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Immobilization of *Bacillus megaterium* in Carrageenan from Maluku Sea and Their Effect on Protease Production

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1. Introduction

Immobilization is a bacterial condition maintained in a matrix. Carrageenan, an isolate from red seaweed (Eucheuma cottoni) largely available in Indonesian waters, can be used as an immobilization matrix. There are three types of carrageenan: kappa-carrageenan, used as gelling agent, lambda-carrageenan, used as thickening agent or viscosity enhancer, and iota-carrageenan, used as gelling agent. Being a stronger gelling agent than other types of carrageenan, kappa-carrageenan is generally used in immobilization matrix (Rowe et al. 2009). Carrageenan used in this study was derived from the seaweed grown in West Seram, a district in Maluku known as the biggest producer of Eucheuma cottoni, which contains approximately 50% of kappa-carrageenan on its basic dry weight (Karyani 2013).

Microbes in immobilized form have advantages in industrial applications since they are easier to handle and they do not die easily. Bacteria as an enzyme resource has several advantages on its production scale; it is easier to increase if desired, the microbial cells are easier to grow with relatively faster growth, it takes lower production cost, it does not depend on seasonal changes and it takes shorter production time. Bacteria from the genus Bacillus are active producers of extracellular proteases which produce proteases with high pH and thermal stability (Chatterjee 2015). *Bacillus megaterium* was used in this study as it didn't produce toxins, didn't need expensive substrates, could survive in high temperature, had no metabolic side effect, was easy to grow and capable of producing a high number of extracellular protein (Poernomo 2003).

Protease enzymes produced from cellular immobilization have advantages such as easy-to-control immobilized cell and stable and non-toxic matrix.

Immobilization can also enhance productivity due to high cell density, the ease in cell isolation and continuable product purification (Riwayanti et al. 2012).

Immobilized *Bacillus megaterium* can produce higher protease enzymes as trapped cells in the matrix (immobilized cells) are reusable, have better stability, higher efficiency, and bigger resistance toward environmental disruption (Mrudula & Shyam 2012).

2. Materials and methods

2.1. Instrumental and apparatus

We used an analytical digital scale (Ohaus), UV-Visible spectrophotometer (Shimadzu), centrifugal (Tomy MX-305), centrifuge (PLC series), magnetic stirrer (Thermo scientific), shaker (Health H-SR-200H), vortex (Barnstead type 37600), Laminar Air Flow (ERSA Scientific), micropipette (Fisherbrand Elite), autoclave (GEA LS-50 LJ My Life MA 678GE), incubator (Memmert), colony counter (Rocker Galaxy 230), pH meter (Mettler Toledo), and general laboratory glassware.

2.2. Material and microorganisms

The material used is bacteria *Bacillus megaterium* ITBCC40, carrageenan isolated at Pharmaceutical Technology Laboratory STFI according to Ferdiansyah, 2017. Other materials used were nutrient broth (Oxoid); nutrient agar (Oxoid); 96% ethanol, distilled water, NaCl 0.9% (Wida R.); KCl 0.3 M (Merck); $CaCl_2$ 0.18 M (Merck); skim milk; peptone (Oxoid); yeast extract (Oxoid); casein; NaCl (Merck); KH_2PO_4 (Merck); $MgSO_4 \cdot 7H_2O$ (Merck); tyrosine; K_2HPO_4 (Merck); NaOH (Merck); and TCA (Trichloroacetic Acid) 0.1 M (Merck).

2.3. Determination of *Bacillus megaterium* growth curve

Determination of growth curves was carried out on *Bacillus megaterium* that had been incubated at 37°C for 24 hours. Bacteria was inoculated into 50 mL of sterile nutrient broth (NB) media and homogenized on a shaker at 100 rpm at room temperature for 24 hours. 10% of the culture was put into 100 mL of sterile NB and had its optical density value measured every hour (for 40 hours) at 600 nm wavelength by UV-Visible spectrophotometer (Shibata 1954).

