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Partial Characterization of Chitosanase from *Bacillus cereus* Strain BFE5400 Isolated from Snakehead Fish Intestine

Karakterisasi Parsial Kitosanase *Bacillus cereus* Strain BFE5400 yang diisolasi dari Saluran Pencernaan Ikan Gabus

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Abstrak Pola makan ikan gabus (*Channa striata*) yang memangsa udang dan krustasea kecil menjadikan saluran pencernaannya sumber yang baik untuk mengisolasi enzim maupun mikroba penghasil enzim kitinase dan kitosanase. Tujuan dari penelitian ini adalah untuk mengisolasi bakteri penghasil enzim kitosanase dari saluran pencernaan ikan gabus, memilih isolat dengan indeks kitosanolitik tertinggi dan menggunakannya dalam proses produksi enzim serta melakukan karakterisasi parsial enzim kitosanase ekstrak kasar yang dihasilkan. Hasil penelitian menunjukkan bahwa isolat I2 memiliki indeks kitosanolitik tertinggi dengan nilai 1,5 dengan aktivitas enzim 0,00331 U/mL setelah 72 jam inkubasi. Densitas bakteri tertinggi berada pada jam ke-24 dengan nilai turbiditas 0,5315. Aktivitas enzim optimal tercapai pada pH 6, dengan nilai 0,00816 U/mL, sedangkan suhu optimum adalah 70°C dengan aktivitas enzim 0,00785 U/mL. Identifikasi genetik isolat I2 menggunakan 16S rRNA menunjukkan bahwa isolat tersebut merupakan *Bacillus cereus* strain BFE 5400.

Kata kunci: *Bacillus cereus;* kitosanase; ikan gabus; saluran pencernaan

Abstract Feeding pattern of wild snakehead fish (*Channa striata*) which includes shrimp and small crustaceans makes it is intestine a good source of chitinase and chitosanase enzymes. This study aimed to isolate chitosanase producing bacteria from snakehead fish intestine, to select isolate with highest chitosanolytic activity, to produce chitosanase using the selected isolate and partially characterize the resulting enzyme. I2 isolate exhibited the highest chitosanolytic index with the value of 1.5 and produced enzyme with an activity of 0.00331 U/mL after 72 hours incubation. The ultimate bacterial density was at the 24th hour with a turbidity value of 0.5315. The enzyme activity was optimum at pH 6, with a value of 0.00816 U/mL, while the optimum temperature was 70°C with the enzyme activity of 0.00785 U/mL. Genetic identification of I2 isolate using 16S rRNA showed that the isolate has a high similarity to *Bacillus cereus* strain BFE 5400.

Key words: *Bacillus cereus;* chitosanase; snakehead fish; intestine

INTRODUCTION

The enzyme technology has developed rapidly and occupies an important position in the catalyst industry. The use of enzymes as biocatalyst offers more advantages as they are safer and have high specificity for the substrate compared to traditional chemical catalysts.

Chitosan oligomers or chitosan oligosaccharides are the hydrolysates of chitosan which show a wide range of bioactivity, including antibacterial, antifungal, antioxidant and antimicrobial activity (Ibrahim *et al*., 2016). Both chemical and physical processes have been used to produce chitosan oligomers by using hydrochloric acid (Kasaai *et al*., 2013) and ultrasonic irradiation (Kasaai *et al*., 2008) or combination between degradation method using hydrogen peroxide and microwave radiation (Sun *et al*., 2007). Employing concentrated hydrochloric acid for chitosan hydrolysis has some drawbacks like harsh conditions, many purification steps to remove the strong acid and low yields of chito-oligomers (Ibrahim *et al*., 2016). On the other hand, the use of ultrasonic irradiation is only suitable to obtain moderate-size macromolecules due to its partial depolymerization ability. The enzymatic process is known to produce more uniform oligomers with a high degree of polymerization (Shahidi *et al*., 1999).

Chitosanase is an enzyme that can cleave chitosan polymers. It is generally an endo splitting enzyme and can hydrolyze chitosan to chitosan oligomers and glucosamine as the product of hydrolysis (Chiang *et al*., 2002). Chitosanase is known to occur widely in various microorganisms such as molds, actinomycetes, and bacteria. The difference between chitosanases from each organism is in their hydrolytic action pattern. This property depends on the degree of acetylation of the substrate. Moreover, the difference in biochemical properties is also observed between chitosanases from various sources (Somashekar & Joseph, 1996). Therefore, the search for a new source of chitosanase is very important to produce specific-size chitosan oligomers.

The digestive tract of snakehead fish (*Channa striata*) is a good source of chitinase and chitosanase enzymes. This

carnivorous fish feeds mainly on live animals such as frogs, small fish, shrimp, and crustaceans. The digestive tract of fish provides a good habitat for bacteria. However, very few reports are available on chitosanase producing bacteria associated with snakehead fish intestine as well as on the characteristics of the enzyme.

MATERIALS AND METHODS

Materials

The materials used in this study were physiological salts (NaCl 0.85%), chitosan from shrimp shell >75% deacetylated (Sigma Aldrich), HCl, NaOH, K_2HPO4, $MgSO4.7H_2O$, NaCl, $(NH4)_2SO4$, phosphate buffer, citrate buffer, citrate-phosphate buffer, borate buffer, and Schales reagent ($K_3[Fe(CN)_6]$ and Na_2CO_3), alcohol, glycerol 85% emsure (Merck), bacteriological agar and yeast extract (Oxoid), aquadest and sintered glass filter.

