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The Role of Calcium Ions to Improve Activity of Chitinase Isolated from *Vibrio* sp.

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Abstract

Chitinase (EC 3.2.1.14) plays a crucial role in chitin degradation, specifically by breaking down the 1→4 β-glycosidic bonds of N-acetyl-D-glucosamine (GlcNAc) to produce its mono- or oligomers. This study aims to study the characteristics of chitinase from *Vibrio* sp. (isolated from tiger shrimp in Indonesia) and to explore the role of calcium ions (Ca^{2+}) in increasing chitinase activity. The optimum condition for chitinase activities is pH 7.5, 45 ^{o}C of temperature, and 120 min of incubation time. The enzyme activity parameters such as K_m and V_{max} values were calculated by varying the concentration of Ca^{2+}, namely: 0 % ; 0.2 % ; 0.4 %; 0.6% ; 0.8 %. The final product of the chitinase reaction, which is the GlcNAc, is then used as a basis for measuring the enzyme activity based on the Somogyi-Nelson method. The results showed that chitinase isolated from *Vibrio* sp. has increasing activity with the addition of Ca^{2+}. Without the addition of Ca^{2+}, the K_m and V_{max} of chitinase were 7.781 µmol mL^{-1} and 0.066 µmol min^{-1}, respectively. The treatment of 0.4 % Ca^{2+} shows optimum activity with the K_m and V_{max} at 6.723 µmol mL^{-1} and 0.079 µmol min^{-1}, respectively. The results showed the potential use of Ca^{2+} as a chitinase activator to fulfill demands for energy-efficient and economically profitable chitinase usage.

Keywords: Eco-friendly waste management, Enzyme activity, Marine waste, Profitable chitinase, Utilization of Agro-industrial waste

1. Introduction

Chitinase is an enzyme that catalyzes the hydrolysis reaction of N-acetyl-D-glucosamine (GlcNAc) polymers. It can be used in the decomposition and re-utilization of chitin, one of the major marine waste problems (Atalla *et al.,* 2020). GlcNAc itself has been widely applied in the health sector, including reducing blood pressure and blood fat levels, suppressing cancer cell development, malignant tumors, and other inflammation, low-calorie sweeteners, cosmetics, biocontrol agents, and packaging materials (Awad *et al.*, 2014; Krolicka *et al.*, 2018; Rathore and Gupta, 2015; Van den Broek *et al.*, 2015; Veliz *et al.*, 2017). Thus, looking for the chitinase can be key to reduce the environmental hazards through eco-friendly waste management, and generate an added-value product which is important to the industry (Hamed *et al.,* 2015; Jahromi and Barzkar, 2018; Sadik *et al.*, 2021).

Chitinase is a chitinolytic enzyme in cell organisms and can be synthesized from various sources such as bacteria, fungi, and various other types of microorganisms. Chitinase synthesized by animals and plants has several functions, including helping the metabolic process and preventing infections, such as preventing the growth of fungi or bacteria that can damage the individual's tissues. Chitinase, which is produced by bacteria, has the function to degrade chitin into compounds that can fulfill the nutritional needs of these bacteria (Adrangi and Faramarzi, 2013). Recent chitinase isolated from several species of *Vibrio sp*. has optimum enzyme activity at pH 5 to pH 7 with incubation temperature at 45 ^{o}C to 50 ^{o}C (He *et al.*, 2020).

