Immobilization of Bacterial Cells and Chitinolytic Activity of *Streptomyces* sp. (PB 2)

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Abstract. This study was aimed to immobilize the chitinolytic bacteria *Streptomyces* sp. PB 2 using sodium alginate as immobilization agent. *Streptomyces* sp. PB 2 was reported have a good chitinolytic activity and immobilization is known to increase the stability of bacteria during repeated used. Sodium alginate has been used on several methods of immobilization and sodium alginate are reported as a good agent. Cell immobilization was done by growing the cell on nutrient broth (NB), mixed with 1% sodium alginate to form the beads, then the mixture were put to a syringe and droped into $CaCl_2$ solution. The beads were washed with 0.85% NaCl solution and filtered. To test the bacterial activity, beads were applied into chitin liquid medium and the chitinolytic activity was observed every 24 hours for 5 days. To test its stability, after the 5th day, the beads was filtered and put in to chitin liquid medium and tested the chitinolytic activity. This experiment was repeated twice. The highest chitinolytic activity of *Streptomyces* sp. PB2 was observed on the 4th day with the value of 0.00014 U/ml and the concentration of NAG of 5.42087 µg/ml with the treatment of immobilized NB medium without the addition of chitin.

1 Introduction

Shrimp are a group of crustaceans which are superior commodities. Indonesia has a high enough potential to produce shrimp. Shrimp production in Indonesia in 2018 will reach 138,000 tons. According to data from BPS (2017) most of the shrimp farming production is exported to several countries such as Japan, Singapore, the United States, the Netherlands, the United Kingdom, and several other countries. Shrimp that are exported are frozen so that the shrimp freezing industry in Indonesia develops. Shrimp freezing industry produces waste in the form of shrimp shells because for shrimp exports only meat is taken. The shrimp freezing process by the shrimp freezing industry produces shrimp shell waste. According to [1] traditionally shrimp shell waste is widely used as a mixture of crackers, petis, shrimp paste, for additional animal feed and returned for shrimp farming itself at a low price. While at this time it is known that one of the utilization of shrimp shell waste that has prospects to be developed and has a high economic value is to process it into chitin, chitosan and glucosamine.

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Chitin is a carbohydrate biopolimer compound contained in crustacean shells . Shrimp is a group of crustaceans which are included in superior commodities. Currently enzymatic processing of shrimp shell waste is widely carried out. Chitin degrading enzymes are produced by micro-organisms. So that immobilization is carried out to get stable micro organisms that produce chitin degrading enzymes.

Streptomyces sp. is one of the chitinolytic bacteria that attracted the attention of many researchers because it was found to be the largest chitinase producer on the ground and its good chitinolytic ability. *Streptomyces* sp. has become a group of microorganisms that are widely exploited in the production of secondary metabolites and enzymes that have commercial interests in medical and agricultural applications [3]. *Streptomyces* sp. is producing chitinase to hydrolyze chitin which is used as a source of carbon and nitrogen [3]. The use of enzymes in hydrolyzing chitin polymers is more developed than using chemical methods because it is more specific in producing products and does not cause environmental pollution.

Immobilization on a particular compound matrix has several advantages, namely protecting microorganisms from environmental conditions that have high levels of pollutants, easily becoming liquid again, having high cell density to increase substrate conversion, and reducing reactor volume [4]. Some techniques for immobilization are attachment, containment, entrapment, carrier binding, adsorption technique, encapsulation, cell coating and self aggregation [5]. Immobilization of microorganisms with entrapment gel beads is a good method to maintain the viability of microorganisms, as a protector so that it prolongs the life of microorganisms and also as a nutrient for microorganisms. The chosen carrier material must be able to provide various conditions for inoculants so that the inoculant can survive and function as well as possible, thereby extending the life span and increasing life skills and inoculant activity [6]. This study aims to determine the effect of immobilization on chitinolytic activity of the *Streptomyces* sp.

2 Materials and methods

The research process was carried out in several stages, namely the stage of making colloidal chitin, making bacterial inoculum, bacterial cell immobilization, immobilized bacterial chitinolytic activity test, and immobilization stability test. The study took place from January to March 2019 at the Fisheries Product Quality and Safety Labotarurium, Nutrition Laboratory, and the Hydrobiology Laboratory of the UGM Fisheries Department.

The tools used in this study include Petridisk, Erlenmeyer, Bunsen, Needle Ose, 1.5 mL microtube, Yellow tip, Blue tip, White tip, Hotplate stirer, magnet stirer, clamp, autoclave, oven, vortex (Thermoscientific), scales analytic, paper weigh, micropipet (Gilson), centrifuge (Thermoscientific) electrophoresis mini-gel set (, Mupid-exU, Takara), incubator, refigerator -30ºC (Sanyo), K3 tool (safety and security).

