Immobilization of *Aeromonas bivalvium* PT2 Cells with Alginate and Measurement of Chitinolytic Activities

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Abstract. *Aeromonas bivalvium* is one of the chitinolytic bacteria that able to degrade chitin into its derivatives. These bacteria can only be used once during the fermentation process, which is less profitable to be applied in industrial scale. This limitation can be solved by bacterial immobilization method. This study aimed to determine the effect of bacterial cell immobilization on chitinolytic activity and to determine the stability of the immobilized bacteria during repeated usage. Bacterial cell immobilization was carried out by entrapment method with 1% sodium alginate matrix. Immobilized bacteria was cultured in two different mediums, namely nutrient broth (NB) and nutrient broth (NB) added with colloidal chitin (NB + K). Tests for chitinolytic activity were carried out in bacteria. In addition, the stability of immobilized bacteria was also tested for chitinolytic activity with repeated removal and use. The result shows that the effectiveness of immobilization on average is 91.8%. Immobilization did not significantly affect chitinolytic activity when compared with bacteria without immobilization. Immobilized bacteria in this study has similar performance as bacteria without immobilization. The results of the stability tests including chitinase activity and NAG released indicated a significant decline during repeated usage with maximum usage of three times.

1 Introduction

Chitin is a group of polysaccharides composed of β -1,4-N-acetyl-D-glucosamine monomers [1]. Chitin is composed of polymeric chain N-acetyl-glucosamine which is bound by a hydrogen bond between the N-H group of one chain and the C=O group from the adjacent chain and forms microfibrils. One of the properties of chitin is insoluble in water due to the presence of hydrogen bonds. Chitin becomes an organic waste when accumulates in nature [6]. Chitin monomers are obtained from the chitinolytic mechanism by hydrolyzing β -1,4-glycoside bonds which can be carried out by the aid of microorganisms such as bacteria [7] [14].

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Chitinases is produced by bacteria with fermentation, but bacteria can only be used in one process. Meanwhile, if in industrial scale it give less profitable, so that a method is needed for some bacteria can be used repeatedly in fermentation medium. One technique can be use is bacterial immobilization. Bacterial immobilization made by maintain microorganisms or certain compounds in a matrix. The treatment for immobilization can affect the performance of bacteria in producing enzymes [4]. Bacterial immobilization can make bacteria more resistant with temperature, pH and certain physical treatments [11]. The immobilization method has been investigated varies, including entrapment (capture), carrier binding, containment (attachment), attachment (attachment), adsorption technique, self aggregation and cell coating[12].

Chitinolytic bacteria (*Aeromonas bivalvium*) used for cell immobilization in this study. In previous study, *Aeromonas bivalvium* reported have good ability to degrade chitin and its almost equivalent with genera from *Serratia*, *Pasteurella*, *Corynebacterium*, *Lactobacillus* and *Nocardia*[9]. Immobilization in this study using entrapment method (catching) and the matrix material using sodium alginate. According by previous study, tahtusing a matrix based on sodium alginate showed a viable count of bacterial cells higher than other matrix materials such as the k-carrageenan and polyester groups[15]. After bacteria are immobilized, the next step is activity test to see how immobilization effect on chitomonolytic activity of *Aeromonas bivalvium* and stability test. The purpose of this study was to determine the effect of bacterial cell immobilization on chitinolytic activity of *Aeromonas bivalvium* (PT2) and find out the stability of immobilized bacteria in repeated usage.

2 Materials and methods

2.1 Preparation

2.2.1 Chitin medium

Chitin medium was made with refers to the modified Hsu & Lockwood (1974) method by removing FeSO₄. In this study using chitin agar and chitin liquid. Chitin medium was made bydissolving KH₂PO₄ (0.03%), K₂HPO₄ (0.07%), MgSO₄.5H₂O (0.05%), ZnSO₄ (0.0001%), MnCl₂ (0.0001%), colloidal chitin (2%), yeast and bacto agar (2%) into distilled water and sterilized at 121°C for 20 minutes. Liquid chitin was made by same materials without the addition of bacto agar. The chitin medium was used for preparing bacterial inoculums.