Vibrio sp. is a bacterium that is often found in prawns bred in brackish water or from the water itself (Felix *et al.*, 2011; Kharisma and Manan, 2012; Kusmarwati *et al.*, 2017). It can infect and cause diseases namely Vibriosis which can cause huge loss in shrimp culture. This disease is caused by bacteria *Vibrio* genera such as *V. harveyi, V. alginolyticus, V. parahaemolyticus,* and *V. penaeicida* (Utami, 2016). Some cofactors such as Na^{+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} can function as chitinase activators (Jahromi and Barzkar, 2018). The presence of

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calcium ions in brackish water is normal due to efforts to regulate salinity conditions, which later relate to the *Vibrio* survivability, osmoregulation, and post-larval metabolic ability of shrimp (Taqwa *et al.*, 2014). Based on these problems, it is necessary to examine the effect of Ca^{2+} in addition to increasing chitinase activity. After finding the chitinase optimum parameter, chitinase activity with and without the addition of Ca^{2+} will be compared. This can be known through the measurement of K_m (Michaelis-Menten coefficient) and V_{max} (maximum V value). The study aims to determine the role of Ca^{2+} ions to increase isolated chitinase activity from *Vibrio* sp.

2. Materials and Method

2.1. Research materials

The research was conducted in the Laboratory of Parasites and Fish Diseases, Department of Aquaculture, Faculty of Fisheries; Biochemistry Laboratory, Biomolecular Laboratory, Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang; Agricultural Product Technology Laboratory, Agricultural Biotechnology Center, Science and Technology of Food Laboratory, University of Muhammadiyah Malang; and Bionutrition and Food Innovation Laboratory, Faculty of Biotechnology, University of Surabaya. The materials and tools used include a tool to characterize chitinase and test the enzyme activity. The *Vibrio* sp. used in this research was obtained from previous work (Zafran *et al.*, 2017) located at Provincial Fish Seed Center (*Balai Benih Ikan Provinsi*-BBIP), Gondol, Bali. The strain was isolated from tiger shrimp (*Penaues monodon* Fabricius, 1798), and the only one which is non-pathogenic was used in this research.

2.2. Preparation of Vibrio culture

The solid medium was made for the propagation of *Vibrio* bacteria in a petri dish (Pyrex 3160-100), wherein how to make it followed previous work by Zarkasi *et al.* (2019) with some modification. Nutrient composition so that TCBSA (Thiosulfate-citrate-bile salts-sucrose agar, Oxoid, United Kingdom) for 1 L was needed as much as 88 g so that for making solid medium as much as 20 mL it was needed TCBSA as much as 1.76 g and chitin (Oxoid, United Kingdom) as much as 0.4 g. Nutrients to be dissolved in distilled water to a volume of 20 mL, heated to boiling for 10 min then put 10 mL each into petri dish that has been sterilized in an autoclave (All-American 25×, USA) at 121 ^oC, pressure 1.5 atm for 15 min (1 atm = 101 325 Pa). Furthermore, it was desirable for cooling it down at room temperature (25 ^oC) to harden. In another way, a liquid medium was used for *Vibrio* growth medium on growth curves and enzyme production. The medium used for making liquid medium was TSB (Tryptone Soya Broth, Oxoid, United Kingdom). To make 100 mL of liquid TSB medium, 3 g TSB, 0.5 g NaCl (Oxoid, United Kingdom), and 2 g chitin were dissolved in 100 mL distilled water and then sterilized in an autoclave at 121 ^oC, 1.5 atm for 15 min.

2.3. Isolation of crude chitinase

Vibrio sp. cultures that had been rejuvenated for 2 d (day) were taken using a loop wire and suspended in 20 mL of the sterile liquid medium in 125 mL Erlenmeyer flask (Pyrex 4450-125) and placed in a shaking incubator at room temperature (25 ^oC) for 16 h (half logarithmic phase). The 20 mL inoculum solution was inoculated into 200 mL of sterile liquid medium and grown at room temperature (25 ^oC) on a shaking incubator (Bionics, BST/MIS-100B, India) at speeds of 125 rpm (1 rpm = 1/60 Hz) to 32 h (stationary phase). Then it was centrifuged at 4 ^oC for 10 min at 3 000 rpm (Denley BR401, United Kingdom) and the supernatant was immediately tested for its enzyme activity. The enzyme was purified by the saturated ammonium sulfate (Oxoid, United Kingdom) precipitation method followed by dialysis and Sephadex 75G (Oxoid, United Kingdom) column chromatography method. Each enzyme fractionation was tested for chitinase activity (Harini and Indranila, 2006).