The materials used in this study include *Streptomyces* sp. culture (PB-2), shrimp shells, 99% commercial chitin (Sigma), Flake NaOH (MKR Chemicals), NaOCl, 37% HCl (Merck), distilled water, K_2HPO_4 (Merck), KH_2PO_4 (Merck), $MgSO_4.5H_2O$ (Merck), $FeSO_4.7H_2O$ (Merck), $ZnSO_4$ (Merck), $MnCl_2$ (Merck), 70% alcohol, standard N-acetyl glucosamine (TCI), pdimetilaminobenzaldehyde (DMAB) (Merck) reagents, TSA (Oxoid), sodium alginate, $CaCl_2$, and NB medium.

2.1 Making colloidal chitin

Chitin is obtained from shrimp skin which has undergone a deproteination and demineralization process and has been tested before. Chitin used for the activity test is colloidal chitin. To make 5% colloidal chitin, 5.0 g of chitin powder was added gradually to

60.0 mL concentrated HCl at 40 ° C accompanied by strong stirring. The stirring process is carried out overnight at 40 ° C. The chitin suspension in concentrated HCl is then added to 2.0 L of 95% ethanol and accompanied by strong stirring. This process is carried out for one night at room temperature. This mixture is then centrifuged at 6000 rpm for 20 minutes at 40 ° C. The precipitate obtained was then washed with distilled water to neutral (pH 7.0). Then add water or buffer that corresponds to 100 mL [7].

2.2 Making bacterial inoculum

Making inoculum of *Streptomyces* sp. bacteria beginning with making work culture. Work culture is a bacterial preparation that is used to supply a single colony during the study. The making of work culture consists of two stages, namely the refreshment stage and the streaking of the bacteria. The refreshment phase was carried out by taking one stock culture (glycerol stock culture) which was previously stored at -24 ° C and then grown on agar chitin medium as much as 1 ose and incubated at room temperature to form a single colony. Then from a single colony formed on chitin medium to be taken 1 ose and grown on 7 ml of liquid chitin medium and incubated for 3 days at 30 ° C on the shaker waterbath speed of 100 rpm. Streaking stage aims to get a single colony. You do this by taking one ose from bacterial culture (liquid chitin) then scraping it on ± 15 ml of chitin agar and incubating it for 3 days at room temperature to optimize bacterial growth. After incubation for 3 days, the work culture is ready to be used for further research and stored at 4 ° C.

2.3 Bacterial cell immobilization

Bacterial cell immobilization was carried out by growing bacteria on Nutrient Broth (NB) and Nutrient Broth (NB) medium which had been added chitin (7 ml each). Then incubated for 72 hours at 30 ° C and taken 1 ml to be absorbed and as much as 5 ml from the culture sodium alginate powder which was dissolved in distilled water with a concentration of 2% as much as 5 ml so that the concentration of the culture mixture with sodium alginate became 1% and is cortex to homogeneous. Then the solution is printed using a syringe and dripped into CaCl2 solution with a concentration of 3% to form beads (bead) that have a size of 2-3 mm. The printed bead will settle and be washed with SSS solution (0.85% NaCl) then filtered aseptically. The waste water from the washing solution is used to calculate the effectiveness of immobilization. Before being used the bead was incubated for 24 hours at 30 ° C or stored at 4 ° C for 12 hours first and put into 20 ml of liquid chitin medium for chitinase activity test.

2.4 Immobilized bacterial chitinolytic activity test

The chitinase activity test was carried out by taking 2 ml of liquid chitin fermentation medium for each treatment. Samples were centrifuged at a speed of 12,000 rpm for 2 minutes and the supernatant was taken free of bacterial and chitin medium. Then as much as 500 µl of the supernatant was reacted with 1 ml of colloidal chitin 1.3% (in posphat 50 mM buffer pH 7.4) with incubation for 30 minutes in a waterbath shaker at 37^0C. After 30 minutes the sample was immersed in boiling water for 3 minutes to stop the reaction, then the sample was cooled and centrifuged at 12,000 rpm for 2 minutes [9]. The results of the reaction were measured by the method of [10]. A total of 0.25 ml of the supernatant from the reaction product was added 0.05 ml of potassium tetraborate pH 9.1 to bind NAG. To accelerate the NAG binding reaction by potassium tetraborate the sample is immersed in boiling water for 3 minutes. Then the refrigerated sample was added 1.25 ml p-dimethylaminobenzaldehyde (DMAB) and incubated at 37^0C for 30 minutes in a waterbath shaker to detect NAG. The absorbance

