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Production and immobilization pectinase from *Bacillus* sp. 2P11 using alginate beads

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Abstract. *Utami AP, Fahrurrozi, Meryandini A. 2022. Production and immobilization pectinase from* Bacillus *sp. 2P11 using alginate beads. Biodiversitas 23: 3960-3966.* Pectinase is one of the enzymes often used in the industrial sector, especially in the food industry, such as extracting and clarifying juices. Previous studies have obtained pectinolytic microbial cultures of *Bacillus* sp. 2P11 isolated from cocoa beans. Pectinase production using pure pectin substrates such as citrus pectin or pectin from apples costs a lot because these pectins are expensive. Liquid substrate from cocoa pods TSH 858 and ICS 60 with a concentration of 10% is a good amount for pectinase production, and the percentage of total pectin from cocoa pods is 0.823%. The amount of activity of crude extract of pectinase enzyme is 416,780 mU/mL. Precipitation with ammonium sulfate and dialysis increased pectinase activity by 452,335 mU/mL and 586.88 mU/mL, respectively. Enzymes can be used repeatedly with enzyme immobilization. In this study, the immobilization of the enzyme entrapment method with sodium alginate and $CaCl^2$ was designed with the response surface method. The optimum concentration of sodium alginate was 0.5% and 0.3 M $CaCl^2$, with the percentage of an immobilized enzyme at 40.782%. Stability and repeated use of immobilized pectinase can be done up to 5 times.

Keywords: Alginate beads, *Bacillus* 2P11, entrapment method, immobilization, pectinase, production from cacao

INTRODUCTION

Special Enzyme Global Market Report, the global industrial enzyme market will generate $5.21 billion in 2021 and is expected to grow by $5.57 billion by 2022 at a compound annual growth rate (CAGR) of 7.1%. This change in growth is due to the companies that have beaten them after the COVID-19 pandemic in 2020. The Global Food Enzyme Market is expected to grow to around 7.35 billion at a 2026 CAGR of 7.1%.

Enzymes are biocatalysts that can be used in various industries. Enzymes are used in industry because of their high catalytic power, specific mode of action, stereospecificity, environmentally friendly, and can reduce energy requirements (Tripathi et al. 2014). Enzymes were discovered in the mid-nineteenth century, and their first industrial application was using enzymes produced by fungi. After 20 years, bacterial enzymes were introduced by Bodin and Effront (Haile and Ayele 2022).

Pectinase, cellulase, and hemicellulase are enzymes that degrade plant cell walls. These enzymes have been widely studied related to the potential of biotechnology, especially in the food industry. Pectinase is an enzyme that hydrolyzes pectin through depolymerization reactions (hydrolases and lyases) and de-esterification (esterases). Enzymes produced by microbes are considered an efficient source for developing environmentally friendly. Pectinase is naturally produced by various microbes such as bacteria, yeast, fungi, actinomycetes, animals and plants (Yopi et al. 2013). Microorganisms are the most widely used source of enzymes compared to plants and animals because they grow fast, can grow on cheap substrates, and are easier to increase yields by adjusting growth conditions. Superior microorganisms are one of the essential factors in the production of an enzyme. The use of pectinase enzymes are widely used in the food industry, such as fruit juice extraction and clarification, vegetable fiber purification, and degumming of natural fiber (Haile et al. 2022).

Aaisha and Barate (2015) reported that bacteria that can produce pectinases, such as *Bacillus* sp, Martos et al. (2013) have conducted a study in which Wickerhamomyces anomalus (*Pichia anomala*) is yeast produces pectinase enzymes and Dange and Harke (2018) isolated fungi in solid-state and found that *Aspergillus oryzae* can produce pectinase. Enzymes produced by microbes are considered an efficient source for developing environmentally friendly biotechnology in today's modern society (Oumer and Abate 2018). Research reveals that strains of bacteria have been shown to produce more pectinase enzymes than the enzymes produced by fungi (Jabeen et al. 2015)

Daud et al. (2013) reported that the cocoa pod contains 35.4% cellulose, 37% hemicellulose, and 14.7% lignin. Berutu et al. (2017) reported on selecting 20 bacterial isolates from cocoa beans and pods, *Bacillus* sp. 2P11 has the best ability to produce pectinase. Bacillus bacteria have various physiological characteristics and the ability to make many enzymes, antibiotics, and metabolites. Bacillus

species are used in many medical, pharmaceutical, agricultural, and industrial processes (Tanaka et al. 2014)

Enzyme purification is a step to increase the specific activity of an enzyme because enzymes taken from natural materials are still mixed with other impurities, so purification is carried out to remove impurities. The enzymes produced from the purification step are enzymes in free form. Free enzymes are unstable to the environment, so obtaining a highly active enzyme from the reaction mixture is not easy.