2.2.2 Bacterial growth curve

Bacterial growth curvewasmade to determine the exponential phase of bacteria before immobilization is carried out. The growth curve was made by entering as much as 0.1% stock of isolates in 25 ml of medium Nutrient Broth (NB) with two replications. The incubation time inthis study was 72 hours at 37°C. The next step is every 12 hours, bacteria was taken 1 ml of the bacterial culture incubated to see the absorbance. Bacteria are measured with spectrophotometer at wavelength 600 nm. The growth curve was determined by a plot between the incubation time and Optical Density (OD) with Microsoft Excel [17].

2.2.3 Bacterial inoculum

Bacterial inoculum methods is conducted [17]. The bacterial inoculum was made by two stages consisting of a refreshment and streak. The refreshment phase was carried out by

taking one stock culture (glycerol stock culture) which was previously stored at -23°C and then inoculated on 7 ml of liquid chitin medium. The bacterial inoculum incubated for 2 days at 37 °C at waterbath shaker (100 rpm). Streaking stage was carried out by one ose from bacterial culture (liquid chitin) and then it scratching at ± 15 ml of chitin agar and incubating for 2 days at room temperature. After that, one ose of single colony was put into 5 ml Nutrient Broth (NB) medium and incubated for 24 hours at room temperature. The next step is as much as 2% of bacteria from work culture was grown in 10 ml of Nutrient Broth (NB) and 10 ml of Broth Nutrient added colloidal chitin 1% (NB + K). After that, incubation time was carried out for 48 hours with waterbath shaker (100 rpm) at 37°C. Meanwhile the parameters was tested by number of bacteria using the Total Plate Count (TPC).

Number of bacteria was calculated by the indirect method of Total Plate Count (TPC). The purpose of this method was determined by number of bacterial colonies in *Aeromonas bivalvium* samples at the stage before the bacteria are immobilized. The results of the TPC show that the number of bacteria in the two different mediums has almost the same amount. Bacterial isolates was cultured in Nutrient Broth (NB) and Nutrient Broth (NB) medium added choloidal chitin1% (NB + K) and from this stage was taken about 100 µl from the bacterial inoculum to be diluted. After that, 4 ml of each treatment was used later for the immobilization method.

2.2 Cell immobilization

The immobilization method in this study refers to the method of Mahbubillah and Shovitri which has been modified[11]. Bacterial cultures that will be immobilized using two medium, namely Nutrient Broth (NB) and Nutrient Broth plus colloidal chitin 1% (NB + K). The reason of using chitin in the NB medium treatment was stimulated by the bacteria to degrade chitin from inside the beads. Besides that bacteria in beads can adapt faster when inserted into the fermentation medium. Before being immobilized, bacterial cultures were incubated at 48 hour before it moved to Nutrient Broth (NB) and Nutrient Broth added 1% (NB + K). After that, every 1 ml bacterial culture was absorbed at a wavelength of 600 nm using a disposable cuvet. The application of absorbance for calculate the effectiveness as X1.

Cultures were taken from each treatment and used for bacterial immobilization. Four mililiters of bacterial culture were added 1% sodium alginate powder. That solution began to be printed using 5 cc syringe by dropping it into 4 ml of CaCl₂ solution with a concentration of 3% to form beads. Beads with 2-3 mm size have been deposited were washed with 4 ml of 0.85% NaCl solution and filtered. All treatments at this stage were carried out sterile conditions. After that, it was placed in a sterile petri dish as a temporary storage container and beads was ready used for the effectiveness test, chitinase activity and stability test. On the other hand the remaining results of the beads were centrifuged and the pellets were taken to calculate the effectiveness test.