2.4. Chitinase Activity Test

The chitinase activity was tested by using Somogyi-Nelson assay. As much as 1 mL of 30 mg L^{-1} N-acetyl-D-glucosamine (Sigma-Aldrich, Germany) standard solution was taken and added with 1 mL of the Somogyi-Nelson cooper reagent (Sigma-Aldrich, Germany). The mouth of the tube was covered with aluminum foil, then heated in boiling water for 10 min. The tube was cooled in ice water and added with 1 mL of arsenomolybdate reagent, shaken, and allowed to stand for several minutes until the foam disappeared. After that, the distilled water was added up to 10 mL in volume and then shaken and measured the absorbance in the wavelength range of 500 nm to 800 nm by UV-vis spectrophotometer (Shimadzu-1601A, Japan), which maximum wavelength (λ_{max}) was 750 nm (Shalaby *et al.*, 2019). The arsenomolybdate reagent was made of 25 g of ammonium molybdate (Sigma-Aldrich, Germany) dissolved in 450 mL distilled water, 21 mL of concentrated H_2SO_4 (JT Baker, United States), and 3 g of $Na_2HAsO_4.7H_2O$ (Sigma-Aldrich, Germany) dissolved in 25 mL of H_2O, mix then place in an incubator (Heraeus B5042, Germany) at 37 ^oC for 24 h to 48 h. The standard N-acetyl-glucosamine curves were prepared by testing several N-acetyl-glucosamine concentrations [10, 20, 30, 40, 50, and 60) mg L^{-1}] following exactly Somogyi-Nelson assay which written above.

A total of 1 mL of 2.5 % (w/v) chitin in 0.1 M phosphate buffer pH 7.5 was added to 1 mL of chitinase solution. Then the mixture was incubated at 45 ^oC and after 120 min of incubation time, it was centrifuged at 5 000 rpm for 10 min. The enzyme activity was stopped by heating it in boiling water for 15 min before it was mixed with the reagent. Furthermore, the supernatant obtained was taken

as much as 1 mL to be tested by Somogyi-Nelson assay. The solution was diluted with distilled water to a final volume of 10 mL and read the absorbance at λ_{max} with a phosphate buffer blank which was treated the same as the sample. Enzyme activity value was measured by the levels of N-acetyl-D-glucosamine obtained from the plot results against the standard curve of N-acetyl-D-glucosamine. The measurement of enzyme activity was done by converting the absorbance value to the concentration of N-acetyl-D-Glucosamine (Yang *et al.*, 2016) and calculating it following equation (1):

$$\text{Enzyme Activity} = \frac{[N-\text{Acetyl}-D-\text{Glucosamine}]\ x\ V}{\text{Mr}\ N-\text{Acetyl}-D-\text{Glucosamine}} x \frac{df}{E\ x\ t} \quad (1)$$

Note: V = total sample volume (mL), E = amount of crude enzyme (mL), t = incubation time (min), df = dilution factor, Enzyme Activity (unit) = µmol N-acetyl-D-glucosamine which is produced by each mL of the enzyme each one minute under certain conditions. The N-acetyl-D-glucosamine obtained was measured absorbance by the UV-Vis spectrophotometer at λ_{max} 750 nm.

2.5. Determination of the optimum pH, temperature and incubation time

The determination of the optimum pH of chitinase was carried out with a variation of pH 6; pH 6.5; pH 7.0; pH 7.5; pH 8; and pH 8.5. Each test tube was provided with 1 mL chitin 2 % (w/v) in a phosphate buffer with variation of pH 6; pH 6.5; pH 7.0; pH 7.5; pH 8; and pH 8.5. Then 0.5 mL of enzyme filtrate was added and incubated at 45 ^{o}C for 60 min. The solution was centrifuged at 5 000 rpm for 10 min, then the filtrate obtained was taken 1 mL and added 1 mL of Somogyi-Nelson reagents and shaken. The mouth of the tube was covered with aluminum foil and heated for 20 min in boiling water. After chilling, 2 mL of arsenomolybdate reagent was added, mixed, and allowed to stand for 4 min. Subsequently, the solution was diluted with distilled water to a volume of 10 mL and the absorption was read at the λ_{max}. Then the activity was determined, where the optimum pH was determined from the graph of the relationship between changes in pH of the enzyme activity.