High production of enzymes requires large production costs, so it is considered a limiting factor for the wide-scale application of enzymes. Immobilization of microbial cells to inert solid matrix provides the advantage of recycling producer strains, thereby substantially reducing production costs (Ejaz et al. 2020). Enzyme immobilization is a technique used to assist the development of sustainable bioprocesses by reusing biocatalysts or using enzymes repeatedly. Based on this, enzyme purification and immobilization need to be done to obtain enzymes with the best activity and repeated use. Alginate.

Enzyme immobilization techniques commonly used are microencapsulation, copolymerization, entrapment, physical adsorption, chemical attachment, and covalent bonding. Among these methods, entrapment with calcium alginate gel has many advantages due to the simple and non-toxic nature of alginate (Geethanjali and Subash 2013). The lack of operational stability often hampers the sound characteristics of enzymes and their industrial application, and long-term storage, recovery, and reuse are complicated. Enzyme immobilization can generally overcome this weakness (Homaei et al. 2013). Immobilized enzymes have several advantages compared to free enzymes, namely reducing the cost of using enzymes, facilitating the separation of enzymes from reaction products and solutions so that they can stop reactions quickly, reducing effluent problems, and can be used repeatedly. In the study of microbes producing pectinase enzyme activity, *Bacillus* sp. 2P11, which has been isolated in previous studies.

MATERIALS AND METHODS

Rejuvenation of *Bacillus* sp.

Rejuvenating bacterial isolates was carried out by growing bacteria into a petri dish containing Nutrient Agar (NA) media by streak plate method, then incubating for 48 hours at room temperature.

Pectinase enzyme production

Cocoa pods separated from the seeds are chopped and dried, then blended and filtered. The sieve results in the form of powder were varied (1-15%) to be used as a substrate for the production media. The liquid production media used is according to Pereira et al. (1999) (3 g $(NH4)_2SO4$, 4.5 g KH_2PO4, 0.25 g $MgSO_4.7H_2O$, 0.25 g $CaCl_2.2H_2O$, and 1 g yeast extract). A total of 1 ose isolates *Bacillus* sp. 2P11 aged 48 hours were grown on pre-culture media to OD_{600} (0.6-0.7) for about 24 hours. Furthermore, 1% of the pre-culture media was transferred to the production medium. The production media were incubated using a shaker incubator at a temperature of 30ºC at a speed of 110 g for 48 hours. The crude extract enzyme was obtained from the supernatant from the culture centrifugation at a rate of 8500 g, at 4ºC for 30 minutes. Enzyme activity was measured using the DNS method.

Enzyme production curve

Pectinase production curves were made to grow on 10 mL of enzyme pre-culture medium according to Pereira et al. (1999) until it reached OD_{600} (0.6-0.7) in about 24 hours, then 1% of the enzyme pre-culture medium was transferred to 50 mL of the pectinase enzyme production medium and incubated at 30ºC with agitation speed of 110 g for 96 hours with observations every 12 hours. Crude enzyme extract was obtained from the culture centrifuged supernatant at a speed of 8500 g, temperature 4°C for 30 minutes. Enzyme activity was measured using the DNS method.

Galacturonic acid standard curve

The stock solution of galacturonic acid was prepared by dissolving 0.01 gram of galacturonic acid monohydrate in 10 mL of distilled water. Then, the stock solution was dissolved in acetate buffer (pH 5) with a different concentration (mg/mL) from 0-0.8 mg/mL. A total of 1 mL of a standard solution of galacturonic acid with various concentrations was put into each test tube. The samples were then incubated at 30ºC for 5 minutes, then 1 mL of DNS solution was added and homogenized using a vortex, then incubated again at 100°C for 5 minutes. The absorbance of the standard solution was measured with a spectrophotometer at a wavelength of 540 nm.

Enzyme specific activity measurement

Pectinase activity was determined using the colorimetric method. The measurement of enzyme activity was based on determining the levels of reducing sugars produced as a result of the enzymatic hydrolysis of pectin using dinitrosalicylic acid (DNS) reagent with galacturonic acid standards. One unit of pectinase activity is the amount of enzyme needed to break down pectin into 1 mol of galacturonic acid per minute under the conditions of the enzyme activity test.