Chitinolytic activity of bacterial was seen by quantitatively tested which one is colorimetric method and tended to absorb it using a spectrophotometer. The chitinase activity test in this study was carried out with 3 replications. A total of 4 ml of the free bacteria was put in 25 ml of liquid chitin medium. On the other hand the bacteria that were immobilized with NB and NB + K treatment were also included in 25 ml of liquid chitin medium. The next step is that the three samples were incubated for 5 days in a shaker waterbath (100 rpm) at 37 °C and taken as much as 2 ml of samples in each treatment to be tested for their activity. After chitinase activity test, beads filtering was carried out which had previously been used for fermentation in liquid chitin medium for 5 days. The beads were then washed, filtered, reused for the fermentation process in the new liquid chitin medium and the stability test was carried out. The stability test was carried out after the beads were incubated in liquid chitin medium for 24 hours. Washing, filtering and reuse treatment in the beads is done 3 times.

2.3 Test parameters

2.3.1 Total plate count (TPC)

The next step is from 100 µl of cultures were put into a microtube containing 900 µl of sterile distilled water as a 10⁻¹ dilution. After that 100 µl from another cultures were taken from 10⁻¹ sprays and it inserted into a microtube containing 900 µl of new sterile distilled water as a 10⁻² dilution. This treatment was repeated until dilutions of 10⁻⁸. After that, 100 µl of 10⁻⁷ and 10⁻⁸ dilutions were taken and grown in Triptone Soya Agar (TSA) medium at 37 ° C for 24 hours. After the bacterial colonies were grown on the TSA medium, then it calculated using a formula (BSN, 2006).

$$N = \frac{\Sigma C}{[(1 \times n1) + (0,1 \times n2)] \times (d)}$$

Information:

N: The number of product colonies, expressed in colonies per ml

ΣC: The number of colonies in all plates is calculated

n1: The number of plates in the first dilution is calculated

n2: The number of bowls in the second dilution is calculated

d: is the first dilution calculated

2.3.2 Effectiveness of bacterial immobilization

The effectiveness of bacterial immobilization was used to calculate the percentage of successful immobilization of NB and NB + K treatments. This method was adopted by the Wardoyo's(2014) research formula for calculating the percentage of enzyme immobilization. From that formula, some modifications were made to be applied to bacterial immobilization. The results of residual filtering solution from 4 ml of CaCl₂ solution and 4 ml of NiCl 0.85% solution were combined into the falcon tube with centrifugation (10,000 rpm). After that the supernatants in the falcon tube were removed until the remaining pellets were left. The pellets were added to sterile distilled water until it reach the same volume as the solution in the falcon before being centrifuged. After that, 1 ml solutions were taken and measured by measuring Optical Density (OD) with a wave of 600 nm. The results of the absorbance as X₂ were used to calculate the effectiveness of immobilization using the following formula.

$$E(\%) = \frac{X_1 - X_2}{X_1} \times 100 \%$$

Information :

E = Effectiveness of immobilization (%)

X₁ = Optical Density (OD) of bacteria before immobilization

X₂ = Optical Density (OD) remaining bacteria from the immobilization process

2.3.3 Test for chitinase activity immobilized and without immobilization

Observation of chitinase activity was carried out every 24 hours and 2 ml of each treatment was taken. The total volume of the medium were taken during the chitinase activity test each

treatment (12 ml) so the remaining medium volume was 13 ml. The 2 ml sample taken from each treatment was put into a microtube and centrifuged at 10,000 rpm for 2 minutes to obtain the supernatant [17]. The supernatant results of the reaction from the three samples were tested for chitinases activity with Reissig method [16]. The 250µl of supernatants reacting with 50µl potassium tetraborate pH 9.1 to bind the NAG were boiled for 3 minutes to accelerate the binding reaction of NAG. The samples were cooled and added 1.25 ml of DMAB reagent and then immediately was incubated in a waterbath shaker (100 rpm) for 30 minutes at 37 °C. Absorbance of samples and standard solutions of NAG (various concentrations) were measured by UV-Vis spectrophotometer at wavelength of 584 nm. The measurement of standard solution absorbance is done to obtain mathematical equations as a standard curve. The absorbance value of the samples were compared with the absorbance value of the standard NAG solutions. Standard curve was made by standard NAG solution at various concentrations (0; 10; 20; 30; 40; 50) (µg/ml). The absorbance value of the NAG standard solution was measured by using the same wavelength as the wavelength to measure the sample absorbance sample at a wavelength of 584 nm so that a mathematical equation was obtained as a standard curve. One unit of chitinase activity was defined as the amount of free enzyme as much as 1 µmol NAG / minute[15]. Calculation of chitinase activity was carried out by using the following formula.