2.4. Immobilization of *Bacillus megaterium*

Bacillus megaterium were cultured on nutrient agar (NA) media and incubated at 37°C for 24 hours, then made into suspension in 0.9% NaCl solution until 22% transmittance was obtained. Carrageenan matrix solution was made with different concentrations, i.e: 1, 1.5, and 2% (w/v) through a dilution process

in aquadest by shaking it on a magnetic stirrer at 80-100°C. Carrageenan solution was sterilized using an autoclave at 121°C at a pressure of 1.5 atm for 15 minutes. Sterile carrageenan solution was maintained in liquid form at around 40°C through heating and shaking on the magnetic stirrer, then a *Bacillus megaterium* suspension was added. The mixture was added drop by drop by using a pipette into a sterile 0.3 M KCl solution (10°C) while being shaken slowly for a few minutes to obtain beads, and let stand for 15-20 minutes. KCl solution was then decanted and the beads were immersed in a sterile 0.18 M $CaCl_2$ solution (10°C), let stand for 1 hour. $CaCl_2$ solution is decanted then beads containing immobilized bacteria were stored in a refrigerator at 4°C (Cassidy 1996).

2.5. Proteolytic test

Proteolytic assays were carried out on both of immobilized and not immobilized *Bacillus megaterium* by culturing it, using a toothpick, on nutrient agar (NA) which contain 2.8% (w/v) skim milk before incubating it for 48 hours at 30°C. Bacterial culture was observed visually; positive results were indicated by the presence of clear zones on media around bacteria colonies. Proteolytic indexes were calculated by comparing diameter of the clear zone with diameter of colony (Omer & Humadi 2013).

2.6. Calculation of total bacteria

Pour plate methods were used to calculate the total bacteria (total plate count) of immobilized *Bacillus megaterium* on different storage times: 4 months (K), 5 months (L), 7 months (M), and 9 months (N). A total of 0.1 gram beads containing immobilized bacteria were put into a tube filled with 10 mL of NaCl 0.9%, then the beads were dissolved by shaking by vortex. The solution was then diluted to 10^{-1}, 10^{-2} and 10^{-3} using 0.9% NaCl. A total of 100 µL of solution from each dilution was put into a Petri dish, then 10 mL NA was added and homogenized, incubated at 37°C for 24 hours. The growth of bacterial colonies was calculated using colony counter. The number of bacteria was calculated based on Bacteriological Analytical Manual (2011), with formula as follows:

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

where:
C – the total number of colonies from all petri dish counted,
N – the number of colonies per ml/gram,
n1 – the number of petri dish on the first dilution,
n2 – the total of dilution on the second dilution,
d – the dilution level obtained from the first petri dish.

2.7. Production and isolation of rough protease

Enzyme production was done by inoculating beads containing immobilized bacteria as much as 100 mg into 50 mL of production media with the composition of casein 0.5% (w/v), peptone 0.01%, yeast extract 0.02%, NaCl 0.1%, K_2HPO_4 0.01%, $MgSO_4 \cdot 7H_2O$ 0.01% and $CaCl_2$ 0.01%, shaken at 150 rpm for 72 hours. The mixture was centrifuged at 6000 rpm at 4°C for 30 minutes. The resulting supernatant was a crude protease enzyme which would then be tested for activity test (Sevinc 2011).

2.8. Determination of activity value in protease enzymes

The Kunitz method was used to determine the activity value of the protease enzyme by extracting 1 ml of an enzyme (supernatant) into a centrifuge tube containing 1 ml of casein 1% (w/v) in dilution of phosphate buffer pH 7, then incubated 37°C for 30 minute. The hydrolysis reaction was stopped by adding 3 ml of TCA (trichloroacetic acid) 0.1 M then allowed to stand for 30 minutes at room temperature. Suspention centrifuged at a speed of 6000 rpm for 10 minutes, supernatant was taken carefully, then measured using a UV spectrophotometer at 288 nm. Enzyme activity was calculated based on the number of amino acids (simple peptides) formed by using a standard tyrosine curve. One unit of protease enzyme activity is defined as the amount of enzyme that liberates 1 μmol tyrosine under test conditions (Mrudula & Shyam 2012).