Determination of the optimum temperature was done by the same procedure as the method used to determine the optimum pH, while the incubation temperature was varied at (30, 35, 40, 45, 50, 55) ^{o}C for 60 min at pH 7. Then the activity was determined based on the absorbance value of the molybdenum blue complexes which is equivalent to the amount of N-acetyl-D-glucosamine produced from the enzymatic reaction of chitinase. The optimum temperature was determined from a graph of the relationship between temperature changes and enzyme activity.

The optimum incubation time was determined by the same procedure as the method used to determine the optimum pH and temperature, while the incubation time variations were (30, 60, 90, 120, 150, 180) min, at 45 ^{o}C and pH 7. Then the activity was determined based on the absorbance value of the molybdenum blue complexes which is equivalent to the amount of N-acetyl-D-glucosamine produced from the enzymatic reaction of chitinase. The optimum incubation time was determined from a graph of the relationship between time changes to enzyme activity.

2.6. Effect of Ca2+ ions

Addition of Ca^{2+} ions to increase chitinase activity was done by checking the chitinase reaction with variations in Ca^{2+} concentration, as follows: (0; 0.2; 0.4; 0.6; 0.8) %. For each kind of treatment, K_m and V_{max} were measured. The enzyme activity and the K_m and V_{max} values were determined employing the linear regression from the graph of the relationship between V^{-1} and $[S]^{-1}$ (or called Lineweaver-Burk curve). Those enzyme reactions were done using the optimum parameter (pH, temperature, incubation time) which was first tested.

3. Results

3.1. Optimum Condition of Chitinase Activity

Based on sequential trials on each reaction parameter, such as pH, temperature, and incubation time, it is shown that chitinase from *Vibrio* sp. has optimum reaction condition at pH 7.5 (Figure 1A), the temperature of 45 ^{o}C (Figure 1B), and 120 min incubation time (Figure 1C).

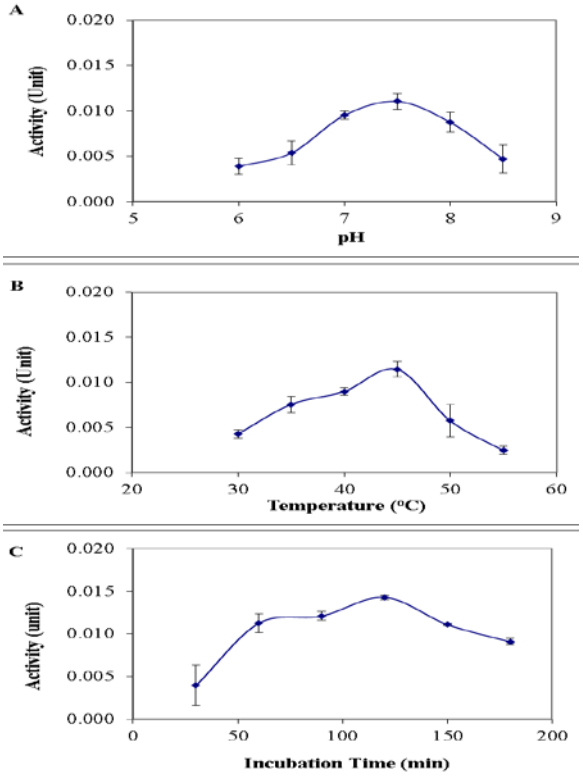

Figure 1. Optimum Condition Chitinase Activity at various pH (A), temperature (B), and incubation time (C).