$$\text{Chitinase activity } \left(\frac{\text{Unit}}{\text{ml}} \right) = \frac{\text{Concentration of NAG} \times \text{Dilution factor (FP)}}{\text{Molecular weight of NAG (221,21 g/mol)} \times \text{incubation time}}$$

2.3.4 Stability of immobilized bacteria

Stability test was carried out to know the stability of the bacteria for produce chitinases which is given a transfer treatment and repeated use. This stability testing refers to the modified Mahbubillah and Shovitri [11]. The displacement carried out in this study was 3 times and carried out three times. Immobilized bacteria that had been tested for chitinolytic activity were washed with 5 ml of 0.85% NaCl solution for 15 minutes and put in 25 ml of liquid chitin medium. The next step was incubated at room temperature with a constant rotation rotary shaker of 120 rpm for 24 hours. After incubation for 24 hours then a chitinase activity test was carried out again.

2.4 Data analysis

This study used descriptive and statistical methods. Descriptive methods are often used to analyze or describe a research result but are not used to make broader conclusions [19]. The chitinase activity test data and the immobilization stability obtained were then tested using a t-test at a 95% confidence level to see whether there were significant differences or not between immobilized bacteria and bacteria without immobilization. Data analysis was carried out by explaining the results of comparison of chitinase activity from immobilized bacteria and bacteria without immobilization and explaining its stability.

3 Results and discussion

3.1 Results

3.1.1 Bacterial inoculum

The results obtained that a single colony formed on liquid chitin medium and showed chitin degradation by *Aeromonas bivalvium*. Diameter of the clear zone of the isolate has a varying size of 7 to 22 mm. In the previous research these bacteria showed that the diameter of the clear zone was 8 mm [9]. The size of the clear zone formed depends on the amount of N-acetylglucosamine monomer and it produced from the hydrolysis process [13]. The single colonies *Aeromonas bivalvium* and clear zones around the colonies are shown in Figure 4.1. The isolate was used as work culture during the research process. On the other side of the single colony is taken to be grow in the NB medium for the immobilization process.

3.1.2 Bacterial growth curve

After the 12th to 36th hours the bacteria experience an exponential phase. The 36th to the 60th hours of the bacteria experiencing stationary phases which are characterized by bacterial growth tend to be stagnant. After the 60th hour the bacteria experience a phase of death. Based on these results it can be used as a reference and it determining the age of bacterial culture for the stage of immobilization. Age of bacterial culture with optimum growth is in the stationary phase (48th hour), in that phase it is assumed that bacteria will also produce optimum chitinase.

Fig. 1. *Aeromonas bivalvium* growth curve with an incubation time of 72 hours at 37°C

3.1.3 Calculation of the number of bacteria with TPC

Calculation of the number of bacteria using dilutions of 10-7 and 10-8 dilutions is due to the growing colonies ranging from 25-250 and included in the TPC calculation requirements [20]. The absorbance observations on NB + K mediums were 0.44 and the NB medium treatment was 0.43. The results of the calculation of the colonies of *Aeromonas bivalvium* bacteria on the treatment of NB medium were 33.7×10^8 CFU / ml and the treatment of chitin-containing Nutrient Broth (NB) plus (NB + K) medium was 31.8×10^8 CFU / ml. Based on the calculation of the bacterial colonies from the two different fermentation mediums it shows almost the same amount. The density of the bacteria was in accordance with the research for chitinase testing can use chitinolytic bacterial isolates with bacterial density of 10⁸ cells / ml [3]. The density of bacteria that will be used for immobilization in this study is also in accordance with previous study [15] who conducted immobilization using an initial bacterial culture of 10⁸ cells/ml.