3. Results and discussion

3.1. Growth curve of *Bacillus megaterium*

The growth rate of *Bacillus megaterium* were measured three times repetation by turbidimetric method, based on the degree of turbidity during storage of bacteria grown on the inoculum medium. Turbidity measured the number of living cells as well as the dead cells (Maier 2008).

Bacillus megaterium underwent a relatively short lag phase (adaptation). This phase occurred at the first 0-2 hours of growth time, this condition similar as growth on peptone, glucose, and yeast extract media (Liu 2011). However, in the exponential phase there was quite difference, the results show that the exponential phase was reached at 15 hours, difference from Liu, 2011 which was reached at 36 hours. The maximum time in the exponential phase was closer to the growth curve under enriched media which was at 22 hours (Nguye 2018).

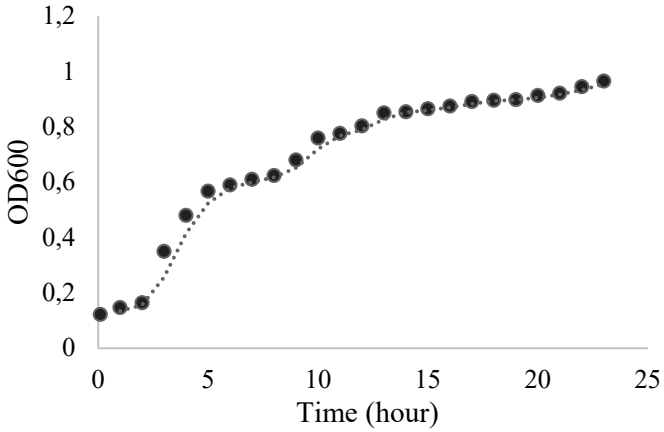

Fig. 1. Growth curve of *Bacillus megaterium*

3.2. Immobilization of *Bacillus megaterium*

The immobilization process was carried out by varying the concentrations of carrageenan to determine the optimal concentration to get the best beads. The concentrations of carrageenan commonly used were 0.2-2%. In this study the concentrations of 1%, 1.5% and 2% were used. The perfectly formed beads were produced in 1.5% concentration of carrageenan. Gel concentrations higher than 2% produced large lump because the matrix with high concentration would very quickly coagulate so that beads did not form. At lower concentrations, the beads produced were too soft and brittle, so the beads would leak easily. According to Kocher and Mishra (2012), the optimum bead diameter was 5.2 mm for protease production and optimum freezing time for protease production was 12 hours.

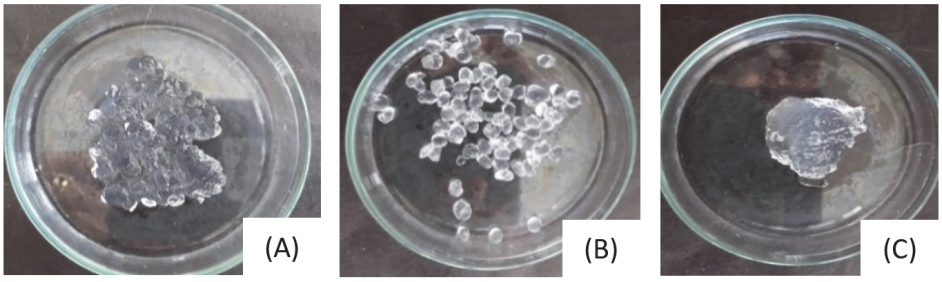

Fig. 2. *Bacillus megaterium* immobilized in Carrageenan. (A) Carrageenan 1%; (B) Carrageenan 1.5%; (C) 2% Carrageenan

The matrix dissolution process was carried out at thermal temperatures (80-100°C) as the matrix can be completely dissolved at this temperature. Under lower temperature, the only dissolved matrix was the salt form. Bacteria were integrated into carrageenan at 400C to prevent bacteria from dying. The use of 0.3 M KCl solution at 10°C in the process of forming beads is meant to induce the formation of 3-dimensional structure of the helical structure to form a strong and durable microcapsule, while immersion in $CaCl_2$ 0.18 M at 10°C was a gelatination stage to compact the beads (Cassidy 1996, Suzana 2013).