of the sample was measured by a UV-Vis spectrophotometer at a wavelength of 600 nm. Then the absorbance value of the sample is compared with the standard NAG solution value. As a control it was made by heating the sample for 3 minutes with boiling water in the same sample. The number of NAG products (μg / ml) formed from the results of chitin hydrolysis by the enzyme chitinase per minute in incubation conditions defines a unit of chitinase activity. NAG concentration was tested by taking samples from liquid chitin fermentation medium for each treatment. Testing of NAG concentrations was carried out simultaneously with the testing of chitinase activity and measured by the method of [10] as explained earlier. Then the absorbance value of the sample is compared with the absorbance value of the standard NAG solution.

2.5 Immobilization stability test

Testing the stability of immobilization is carried out to determine the activity of immobilized bacteria when used repeatedly. Tests were carried out by measuring the immobilized bacterial chitinase activity after being used in the fermentation medium. After being used for fermentation of beads containing immobilized bacteria filtered aseptically and washed with SSS solution, then put it back into the new fermentation medium to be fermented until the 3rd use. On the 1st day a chitinase activity was tested using the method of [10].

3 Results and discussion

3.1 Bacterial cell immobilization

Imobilization of bacterial cells is done by capturing cells in the matrix that have the ability to protect cells to maintain stability. In this study, immobilized sodium alginate was applied to $CaCl_2$ which will form beads that are 2-3 mm in size. The media used as immobility in this study was alginate so that the method of cell immobilization used in this research was entrapment method. This method is based on the placement of cells in the lattice of a polymer. The lattice structure of the gel from alginate will protect cells from environmental factors that cause cell damage. The gel structure provides enough space for the cell to move, but still allows the exchange of substrates and products [11].

Before immobilization bacterial culture was prepared by growing bacteria on NB medium, and NB + chitin. The use of chitin in the NB + chitin medium aims to stimulate the chitinolytic ability possessed by bacteria so that it can accelerate the active chitinolytic ability of bacteria. In addition to preparing the culture, alginate solution is also prepared to make it easier in the homogenizing process of the culture mixture with alginate. In this study the formation of alginate gel was carried out by adding bacterial culture mixture and alginate solution into $CaCl_2$ solution. Crosslinking will occur due to the presence of complexes between $Ca2^+$ ions and carboxylic anions present in alginate [11].

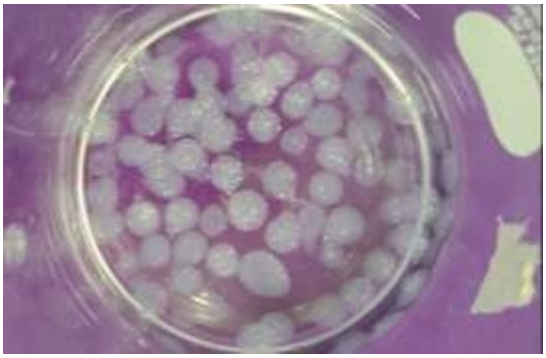

Fig. 1. Bacterial beads immobilized in sodium alginate

In these beads the bacteria are trapped so that they can be separated from the fermentation medium and can be used for several replications. In addition, bacterial cells will experience a smaller effect from the environment because they are protected by alginate beads.

3.3 Chitinase activity test

According to [12], chitinase is mentioned as a growth enzyme that can be secreted by bacteria to degrade chitin compounds to be simpler, such as NAG which can be used as a single source of carbon and nitrogen for microbial growth. In producing NAG each bacterium has different abilities and is influenced by environmental factors such as temperature, pH and nutrient availability.