A total of 1 mL of 1% citrus pectin in 9 mL of a solution of 0.05 M acetate buffer pH 5 was mixed into 1 mL of the crude enzyme. The mixture was then incubated in a 30 °C water bath for 5 minutes. The enzyme activity was stopped by mixing 1 mL of the reaction mixture with 1 mL of DNS, then stored in a water bath at 100°C for 5 minutes. The absorbance value of the sample was measured using a spectrophotometer at a wavelength of 540 nm. The absorbance value was then converted based on the standard curve to get the enzyme activity value.

Crude enzyme extract purification

The initial step of enzyme purification is concentration using ammonium sulfate salt. The crude extract enzyme was precipitated gradually at 4°C with ammonium sulfate to a saturation level of 80%. The required ammonium

sulfate was weighed from 0% to 80% saturation level, then added to the crude extract enzyme little until dissolved. Then the precipitate was centrifuged at 6,000 g at 4°C for 20 minutes. The supernatant was taken and used for further precipitation, while the pellet was dissolved with acetate buffer pH 5.

Dialysis was carried out by inserting the enzyme fraction with optimum enzyme activity into a cellophane tube with a knot at one end. Next, the cellophane tube filled with the sample was tied tightly at both ends, put into a 0.05 mM acetate buffer, allowed to stand overnight, and stirred slowly using a magnetic stirrer at 4°C, and the buffer was replaced periodically (6-8 times) for 24 hours. Before use, the cellophane tube was prepared by heating the cellophane tube into 200 mL of a boiling washing solution, namely 10 mM $NaHCO_3$ and 1 mM EDTA (Amid et al. 2014).

Enzyme immobilization entrapment method with alginate beads

Enzyme immobilization was carried out based on Bickerstaff (1997) with several modifications. The enzyme was added to the sodium alginate solution using a syringe, then the enzyme and alginate mixture (1:4) was slowly dripped into the calcium chloride solution (1:4), which would form alginate beads. Furthermore, the immobilized enzyme was stored in $CaCl_2$ solution for 20 minutes and then washed with 0.1 M acetate buffer solution pH 4.5 to remove unbound enzyme molecules. Alginate beads were stored in 0.05 M acetate buffer at 4°C for 14 days.

Optimizing enzyme conditions in immobilization were designed using the Response Surface Method (RSM) with Minitab or expert design. The variables analyzed were: Sodium alginate (0.5; 1; and 1.5%) and calcium chloride concentration (0.1; 0.2, and 0.3 M). The results of immobilization were taken as a response to the experimental design and calculated by the following equation:

$$IY\ (\%) = \frac{\text{Activity of immobilzed enzyme}}{\text{Acitivity of free enzyme}} \times 100$$

Where:

IY: Immobilzation Yield

Measurement of enzyme activity using the DNS method and measurement of protein content using the Bradford method.

The efficiency of storage and reuse of immobilized pectinase

Repeat use was calculated by determining the immobilized pectinase activity of the repeated use of the immobilized enzyme. The immobilized pectinase enzyme stored for 14 days was filtered using filter paper. The immobilized enzyme was added with substrate and buffer and then filtered again. The results of the precipitate were measured for the immobilized enzyme activity, and the alginate beads were used for the next hydrolysis cycle.

RESULTS AND DISCUSSION

Pectinase production curve

Determination of enzyme production time was carried out in order to obtain the highest pectinase activity. Observations were made for 96 hours, and samples were taken every 12 hours. Observations were made for 96 hours, and samples were taken every 12 hours. Pectinase activity (Figure 1) shows that at 12 to 36 hours, the pectinase activity produced is still deficient, with a value of 0.052 U/mL at 12 hours and 0.129 U/mL at 36 hours. The increasing incubation time of enzyme production caused the pectinase activity of *Bacillus* sp. 2P11 continued to increase and reached its optimum value at 60 hours with an enzyme activity of 0.391 U/mL. At 72 hours of incubation time, the activity of the pectinase enzyme decreased to 0.016 U/mL.

Enzyme purification by precipitation using ammonium sulfate and dialysis

The pectinase enzyme (Figure 2) was precipitated with ammonium sulfate salt, and the enzyme activity produced was 0.452 U/mL. The enzyme activity value was higher than the crude extract enzyme, which was 0.416 U/mL. Dialysis is a step that is carried out after the enzyme is concentrated with ammonium sulfate. The activity of the pure enzyme (Figure 2), which is the enzyme from dialysis, increased in value to 0.586 U/mL.