3.3. Proteolytic assay

Proteolytic indexing was carried out by culturing *Bacillus megaterium* immobilized into NA media contained skim milk, and positive result can be seen by the presence of clear zones around the colony, while observations can be seen in Figure 3. The addition of skim milk in the growth media functioned as an enzyme substrate. Casein hydrolysis reaction served to show proteolytic hydrolysis activity. Protease catalyzed casein degradation by breaking the peptide bonds in CO-NH as water entered the molecule; the hydrolysis reaction produced amino acids (Joseph 2006).

The results of proteoliytic index shown in Table 1 stated that *Bacillus megaterium* produced the same proteolytic index as K immobilized bacteria (4 months storage) which was 1.1 cm. Proteolytic index increased along the immobilization storage time, namely L (storage 5 months) 1.2 cm, M (storage 7 months) 1.8 cm, and N (storage 9 months) 2.7 cm. However, the clear area around the growth of bacteria did not represent the number of proteases produced by microorganisms as the clear area would grow larger along with the increased length of the incubation time (Figure 3). Therefore, the protease content in immobilized *Bacillus megaterium* had to be determined quantitatively.

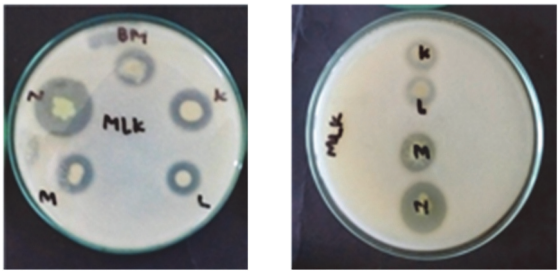

Fig. 3. Proteolytic tests of *Bacillus megaterium* unimmobilized and Immobilized. K (4 months), L (5 months), M (7 months), N (9 months), Bm (unimmobilized)

Table 1. Proteolytic index

Description	Clear zone diameter (mm)			
	Dish 1	Dish 2	Dish 3	Average
Bacillus megaterium	12	11	11	11
K (4 months)	11	11	11	11
L (5 months)	11	12	13	12
M (7 months)	11	16	25	18
N (9 months)	25	27	30	27

3.4. Calculation of total bacteria

Bacillus megaterium is an anaerobe facultative bacteria capable of growing on parts of the media that are indirectly exposed to the oxygen, therefore pour plate method is used to determine the number of bacteria on Total Plate Count. Dilution is a means to reduce the number of microbes to enable observation and determination of specific number of microorganism for accurate calculation. The study showed that the number of bacteria in storage were relatively stable, indicated by the number of bacteria increase that didn't go higher than 101 CFU/100 mg (Fig. 4).

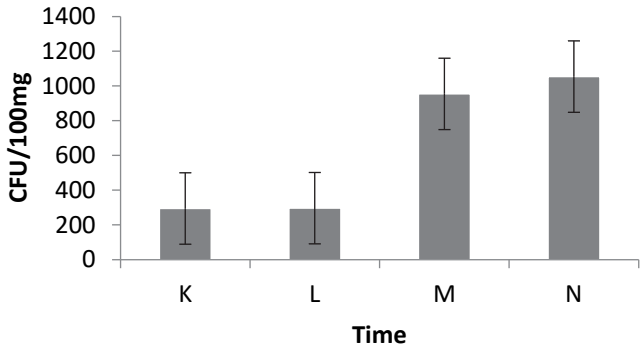

Fig. 4. Results of Total Plate Count (TPC) *Bacillus megaterium* immobilized
Description: K (4 months), L (5 months), M (7 months), and N (9 months)

3.5. Determination of protease activity enzymes

Determination of protease enzyme activity value was carried out by reacting 1 mL of casein solution dissolved in phosphate buffer pH 7 and 1 mL of enzyme solution from the sample. Casein was used to measure the size of enzyme

activity by measuring the amount of tyrosine in said protease. Phosphate buffer pH 7 used to dissolve casein was also used to maintain the stability of enzyme pH as pH changes can affect enzyme activity and extreme pH can ruin enzyme. Next, the solution was incubated at 37°C for 30 minutes to activate the protease enzyme in decomposing casein. The addition of TCA will denature the casein due to the formation of an acidic environment and form a white complex, tyrosine was then separated from other components by centrifugation. Casein with its high density would settle to form pellets, and tyrosine, enzymes, and buffers would be found in the supernatant. This supernatant was then measured at 288 nm wavelength, which is the maximum absorption of tyrosine (Kocher 2009).