3.1.4 Immobilization of *Aeromonas bivalvium*

Cells immobilization was carried out by entrapment method using 1% sodium alginate matrix and dripped in 3% CaCl₂ solution. The results of the Ca-alginate beads can be seen in Figure 2 where the resulting beads are bone white and measuring 2-3 mm, smooth and chewy in texture.

Fig. 2. Size of immobilized beads with 1% alginate matrix

3.1.5 Effectiveness of bacterial immobilization

Table 1. Effectiveness of immobilization of *Aeromonas bivalvium*

Informatio n	Repetitio n	X ₁	X ₂	Effectiveness (%)	$\bar{x}$ Effectiveness (%)
NB + K	I	0.821	0.000	100.0	88.6 ± 18.4
	II	0.181	0.059	67.4	
	III	0.338	0.005	98.5	
NB	I	0.311	0.001	99.7	95.0 ± 8.3
	II	0.441	0.000	100.0	
	III	0.565	0.083	85.3	
$\bar{x}$ Total					91.8

Effectiveness of immobilization is a method that can be used to see the percentage or success of immobilization in absorbing bacterial cells. Effectiveness was calculated based on the reduction of Optical Density (OD) of bacteria before immobilized with Optical Density (OD) the remaining bacteria from the immobilization process then multiplied by 100%. The results of the effectiveness calculation in Table 1 show that immobilization in Nutrient Broth (NB) medium with colloidal chitin has an average effectiveness of 88.6%, while immobilization in Nutrient Broth (NB) medium has an average effectiveness of 95%. The immobilization in the solid-chitin chitin (NB) Nutrient Broth (NB + K) tends to have a lower effectiveness compared to the immobilization treatment in the Nutrient Broth (NB) medium. The overall results of the effectiveness of bacterial cell immobilization showed a successful immobilization of 91.8%.

3.1.6 Chitinolytic bacterial activity immobilized and without immobilization

Based on the results of *Aeromonas bivalvium* chitinolytic activity in all three immobilization treatments, namely in Nutrient Broth (NB), Nutrient Broth (NB) added colloidal chitin (NB + K) and free cells are shown in Figure 3. Chitinase activity in the immobilization treatment (NB) increased from day 1 to day 2 respectively by 0,00005 U / ml and 0,000274 U / ml. The highest chitinase activity was treated on the second day of incubation. After that, on the 3rd day it decreased to 0.00003 U / ml. Chitinase activity began to increase on day 4 to 0.000073 U / ml and decreased on day 5 to 0.000014 U / ml. Chitinase activity in the treatment of

immobilization (NB) plus colloidal chitin (NB + K) increased from day 1 to day 2 respectively by 0,000029 U / ml and 0,000121 U / ml. After that, on the 3rd day it decreased to 0.00003 U / ml. Chitinase activity increased while the highest in the treatment was 0,00016 U / ml on the 4th day of incubation. After that it decreased on day 5 to 0,00014 U / ml. Chitinase activity in the treatment without immobilization increased from day 1 to day 2 respectively by 0.000059 U / ml and 0.00007 U / ml. The highest chitinase activity was treated on the second day of incubation. After that, on the 3rd day it decreased to 0.000004 U / ml. Chitinase activity began to increase on the 4th and 5th day respectively to 0.000008 U / ml and 0.00001 U / ml.

Fig. 3. Chitinase activity of *Aeromonas bivalvium* in liquid chitin medium and 37°C temperature

Fig. 4. NAG concentration of *Aeromonas bivalvium* in liquid chitin medium and 37°C temperature