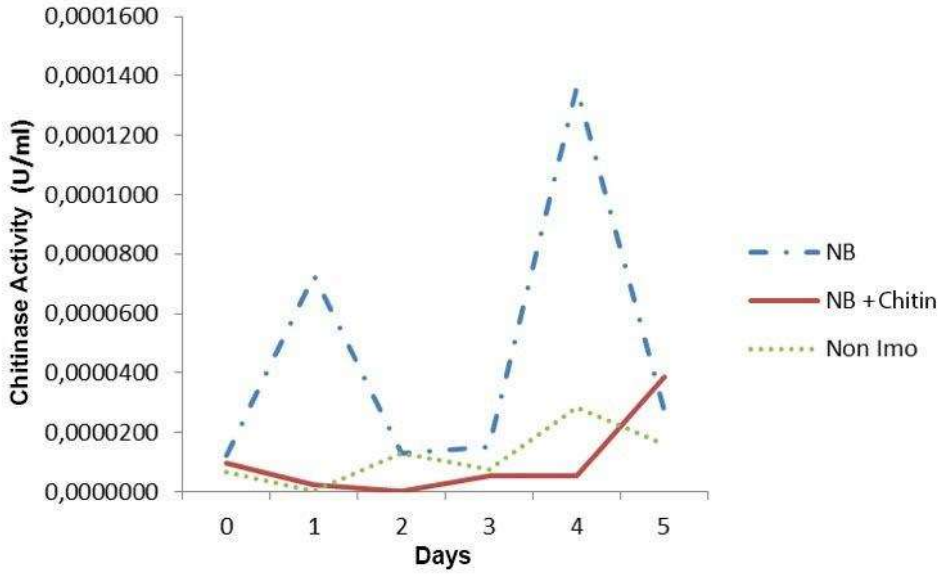

Fig. 2. Graph of chitinase activity

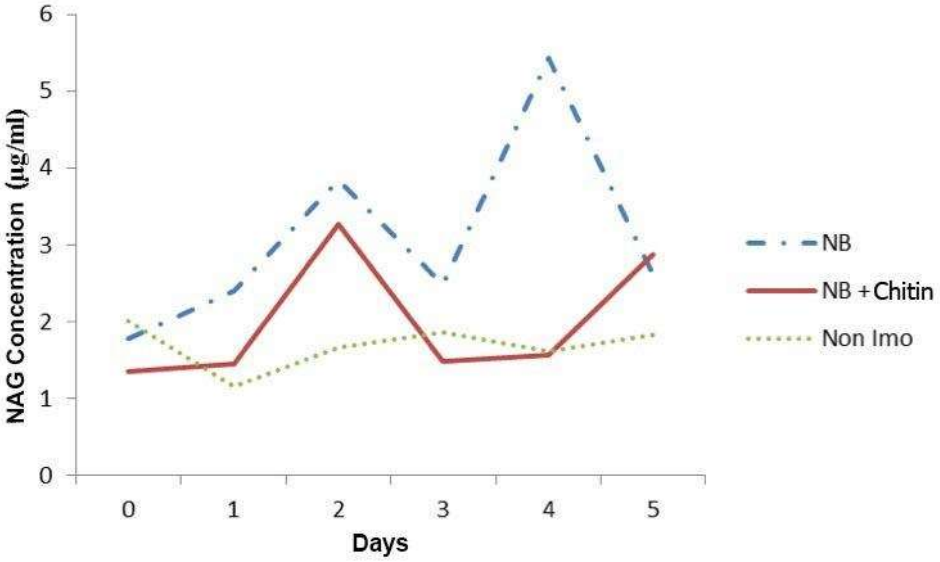

Fig. 3. NAG concentration chart

Based on the picture above it can be seen that the bacteria Streptomyces sp. who were treated with immobilization with the initial NB culture medium had higher activity than bacteria treated with immobilization with NB initial culture media which added chitin and bacteria that were not immobilized. Chitinase activity from bacteria with immobilization treatment with NB media increased on day 1 and decreased on days 2 and 3. This was due to an increase in the concentration of NAG on the substrate on day 2 which might be an inhibitor for the enzyme chitinase. On the 4th day chitinase activity increased which was accompanied by a high concentration of NAG. This happens because of the relationship that is in line between cell growth and the products produced [13]. Then on the 5th day chitinase activity and NAG concentration decreased dramatically due to a decrease in chitin concentration on the substrate, reduced energy sources, changes in pH in the media that can damage bacterial cells and also the accumulation of metabolic processes that can become inhibitors [14] While for bekteri immobilized with the initial NB culture media added chitin has a low activity. Viewed from Figure 2 chitinase activity from these bacteria experienced activities that tended to be constant on day 0 to day 4 and experienced an increase on the 5th day. In the [15] study it was found that Streptomyces sp. increased activity after incubation on day 4. However, from Figure 3 it can be seen that the bacteria treated with immobilization with the initial NB culture media added by chitin experienced an increase in the concentration of NAG on the second day. This can occur because of the accumulation of NAG from chitinase activity on day 0 and day 1. Then the concentration of NAG on days 3 and 4 decreased and again increased at the 4th day incubation because chitinase activity on day 5 also increased.