3.2. Determination of Km and Vmax

In this study, the determination of K_m and V_{max} of chitinase were carried out with variations in substrate (chitin) concentration as follows: (1; 1.5; 2; 2.5; 3; 3.5) % (w/v). The relationship of enzyme activity to substrate concentration is shown in Figure 2. It shows that the greater concentration of chitin, the greater the speed of the enzymatic reaction to a point where an increase in substrate concentration does not significantly increase the reaction speed.

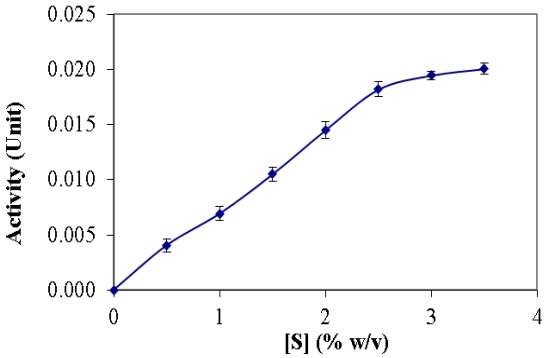

Figure 2. Relationship Curve of Chitinase Activity by Various Chitin Concentrations

Based on the Lineweaver-Burk curve shown in Figure 3 the values of K_m and V_{max} can be determined from the equation Y = aX + b. The value of V_{max}^{-1} is 15.145 and K_m V_{max}^{-1} is 117.84, so the V_{max} is 0.066 µmol min^{-1} and K_m is 7.781 µmol mL^{-1}.

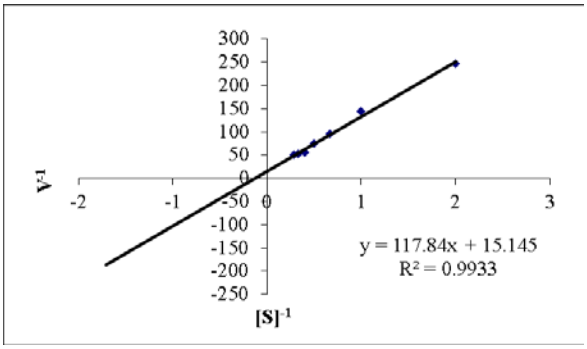

Figure 3. Lineweaver-Burk Curve of Chitinase Activity Isolated from *Vibrio sp.*

3.3. The Role of Calcium Ions on Chitinase Enzyme Activity

Figure 4. shows that Ca^{2+} increases enzyme activity until the addition of 0.4 % (w/v), whereas above 0.4 %, the enzyme activity starts to decline. The enzyme can be saturated with Ca^{2+} because the ability of the allosteric site to bind Ca^{2+} decreases. Thus, the addition of excess Ca^{2+} may cause denaturation on the enzyme which is characterized by decreased enzyme activity (Kumari *et al.*, 2010).

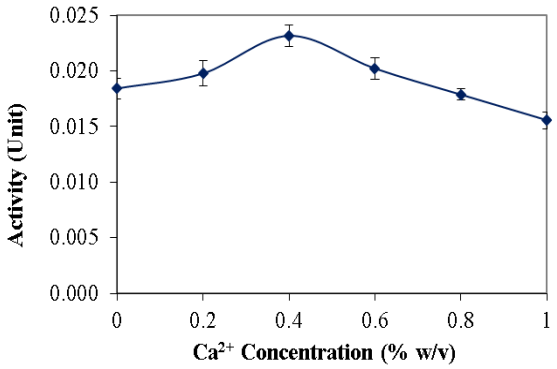

Figure 4. Chitinase Activity with the Addition of Ca^{2+}

The effect of chitinase activity in the presence of Ca^{2+} ions on various variations is shown in Figure 5. Changes in activity caused the price of K_m and V_{max} to change. The value of K_m obtained is 6.723 µmol mL^{-1} and V_{max} is 0.079 µmol min^{-1} (Figure 6).