Determination of protease activity was calculated based on the equation obtained from the standard tyrosine curve which was the line equation $y = 0.1595x + 0.0596$. The results of the observations shown in Figure 5 state that the increase of protease activity value is 0.0271 U/Gram for immobilization sample after being stored for 4 months (K), 0.0489 U/Gram for 5 months (L), and 0.1372 U/Gram for 7 months (M), and the activity decreases after being stored for 9 months (N) which is 0.0501 U/Gram immobilization.

Immobilized bacteria in carrageenan from Maluku sea are able to produce proteases with different activities influenced by storage time. The activity of the crude protease enzyme is shown in Figure 5, the immobilized bacteria stored provide higher enzyme activity compared to wild bacteria. Storage time that gave the highest effect on enzyme activity for storage up to 7 months but at 9 months storage the enzymes produced shown decrease activity, this condition is possible due to decreased physiological activity of bacteria due to growing environmental conditions that are increasingly unsupportive.

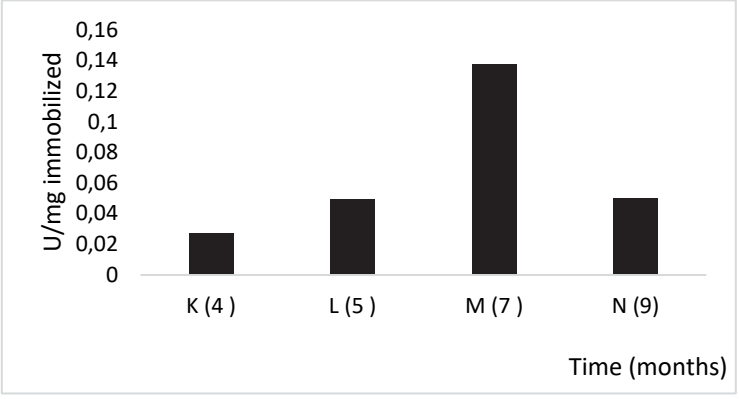

Fig. 5. Activity Value of Protease Enzyme

4. Conclusion

The study concluded that immobilized bacteria in carrageenan from Maluku sea are able to produce proteases with different activities influenced by storage time. The immobilized bacteria stored provide higher enzyme activity compared to wild bacteria. 7 months storage time gave the highest effect on enzyme activity with the activity value of 0.1372 U/Gram. Immobilized bacteria in storage for 9 months shows decreased activity of the enzymes produced, this condition was possible due to decreased physiological activity of bacteria at the environmental conditions that were increasingly unsupportive.

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Abstract

Bacteria immobilized in carrageenan are widely used in industry to facilitate bacterial handling and storage. Carrageenan is derived from seaweed and its nature is influenced by the condition of the origin of the sea where seaweed grows, one of the Indonesia sea territories that has seaweed that contains caraganen with good properties is Maluku. This study was conducted to determine the effect of storage time of bacteria immobilized in Maluku sea's carrageenan on proteolytic activity, the bacteria used were Bacillus megaterium. Bacterial immobilization of carrageenan was made at concentrations of 1%, 1.5%, and 2%, storage in cold conditions for up to 9 months. Protease activity was tested using Kunitz method by adding casein as a substrate. The optimal concentration of carrageenan for immobilization of Bacillus megaterium was obtained at a concentration of 1.5%. Protease isolated from immobilized Bacillus megaterium showed increased activity value from storage for 4 months (0.0489 Ug-1) to 7 months (0.1372 Ug-1), and decreased activity after being stored for 9 months (0.0501 Ug-1).

Keywords:
carrageenan, immobilization, protease activity, *Bacillus megaterium*