 0 (A0) of 7,18 U/ml on day 10, more data can be seen in Figure 1.

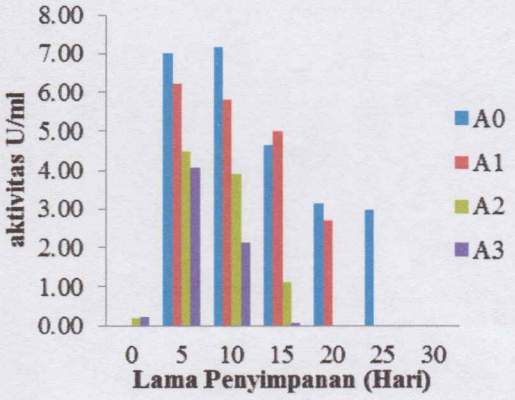


Figure 1. Protease Activity Of Psychotrophic Bacteria Average Crude Extracts Yogurt During Storage.

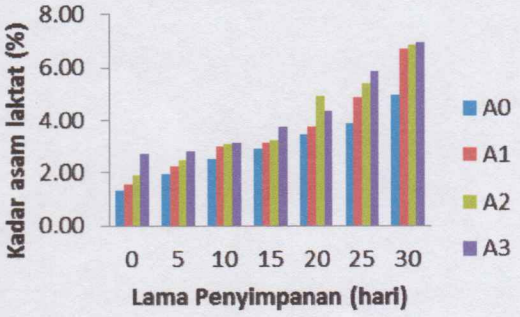


Figure 2. Lactic Acid Content Average Concentration range of Yogurt Starter saved for 30 days

The lowest content of lactic acid starter formula is obtained at 1: 1: 0 (A0) is 4.95%, lactic acid content on average the highest obtained at the yogurt with formula 1: 1: 1.5, namely of 6,93%.

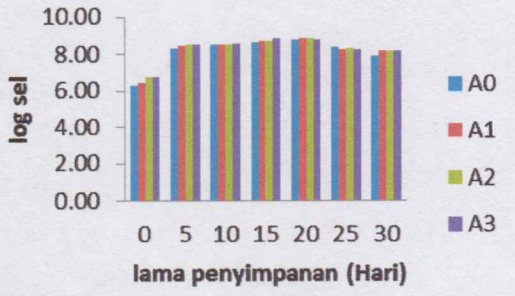
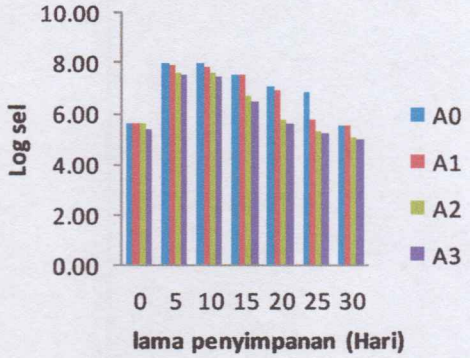


Figure 3. Total Average Lactic acid bacteria Yoghurt range Starter Concentrations that stored for 30 days.



Yogurt-control (A0) has a total of lactic acid bacteria is the lowest of 1.9 x 106 CFU/ml. Total lactic acid bacteria high generated by a formula with yogurt starter 1: 1: 1.5 (A3) which is equal to 7.8 x 10^8 CFU/ml.

Figure 4. Total Average Psychrophile Bacteria Yoghurt range Starter Concentrations that stored for 30 days.

Description

A0: 3% Starter *S. thermophillus*: 3% *L. bulgaricus*: 0% *B. bifidum* (1: 1: 0)

A1: 3% Starter *S.thermophillus*: 3% *L. bulgaricus*: 1.5% *B. bifidum* (1: 1: 0.5)

A2: 3% Starter *S. thermophillus*: 3% *L. bulgaricus*: 3% *B. bifidum* (1: 1: 1)

A3: 3% Starter *S. thermophillus*: 3% *L. bulgaricus*: 4.5% *B. bifidum* (1: 1: 1.5)

Yogurt starter formula 1: 1: 0 (A0) had a total average of psychrophile bacteria highest equal is to 9.8 x 10^7 CFU/ml on day 10. Total average psychrophile bacteria lowest generated by yogurt with starter formula 1: 1: 1.5 (A3) equal is to 1x10^5 CFU/ml on day 30.

Discussion

Based on ANOVA table. be aware that treatment of day (T), concentration (A) and combination of non significant (ns), meaning that the treatment effect is not real against protease activity psychrophile bacterial is effect is small. Based on the results of the

calculation protease activity in each treatment (Figure 1) can be known that yogurt with formula starter 1: 1: 0 (A0) and 1: 1: 0.5 (A1) on protease activity of storage 0 zero, whereas the yoghurt with the starter formula 1: 1: 1 (A2) and 1: 1: 1.5 (A3) has a value of protease activity an average higher than A0 and A1 is equal to 0,19 Units/ml and 0.24 Units/ml. it is caused by several possibilities among others the first, BAL contained in yoghurt A0 and A1 is still utilizing acid amino in substrates for growth, so yet protease activity consequently zero. According to Chotimah (2009), at the beginning of the growth of *L. bulgaricus*, *S. thermophillus* and *B. bifidum* using acid-free amino acids contained in milk, after these amino acids in the media up, BAL recently will synthesize proteases to hydrolyzed protein into amino acids needed by BAL to its growth.