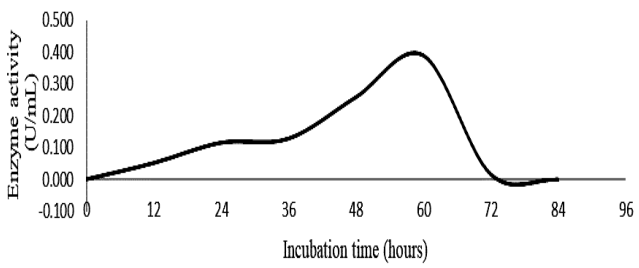

Figure 1. Pectinase production curve of 2P11 isolate at a substrate concentration of 10% pH 5 and a temperature of 30°C

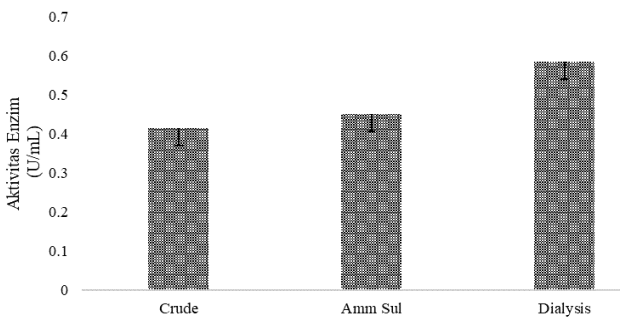

Figure 2. Pectinase activity of crude enzyme extract and after purification

Pectinase immobilization alginate beads entrapment method

The statistical analysis results for the surface model of enzyme immobilization response (Table 1) show that the p-value obtained for the parameters of sodium alginate concentration and $CaCl_2$ concentration is less than 0.05, indicates that the parameters of sodium alginate concentration and $CaCl_2$ concentration affect the enzyme immobilization response in immobilization response surface model equation. The combination of sodium alginate concentration and CaC_{l2} concentration showed a p-value greater than 0.05 means that they do not affect the immobilization response in the response equation.

The p-value for lack of fit is less than 0.05, which is 0.0016 indicates a lack of fit, so this model must be reconsidered if it is to be used. Meanwhile, the R^2 (coefficient determination) value explains that 73.3% of the data generated from the study influences factors from the treatment. At the same time, the rest comes from factors outside the experimental treatment. In this model, the R2 value is significant, and the F value in the model is 3.16, indicating that the model is significant (appropriate). The p-value in the model is 0.0322, indicating that only 3.23% is possible that the model's F value will cause interference.

The immobilization results showed that the parameters of sodium alginate concentration and $CaCl_2$ concentration had an influence on the enzyme immobilization response in the equation of the immobilization response surface model from the Response Surface Method (RSM), and the alginate concentrations of 0.5% and 0.3 M $CaCl_2$ which had high enzyme activity were in the third treatment (Figure 3) with an enzyme activity value of 0.239 U/mL. The percentage of immobilized enzymes is 40.782%.

Storage and reuse of immobilized pectinase

The immobilized enzyme was stored for 14 days, then repeated hydrolysis of the immobilized pectinase enzyme. Repeated immobilized pectinase enzymes (Figure 4) in this study were carried out by repeating the hydrolysis reaction of the immobilized enzyme five times. The results of repeated use of immobilized pectinase from *Bacillus* sp. 2P11 maintained more than 70% residual activity in cycle three and less than 20% repeated use in cycle five.