Based on the results of *Aeromonas bivalvium* NAG concentration in all three immobilization treatments, namely in Nutrient Broth (NB), Nutrient Broth (NB) added colloidal chitin (NB + K) and free cell bacteria are shown in Figure 4. NAG concentration in the immobilization treatment (NB) decreased on day 1 by 2.25 µg / ml, then increased on day 2 by 3.86 µg/ml. NAG concentration decreased on day 3 to 1.74 µg/ml then increased on day 4 to 2.87 µg/ml and decreased on day 5 to 1.65 µg / ml. The NAG concentration in the colloidal plus chitin (NB) plus immobilization (NB + K) decreased on day 1 by 1.59 µg / ml, then increased on day 2 by 2.97 µg / ml. NAG concentration decreased on the 3rd and 4th

day respectively by 2.50 µg/ml and 1.74 µg/ml. After that, NAG concentration increased on day 5 to 1.75 µg/ml. NAG concentration in the treatment of free cell bacteria decreased on day 1 by 1.25 µg/ml, then increased on day 2 by 2.25 µg/ml. NAG concentration decreased on day 3 by 1.72 µg/ml. After that, there was an increase on the 4th and 5th day respectively 1.72 µg/ml and 1.80 µg/ml.

3.1.7 Stability of *Aeromonas bivalvium* immobilized

Stability was calculated based on chitinase activity and NAG concentration (Figure 5). Displacement is carried out every 24 hours, as many as 3 times. The treatment of immobilized bacteria in the Nutrient Broth medium (NB) on the first transfer had chitinase activity of 0.00005 U / ml, the second removal was 0.000024 U / ml and the third transfer was 0.000002 U / ml. The treatment of bacteria immobilized in Nutrient Broth (NB) plus colloidal chitin (NB + K) medium. The first transfer had chitinase activity of 0.000059 U / ml, the second removal was 0.000016 U / ml and the third transfer was 0.00000 U / ml. It is also seen from the resulting NAG concentration decreasing from the first transfer to the third transfer (Figure 6) Immobilized bacteria in the treatment of Nutrient Broth (NB) the first displacement of 1.59 µg / ml, the second removal of 1.14 µg/ml and the third transfer is 1.10 µg / ml. Immobilized bacteria in the Nutrient Broth (NB) treatment plus chitin (NB + K) in the first transfer amounted to 2.049 µg / ml, the second removal was 1.41 µg/ml and the third transfer was 1.24 µg / ml.

Fig. 5. *Aeromonas bivalvium* stability immobilized based on chitinolytic activity at 24 hour of incubation

Fig. 6. Stability of *Aeromonas bivalvium* immobilized based on NAG concentration in the 24 hour of incubation

3.1.7 Data analysis

The first paired sample t-test was compared between *Aeromonas bivalvium* without immobilization (N) and *Aeromonas bivalvium* immobilized in Nutrient Brtoth (NB) medium which showed a probability value or Sig. (2 tailed) of 0.656. The result of this value is greater than 0.05 so there is no significant difference between the two treatments. The second t-test test was comparing *Aeromonas bivalvium* without immobilization (N) and *Aeromonas bivalvium* immobilized in Nutrient Broth medium (NB) added colloidal chitin (NB + K) which showed a probability value or Sig. (2 tailed) of 0.096. The result of this value is greater than 0.05 so there is no significant difference between the two treatments. Based on the above output, it can be concluded that there is no significant difference between immobilized bacteria and bacteria without immobilization. Immobilized bacteria have almost the same performance as bacteria without being immobilized to degrade chitin. The paired sample t-test test results can be seen in Table 2 below.

Table 2. The results of chitinase activity testing using paired sample t-test with SPSS

Comparison		Paired Differences					T	Df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
1	N & NB	,0000196 7	,0001018 7	,0000415 9	-,00008724	,00012657	,473	5	,656
2	N & NB+K	,0000640 0	,0000765 2	,0000312 4	-,00001631	,00014431	2,049	5	,096

Note :
N = *Aeromonas bivalvium* without immobilization
NB = Immobilized *Aeromonas bivalvium* in NB medium
NB+K = Immobilized *Aeromonas bivalvium* in NB+K medium