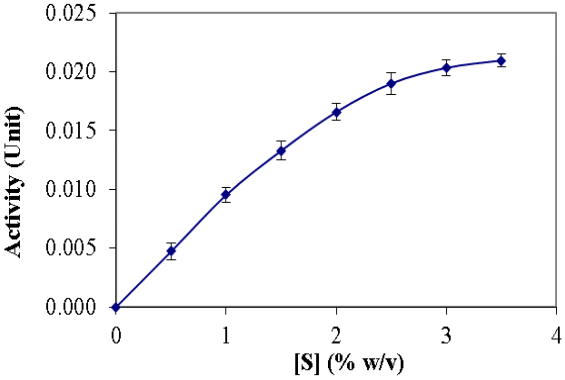

Figure 5. Chitinase Activity in the Various Concentration of the Substrate with Ca^{2+} Addition

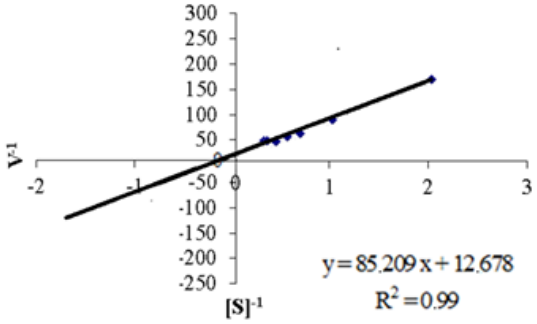

Figure 6. Lineweaver-Burk Curve Chitinase Activity by Addition of Ca^{2+}

4. Discussion

The optimization results of chitinase activity (Figure 1) are concurrent with the previously reported study of He *et al.* (2020), which showed optimum chitinase activity at pH 5 to pH 7 with incubation temperature at 45 °C to 50 °C. However, it quite a different condition compared to other

work of Nguyen and Nguyen (2020), which stated warm temperature (30 ^{o}C or 35 ^{o}C) and mildly alkaline pH (8.0) are the best conditions of chitinase from *V. parahaemolyticus*, one of the pathogenic species which infect shrimp.

One of the important things to know about the characteristics of an enzyme is the determination of K_m and V_{max}. The Michaelis-Menten constant (K_m) is a certain substrate concentration when the enzyme reaches half the maximum speed. Whereas Vmax is the maximum speed of an enzyme. To determine the value of K_m and V_{max}, measurement of enzyme activity was done at various concentrations of substrate, under optimum conditions (pH 7.5, temperature 45 ^{o}C, and 120 min incubation time). Thus, the K_m indicates the amount of substrate needed to obtain high chitinase activity as indicated by V_{max}, which is the fastest enzyme reaction achieved at the optimum concentration (Nakamura *et al.*, 2018). According to Robinson (2015), the speed of enzymatic reactions will increase with increasing substrate concentration until finally reaching a stationary point. After exceeding that point, although the substrate amount is increased, the increase of enzyme reaction speed is very small (almost constant), but will never reach the maximum condition. This condition limit is called the maximum speed (V_{max}) where the enzyme becomes saturated by its substrate, as shown in Figure 2.

The Michaelis-Menten equation for precisely determining K_m and V_{max} is quite difficult. For this reason, the Michaelis-Menten equation is transformed into the Lineweaver-Burk equation. The Lineweaver-Burk equation is the opposite of the Michaelis-Menten equation which can determine K_m and V_{max} precisely, as shown in Figure 3. Thus the substrate concentration added was equal to the value of K_m. Whereas the V_{max} value shows that the speed of the formation of the final product (N-acetyl-D-glucosamine) and the speed of returning to the enzyme chitinase must be the same as the speed of the breakdown of chitin that is equal to 0.066 µmol min^{-1}.