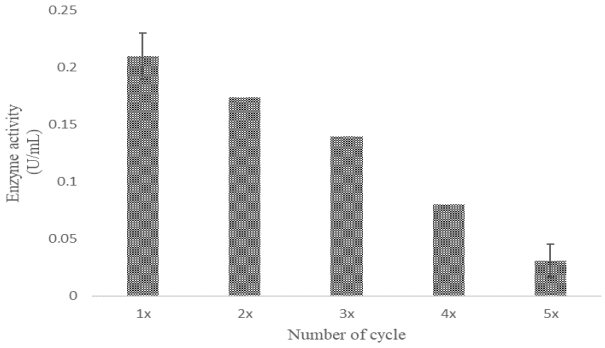

Figure 4. Reuse of immobilized pectinase enzymes

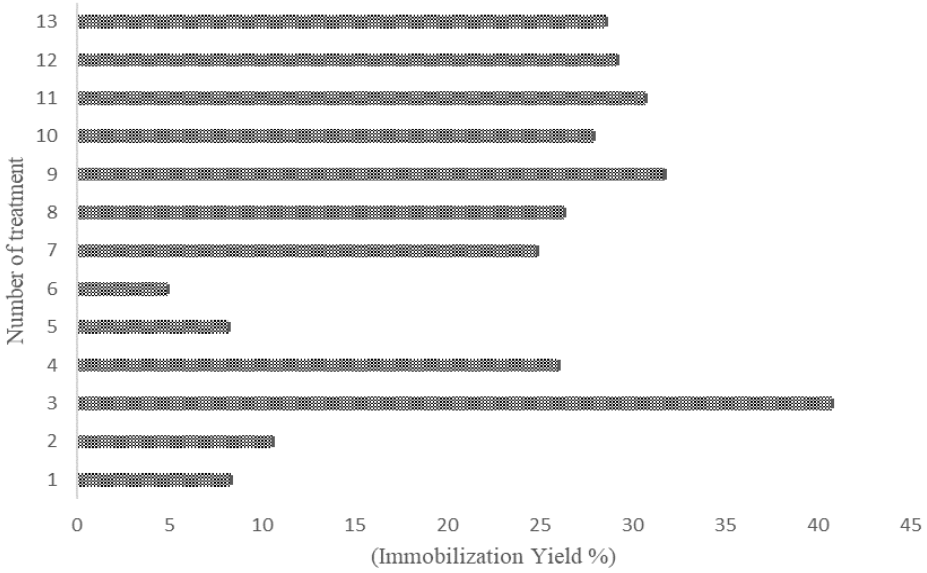

Figure 3. The activity of immobilized pectinase enzyme entrapment method using alginate beads

Table 1. Enzyme Immobilization ANOVA Regression

Source	ANOVA Regression Analysis Results Regresi					
	Sum of square	**db**	**Mean square**	**F-value**	**P-value**	
Model	1147.83	5	229.57	4.78	0.0322	significant
A-Na Alginate	37.16	1	37.16	0.7732	0.4084	
B-Cacl2	311.58	1	311.58	6.48	0.0383	
AB	72.55	1	72.55	1.51	0.2589	
A^2	723.48	1	723.48	15.05	0.0061	
B^2	3.14	1	3.14	0.0654	0.8056	
Lack of Fit	326.54	3	108.85	44.01	0.0016	significant
Pure Error	9.89	4	2.47			
Total	1484.27	12				
R2						73.3%

Discussion

The low enzyme activity during incubation could be due to the microbe *Bacillus* sp. 2P11 is in the lag phase of bacterial growth where bacteria adapt to their growth environment, namely production media containing pectin as a substrate from cocoa shells as a carbon source. The increase in incubation time for enzyme production causes the pectinase activity to increase and reach its optimum value at 60 hours. At 72 hours of incubation, there was a decrease in pectinase activity. This decrease in enzyme activity can be caused by a decrease in the nutrient content in the production medium, which causes the secretion of microbes to be increasingly limited, thereby inhibiting the formation of the enzyme-substrate complex. The results of measurements of pectinase activity that have been carried out for 80 hours are used as the incubation period of isolates on the production media.

The incubation time required to produce pectinase from *Bacillus sp.* 2P11, which is for 60 hours. The crude extract increases because the enzyme-producing microbes are already in the exponential phase, which is the phase where the cells have adapted to the environment. The production of pectinase between one microbe and another requires different times, depending on the growth medium and environmental conditions. Berutu et al. (2017) produced pectinase with the same isolate, *Bacillus* sp. 2P11 reported that with pectinase in these isolates, the optimum time for pectinase production was 48 hours. Several studies also reported that the production of pectinase by *Bacillus* could be carried out for 48 to 96 hours, including Namasivayam et al. (2011) reported that pectinase production was carried out during 48 hours of incubation, Tripathi et al. (2014) for 48 hours, Kaur et al. (2016) for 72 hours, Kumari et al. (2014) for 96 hours and Mukesh et al. (2012) which isolates *Bacillus* sp. MFW7 with optimum time to produce pectinase 96 hours. Alqahtani et al. (2022) reported that A short incubation time at the desired temperature makes the production cost-effective

The value of the crude extract activity of the pectinase enzyme produced was increased by purification. Enzyme purification consists of concentration with salt, dialysis and chromatography. The initial step of enzyme purification is concentration with ammonium sulfate. This concentration aims to reduce the crude enzyme extract of the pectinase enzyme produced from the content of other compounds.