Both of these amino acids contained in the substrate has exhausted used by BAL on yoghurt A2 and A3 totaling more than A0 and A1 (Figure 3.). Therefore, BAL on yoghurt A2 and A3 protease yield faster than A0 and A1. This is in accordance with statement Muholland (1994) that the greater number of BAL in the yogurt, then a growing number of protease produced by BAL to hydrolized protein into amino acids. Widodo (2003) Stating that lactic acid bacteria able to degrade casein into amino acids through a complex system of proteolytic enzyme protease, by involving the process takes place after all peptides and free amino acids in the milk consumed by bacterial cells.

Protease activity yogurt with starter formula 1: 1: 0, 1: 1: 0.5, 1: 1: 1 and 1: 1: 1.5 stored for 5 days has increased to 7.02 U/ml; 6,23 U/ml; 4,48 U/ml U/ml and 4.07. Increased protease activity that occur due to increasing number of BAL (Figure 3.) and psychrophile bacterial (*P. flourescens*) (Figure 4.). It shows that addition of *B. bifidum* in yogurt are kept day 5 hasn't been able to inhibit the growth of *P. flourescens*, whereas two bacterial are still able to produce proteases, as a results protease activities increased. The increase number of *B. bifidum* in yogurt because *B. bifidum* has entered a phase of logarithmic, so not yet produce antibacterial which are bacteriostatic or bactericidal as a result the number of cells of *P. flourescens* is still growing. According to Neneng (2008) that the antibacterial compounds will be produced by cells of microorganisms on the condition of nutritional deficiencies in the substrate and its growth has entered a stationary phase.

Protease activity yogurt with starter formula 1: 1: 0.5 , 1: 1: 1 and 1: 1: 1.5 suffered a loss on day 10 respectively become 5.80 U/ml; 3,91 U/ml; 2.10 U/ml. Yogurt control decline on 15th day of 4.64 U/ml. Protease activity decreased due to the reduced number of *P. flourescens* (Figure 4.) and amount of BAL that remains constant (Figure 3.). Reduced amount of *P. flourescens* cells due inhibition by *B. bifidum* whereas *P. flourescens* incapable of producing protease activity has decreased result. Amount of *B. bifidum* which remain constant in yogurt because of *B. bifidum* in stationary phase, and the bacteria started producing antibacterial substances so that his amount of *P. flourescens* cell declined. Ramazdianti (2006) Stating that *B. bifidum* will result in antibacterial (bifidin) at the time of stationary phase, which can inhibit the growth of *P. flourescens*.

Akmar (2006) in the results of his research indicate that *B. bifidum* has antagonism with *P. flourescens*, in concentrations of 2%. It is also supported by the results of research Arun (2013) that yoghurt with addition of Bifidobacetrium BBIV have antibacterial activity against *Salmonella typhi* because Bifidobacterium BBIV produce lactic acid and bakteriosin which can inhibit the growth of other bacteria. The inhibition was also effected by several factors, namely the storage and concentration of old starter is added in yogurt. The mechanism of inhibition of p. flourescens by bifidin according to Nakazawa and Hasono (1992) is through destruction of bacterial cell membrane structure by way of increasing membrane permeability. Asriayani et al. (2007) suggested that increasin permeability of cell membrane causing membrane pores become larger, whereas it easier for the influx into the cell antibacterial components and discharge of substance cells. discharge of substance cells like proteins and nucleic acids can lead to bacterial cell lysis that lead to death (Yudhisthira et al., 2011).

Decrease in number of *P. flourescens* cells on day 10th was also caused by the content of lactic acid yogurt that is higher. The longer storage the high content of lactic acid in yogurt are produced. Content of lactic acid in yogurt is high causing the pH of yogurt declined. This is in accordance with statement of Amin and Lekson (2004) lactic acid that have the effect of bakteriostal associated with decrease pH

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environment into 3-4.5 so that the growth of bacteria other including contaminant will be hampered. Decrease pH of yogurt also can affect the work of enzyme protease which causes activity to be hampered. Kusniawati (2004) Stating that pH greatly affect enzyme activity, since pH changes will alter the tertiary casein which serves as a substrate for the enzyme. Changes in structure of substrate occurs resulting in enzyme cannot be glued exactly in substrate active side, whereas the enzyme will work hampered. Protease produced by *P. flourecens* will work in optimum conditions of pH around 5-7, when the condition substrate pH is lower than optimum pH, it will cause denaturation protease and protease activity resulted in loss of (Doyle, 2007).

Conclusion

Based on the results and discussion description, then it can be concluded that: the addition of *B. bifidum* culture on yoghurt with comparison of different starter concentration effect protease activity *P. flourescens*, concentration of *B. bifidum* starter on ratio 1:1:1.5 is able to decrese of protease activity fastest and have protease activity is low. Suggestions for further research that is needed to use the addition of *B. bifidum* with concentrations greater than 4.5% so that yogurt can be consumed in a long time period, and need for antagonism test *P. flourescens* with yogurt *B. bifidum* neutralized in order to know that inhibition of *P. flourescens* caused only by bifidin not because of other factors.

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