Chitinase activity from the bacterium Streptomyces sp. those who are not immobilized based on Figure 2 can be seen that their activities have increased and decreased. In the 2nd and 4th day incubation, the activity increased and declined again on the 5th day. While the concentration of NAG produced based on Figure 3 decreased on day 1 and then continued to increase until the third day. In this sample there was a correlation between the chitinase activity and the concentration of NAG produced. However, on the 3rd day the concentration

of NAG which experienced an increase was possible due to the accumulation of NAG in the previous days.

3.4 Immobilization stability

One of the objectives of immobilization is to maintain the stability of bacterial cells from changing environmental conditions and to make bacterial cells not mixed with fermentation media so that they can be used on new fermentation media after being used for previous fermentation. In this study to test the stability of bacteria in the first use and then carried out by testing the activity of chitinase in the incubation day 1.

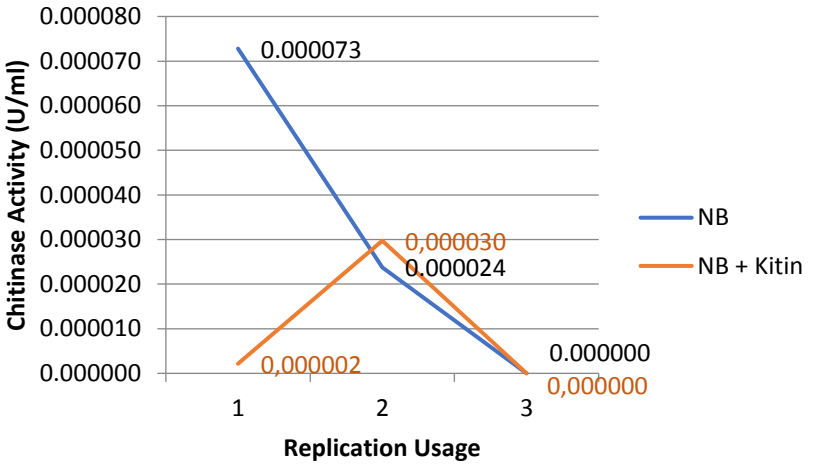

Fig. 4. Stability chart of chitinase activity

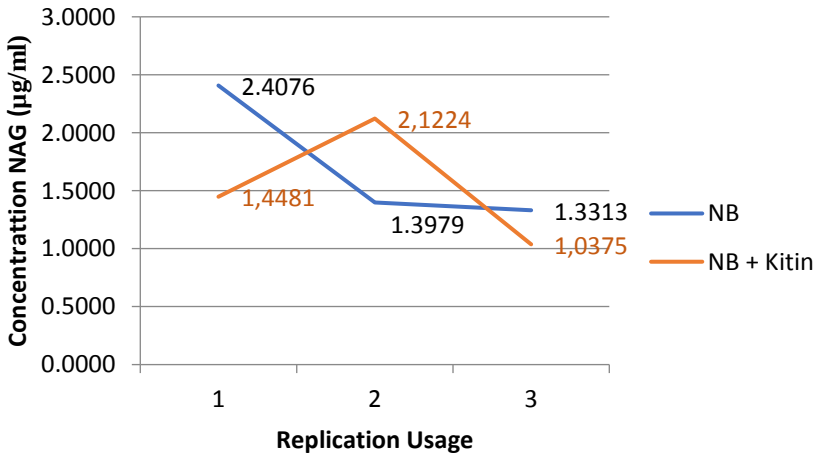

Fig. 5. NAG stability concentration chart

Based on Figures 4 and 5, it can be seen that in the immobilization treatment sample with the initial culture media NB in the first use up to the 3rd use decreased chitinase activity and NAG concentration. This is due to a decrease in biomass from bacteria due to the death phase. In addition to the fermentation, the first use of some immobilized beads is damaged so that the bacteria are released and escaped during filtration. Whereas for samples with immobilization treatment with initial NB culture media which added chitin, there was an increase in activity and the concentration of NAG in the second use then decreased in the 3rd use. This can occur because the second use after the first fermentation carried out with incubation for 5 days is possible in the incubation of the 1st day the bacteria are still in the optimum phase to metabolize as known in the study of [15] which said *Streptomyces* sp. increased activity after incubation on day 4.

4 Conclussion

Immobilization of *Streptomyces* sp. can be done using alginate. From this study it was found that the best immobilization that produced the highest chitinase activity using immobilization with the initial culture medium NB medium was 0.00014 U / ml and NAG concentration 5.42087 µg / ml. The *Streptomyces* sp. bacteria immobilized can be used until the second use but experienced a decrease in chitinolytic ability because the immobilized beads were physically damaged. It is necessary to optimize the pH, temperature, inoculum, additional nutrient sources, or substrate concentration before immobilization to obtain immobilized bacteria that can produce chitinase maximally

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