The enzyme concentration with 80% ammonium sulfate was used because it is a simple, inexpensive method and does not damage the enzyme's structure. The dissolved ammonium sulfate will cause a salting-out process so that the protein will precipitate. A high concentration of ammonium sulfate is used based on a salting-out process that depends on the hydrophobicity of the protein, which causes a high salt concentration to encourage hydrophobic deposition on the protein surface (Jalil and Ibrahim 2021). Salting out is a separation process by changing the interaction of the stationary protein phase through ion protection. Alternatively, the deposition of proteins from the mobile phase leads to faster elution. Occurs due to competition between ions of an ammonium salt and enzyme molecules in interacting with water molecules (Moringo et al. 2019). The protein precipitation method using ammonium sulfate salt is more soluble at low temperatures (around 4ºC).

Dialysis is a process to separate or remove contaminants with a lower molecular weight than enzyme molecules. The principle of dialysis is the transfer of smaller molecules, where these molecules pass through a semi-permeable membrane by diffusion. Sample molecules larger than membrane pores will be retained on the sample side of the membrane. However, smaller molecules and buffers will pass through the membrane freely, reducing the concentration of molecules in the sample (Wasilah et al. 2020). The dialysis step was carried out in a cold environment at 4ºC to avoid decreased enzyme activity. The membrane for dialysis before use was treated by washing under running water for 3-4 hours, then soaking with sodium sulfide at 80ºC and washing with Milli q and sulfuric acid, which aims to avoid metal contamination. Research from Yopi et al. (2013) stated that the enzyme activity of crude extract from *Aspergillus ustus* was BL5 3.45 U/mL after purification of enzyme activity was 5.77 U/mL (Yopi et al. 2013).

Immobilization in this study uses the entrapment method with alginate beads as the polymer and $CaCl_2$ to form the alginate beads. This research was designed with the Response Surface Method (RSM) to optimize the amount of alginate and $CaCl_2$ needed so that the immobilized enzyme produced is optimal.

Immobilization is the physical binding to an enzyme phase that uses a matrix different from the substrates and

product. Inert polymers and inorganic materials are usually used as carrier matrices (Datta et al. 2013). In (Figure 3) shows that the two variables used have a statistical effect on the activity of the immobilized enzyme and shows that decreasing the concentration of sodium alginate can increase the yield of immobilized pectinase and increase the concentration of sodium alginate, decrease the activity of the immobilized enzyme. The activity of the immobilized enzyme was lower than that of the free enzyme because the alginate beads were hydrophilic, which resulted in a decrease in the ability of the hydrophobic substrate to reach the active site of the free pectinase enzyme. The decrease in immobilized enzyme activity was due to a decrease in the interaction between enzyme and substrate, which had to pass through the pores of the alginate beads to meet the substrate. Alginate beads have a relatively high molecular weight that interferes with the interaction between the substrate and the enzyme.

The use of alginate beads as a polymer in the entrapment method is due to the affordable price, cheap, and safe to use for food because it is an extraction from brown seaweed and can bind water and form a gel. According to Bucke (1982) entrapment method is an immobilization method that is often used for research due to its simplicity and good enzyme retention. In the entrapment method, the enzyme remains in its active form without any risk of closure of the active site.

Oliveira et al. (2018) research stated that the immobilized pectinase of *Aspergillus aculeatus* maintained more than 80% residual activity in the third cycle and more than 30% residual activity after repeated use by repeating the pectin hydrolysis reaction eight times. In this study, the immobilized activity showed a drastic decrease, and this may be due to leakage in the alginate beads, which can be caused by a mechanical movement and can also be caused when washing the alginate beads using distilled water.

This research concludes that the time required for the optimum production of the enzyme pectinase is 60 hours. Pectinase from dialysis had better enzyme activity than crude extract enzyme pectinase. Immobilization with sodium alginate and $CaCl_2$ will form alginate beads at a concentration of 0.5% sodium alginate and 0.3 M $CaCl_2$, which is optimum for immobilization and can achieve hydrolysis five times.

The suggestion for this research is that producing pectinase enzymes using substrates derived from natural ingredients of cocoa pods produces pectinase with better activity after undergoing the enzyme purification stage. Therefore, further purification steps such as chromatography can be carried out to get better pectinase activity. The immobilization of the pectinase enzyme using a design with the Response Surface Method (RSM) can be redesigned to obtain a better-immobilized enzyme.

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