3.2 Discussion

Lag phase is the phase where bacteria are still preparing and adjusting to grow in a new environment so that there is no significant amount of increase. After the 12th to 36th hours the bacteria experience an exponential phase, which is the phase in which cells undergo significant changes and chemical composition but there is no increase in the number of bacteria. The 36th to 60th hours of the bacteria experienced a stationary phase, namely bacterial growth tended to stagnate. After the 60th hour the bacteria decrease in number due to the available nutrients decreasing after being used for metabolism [10]. The growth of *Aeromonas bivalvium* is optimal or reaches the highest number at 48 hours. This is in accordance with the research which stated that this bacterium had optimum growth at 48 hours and was marked by changes in the color of the colony on selective Mac Conkey medium [5]. In addition, it is supported by previous research [18] which states that *Aeromonas* sp. experienced the highest growth at 48 hours at 1.21x10¹⁰ CFU / mL. According to Mangunwardoyo *et al.* (2009) bacterial growth of the *Aeromonas* genus, namely experiencing the stationary phase at the 48th hour. Then a study of the bacteria of the genus *Aeromonas* namely *Aeromonas hydrophila* which stated that the optimum incubation time was at the 48th hour. In this study *Aeromonas bivalvium* which will be immobilized is

in the stationary phase because in that phase bacterial growth has been optimal and assumed to be ready to be immobilized [2].

The density of bacteria to be immobilized was 33.7×10^8 CFU / ml (NB medium) with OD of 0.44 and 31.8×10^8 CFU / ml (medium NB + K) with OD of 0.43. The matrix material used is sodium alginate because it is safe and has no toxic properties. In addition, in a study conducted by previous study [15] using a matrix based on sodium alginate showed a viable count of bacterial cells higher than the matrix material from the k-carrageenan and polyester groups. The use of sodium alginate with a concentration of 1% is in accordance with the previous study which states that immobilization with this concentration has a reduction efficiency of 100% [11]. From the statement, it can be assumed that the smaller the concentration of sodium alginate used for cell immobilization, the performance of chitin reduction is the same as the bacteria that are not immobilized.

CaCl₂ solution (3%) was used to condense beads with the gelatinization process of sodium alginate. The gelatinization process occurs because it is the exchange of monovalent from sodium with divalent calcium cations which then reacts with monovalent carboxylic anions from alginate to form calcium (Figure 4.9). Examples of Ca-alginate beads in this study can be seen in Figure 4.3 where the resulting beads are bone white and are 2-3 mm in size and smooth and chewy in texture. The size of the beads is in accordance with the research conducted by previous study [11] namely the size of beads is not more than 6 mm, because if it exceeds this size the large volume of space can inhibit the process of nutrient diffusion. The beads size varies depending on the size of the mouth of the syringe and the strength of the pressure when removing the mixture of bacteria with sodium alginate into CaCl₂ solution. After Ca-alginate beads are completely formed, then filtered and washed using 0.85% NaCl solution which serves to remove impurities and remnants of bacteria attached to beads. The next step is NaCl 0.85% remaining beads washing is used to calculate the effectiveness of immobilization.

After the beads are formed, then the activity test of the bacterium *Aeromonas bivalvium* is carried out quantitatively by the colorimetric method which is examined by its absorption using a spectrophotometer. The type of chitinase bacterium *Aeromonas bivalvium* is included in the genus *Aeromonas* which has the potential to produce endocytinase [8]. Endocytinase is an enzyme that degrades chitin randomly from internal microfibrils and produces chitin oligosaccharides. The chitinase activity test was carried out for 5 days in accordance with Kholifah's study [9]. Meanwhile the sample tested for its activity was culture of immobilized bacteria and non-immobilized bacteria. The fermentation medium used was liquid chitin medium and incubated at a 37 ° C temperature waterbath shaker. The use of waterbath shaker serves to stabilize the temperature during incubation and the liquid chitin medium during incubation is always homogeneous so that chitin degradation is more optimal. The temperature used is at the optimum temperature range of *Aeromonas bivalvium*, which is 30-37 ° C [5]. Chitinase activity in the sample was seen from the formation of a pink complex after reacting with the p-dimethylamino-benzaldehyde (DMAB) reagent. The resulting pink color is formed because of two factors including the formation of glucosazo compounds with dimethylamine-benzaldehyde and the second factor is due to the warming of acetylhexoamine with alkali so that the compound acts with DMAB [16].