Calcium ions can form bridge complexes with enzymes and substrates according to their role in enzymatic reactions. Ca^{2+} can function as an enzyme inhibitor but can also be an enzyme activator. Enzymes that require Ca^{2+} as activators especially in extracellular enzymes (Bilecen and Yildiz, 2009; Garrison-Schilling *et al.*, 2011). It plays an important role in modifying the structure needed for their catalytic activity. With the addition of substrate concentration, chitinase activity also increases with a fairly high increase in the concentration of 2.5 % (w/v). Calcium ion acts as an activator for enzymes that work to hydrolyze a macromolecule. The Ca^{2+} ions change the conformation and orientation of the active site of the enzyme, also increase the activeness of the enzyme to bind to the substrate to form an Enzyme-Substrate complex. The added Ca^{2+} influences chitinase activity because Ca^{2+} act as cofactors so that they can increase chitinase activity. The effect of Ca^{2+} on the chitinase isolated from *Vibrio* sp. is shown in Figure 4.

The presence of Ca^{2+} ions causes an increasing number of enzymes that bind to the substrate. This causes a greater level of substrate saturation as indicated by changes in the values of K_m and V_{max}. Determination of K_m and V_{max} values was carried out by measuring chitinase activity with an optimum Ca^{2+} concentration of 0.4 % in each variation of substrate concentration (Figure 5). The value of K_m and V_{max} (Figure 6) provides information that the substrate concentration needed to reach half of the maximum speed becomes smaller (13.5 % fewer) and the enzymatic reaction becomes faster (19.7 % faster) at the optimum concentration with the addition of Ca^{2+} ions. In the other words, additional Ca^{2+} up to 0.4 % can provide benefits of larger chitinase activity and lesser required chitin.

Hydrolysis of chitin by chitinase with Ca^{2+} shows that the Calcium on the allosteric site (Asp-140) binds the substrate which can then change the active site conformation (Asp-142) to become active and bind to the substrate. When the conformation Asp-142 corresponds to the substrate and there is an interaction between those two, Ca^{2+} ions are released. The release of Ca^{2+} causes Asp-142 to rotate so that it is oriented closer to Glu-144 and hydrogen bonds occur. The interaction between Glu-144 and chitin causes the breakdown of glycosidic bonds and N-acetyl-D-glucosamine is formed. The interaction between Asp-142 and the amine group chitin and chitin with water also causes the formation of N-acetyl-D-glucosamine (Paknisa, 2014).

The presence of Ca^{2+} ions causes an increasingly enzymatic reaction which is characterized by an increase in chitinase activity after adding Ca^{2+} ions. The increase in chitinase enzyme activity is still relatively smaller compared to the research of Park *et al.* (2000) after adding Ca^{2+} ions. This is allegedly due to the concentration of the addition of Ca^{2+} ions which are relatively small and the use of chitin substrate in the form of powder, so that chitinase work is less optimal than the use of chitin substrate in the form of colloids and the addition of greater Ca^{2+} ion concentrations. The presence of Ca^{2+} causes conformation changes that make enzymes bind easier with the substrate, thereby increasing the saturation of the enzyme to the substrate as indicated by the increase in K_m and V_{max} values.

5. Conclusion

The optimum reaction parameters for the chitinase isolated from *Vibrio sp*. are pH 7.5, temperature 45 ^{o}C, and 120 min incubation time. The chitinase activity increases until the addition of Ca^{2+} 0.4 % and decreases with an increasing concentration of Ca^{2+} above 0.4 %. The value of K_m and V_{max} before adding Ca^{2+} was 7.781 µmol mL^{-1} and 0.066 µmol min^{-1}, respectively. After adding Ca^{2+}, the value of K_m and V_{max} was 6.723 µmol mL^{-1} and 0.079

µmol min^{-1}. In conclusion, chitinase kinetic parameters with the addition of Ca^{2+} affects K_m (13.5 % fewer substrate) and V_{max} (19.7 % faster), which means its reaction efficiency is improved.

6. Competing interest statement

The authors have declared that no competing interest exists in the manuscript.

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