Based on results of chitinase activity from the 1st to the 3rd displacement tends to decrease, so it can be said that the stability of bacteria in degrading the substrate also decreased. It is also seen from the levels of NAG produced which is decreasing from the first transfer to the third displacement (Figure 4.7). The decrease in activity is possible because of several factors including the beads that leak until they break during the transfer process, so that when the second and third transplants the number of intact beads is reduced. The broken beads are caused by the use of alginate is only 1% so that the process of absorbing bacterial cells is not maximal. The pores formed in beads are also wider, causing high cell leakage [11]. Therefore

a higher concentration needs to be carried out as done used sodium alginate by 1.5% on immobilization of *Aspergillus niger* cells, so that the results obtained can last up to 4 times [4]. Beads can only be used for 2 times because of the lack of incubation time used, the incubation time used should be 48 hours, according to the optimum growth time of *Aeromonas bivalvium* so that the resulting chitinase is also more optimal. Although chitinase activity decreases but the bacteria can still be used for 2 times, when compared to free cell bacteria, immobilized bacteria are certainly more beneficial because immobilized bacteria can be used not only once.

The next test was a stability test on immobilized *Aeromonas bivalvium* bacteria. This test is carried out based on the testing of chitinase activity carried out on immobilized bacteria in NB and NB + K. The physical treatment provided is in the form of washing, filtering, transfer and use of immobilized bacteria repeatedly. The physical treatment was carried out to test the resistance of immobilized bacteria whether or not the bacteria had chitinolytic activity after repeated removal and use. This test is carried out by removing immobilized bacteria (bead Ca-alginate) that have been used to test the activity (transfer 1) into the new fermentation medium. Before transferring the beads were washed using a 0.85% saline solution and filtered with a stainless steel filter in a sterile manner. Beads are washed to clean the rest of the fermentation medium beforehand and remove other bacteria that attach to prevent contamination. Beads that are clean, ready to be used again in the new fermentation medium as the second transfer, the step is repeated again until the third transfer. Immobilized bacteria incubated with a 37° C temperature waterbath for 24 hours. The fermentation medium used during transfer was liquid chitin medium, the reason for using the medium was to test chitinase activity and NAG concentration.

Based on the results of research obtained from the immobilization of *Aeromonas bivalvium* bacteria, the level of success or effectiveness of immobilization was 91.8%. The results of chitinase activity testing from the three samples showed no significant differences or no real differences. The graph of chitinase activity showed that the three samples had almost the same pattern, especially at the 48th hour chitinase activity was higher than the other incubation times. This is in accordance with previous research [9]. However, of the three who had the highest chitinolytic activity at the 48th hour, they were treated with immobilization in the Nutrient Broth medium (NB), as well as the NAG levels produced. Stability testing for immobilized bacteria was also observed based on chitinase activity and NAG levels produced. The results of the stability test of the two samples had the same tendency of decreasing as the displacement was carried out. The two samples that still had chitinase activity until the third transfer were in the treatment of immobilized bacteria in the Nutrient Broth (NB) medium.

Based on the results of this study, it can be said that the entrapment method using sodium alginate matrix has the potential to be a good method of immobilization. This is based on the advantages of a simple and easy method. 1% sodium alginate which is used as an immobilization matrix can provide almost the same performance as bacteria without immobilization although there are still weaknesses, namely the use of 1% sodium alginate concentration resulting in less perfectly round beads and not too rigid [11]. The shape of the beads has the potential to make cell leakage (leakage) thus causing broken beads and bacterial density in beads to decrease. Leakage can occur if the concentration of the matrix used is not suitable, so that the right combination of concentrations is needed and an additional layer as protective beads is an example of chitosan [12].

4 Conclusion

Immobilization of *Aeromonas bivalvium* cells with 1% alginate matrix does not significantly affect to chitinolytic activity when it was compared with bacteria without immobilization. The stability of *Aeromonas bivalvium* immobilized based on chitinase

activity and NAG levels decreases with the transfer was carried out, but that bacteria still have chitinase activity until the second transfer and use.

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