Equipment used in the study consisted of vortex, laboratory glassware, autoclave (Hirayama HL36AE, Japan), analytical scales (Ohaus), spectrophotometer (Shimadzu UV1800, Japan), pH meter (Hanna HI98107, USA), incubator shaker, micropipette (Eppendorf), incubator (Memmert IN55, Germany), microtube (Eppendorf), centrifuge (Hettich Universal 320R Benchtop Centrifuge, Germany) and bunsen burner.

Methods

Media preparation

Colloidal chitosan was prepared according to Pagnoncelli *et al.* (2010). Ten grams of chitosan powder was dissolved in 1 L of HCl solution (0.1 M) with continuous stirring. Insoluble materials in the suspension were separated by filtration through sintered glass filters. The pH of the suspension was adjusted to 7.0 by the addition of 10 N NaOH.

The chitosan broth was prepared by supplementing the Minimal Synthetic Medium which consists of 0,1% K_2HPO4, 0,01% $MgSO4.7H_2O$, 0,1% NaCl, 0,7% $(NH4)_2SO4$ and 0,05% yeast extract with 1% colloidal chitosan (Chasanah *et al*., 2011). As for chitosan agar, 2% of bacteriological agar was added to the medium. The media solution was sterilized by autoclaving at 121°C for 15 min.

Schales' reagent was prepared by diluting 0.5 g K_3 [Fe (CN)$_6$] in 1 L Na_2CO_3 0.5 M to form a 0.5 M sodium carbonate solution containing 0.05% potassium ferricyanide.

Sample preparation

Snakehead fish weighing ±1,5 kg each and a length of ±60 cm were obtained from fish traders at the Indralaya market, Ogan Ilir, South Sumatera. The gut was dissected out from the animal in a-septic condition. The digestive tracts (25 g) were homogenized in 225 mL of sterile (pre-autoclaved 121°C, 15 min) physiological saline solution (0.85% NaCl) and used as a bacterial stock solution.

Isolation of chitosanase producing bacteria (Setia & Suharjono, 2015)

Instead of plating directly on chitosan agar, the bacterial stock solution was firstly inoculated into 100 mL of chitosan broth media in 250 mL closed laboratory flasks and incubated in incubator shaker (30°C, 150 rpm) for 72 h. This aimed to support the growth of chitosanase-producing bacteria. After 72 h, 0.1 mL of the sample was transferred into the chitosan agar medium by using the spread plate technique and incubated at 37°C for 48 h. Chitosan hydrolysis was indicated by zones of clearing around the colonies. Individual colonies with clear zones were picked from each plate and transferred to subculture. The final quadrant streak was done to obtain pure culture in a single colony.

Chitosanolytic index measurement

Bacterial isolates with chitosanolytic activity can show clear zones on chitosan agar plates containing colloidal chitosan. The ratio between clear zones diameter and colony diameter is referred to chitosanolytic index. Both clear zones and colony diameter were measured three times by including the longest and the shortest diameter. The average diameter value was used for chitosanolytic index measurement.

Production of crude enzymes

An isolate giving the highest chitosanolytic index was used in enzyme production. The production of chitosanase enzyme followed the method proposed by Chasanah *et al.* (2011) with slight modification. One loop of selected isolate colony was grown in a laboratory flask containing 20 mL of chitosan broth medium. The flask was tightly closed and incubated in an incubator shaker at 37°C, 100 rpm up to 96 h. Culture media was collected and centrifuged for 20 minutes (4°C, 9,000 rpm) using Hettich 320R centrifuge with angle rotor 6-place. The supernatant produced after centrifugation was considered a crude enzyme solution that will be used in the chitosanase activity test. Duplicate flasks were withdrawn at 24 h intervals and analyzed for cell growth and enzyme activity. Cell growth was measured by absorbance at 660 nm.

Chitosanase activity (Chasanah et al., 2011)

Chitosanase activity was assessed by measuring the reducing sugars produced from chitosan hydrolysis. A sample mixture consisting of 150 µL of 1% colloidal chitosan, 150 µL of 0.05 M phosphate buffer pH 6.0 and 150 µL of enzyme solution was prepared. Separately, a control mixture was made of 150 µL of colloidal chitosan and 150 µL of 0.05 M phosphate buffer pH 6.0. Both samples and controls were then incubated for 30 minutes at 37 °C followed by enzyme inactivation by boiling for 3 minutes. Subsequently, as much as 200 µL of both samples and control were added with 1000 µL of Schales' reagent and 800 µL of distilled water in 2 mL microtubes, wrapped in aluminum foil, incubated a water bath (100 °C, 15 min) and cooled. The solution was centrifuged (8000 rpm, 10 min) and the reducing sugar formed in the supernatant were estimated spectrophotometrically by measuring absorbance at 420 nm. The value recorded was converted into an amount of glucosamine (GlcN) by the standard curve prepared using GlcN.

According to Ferrari *et al.* (2014), the reaction between reducing sugars obtained from chitinase activity and Schales' reagent will contribute to the fading of yellow color which can be monitored at 420 nm. One unit of chitosanase activity was taken as the amount of enzyme

that could liberate 1 µmol of reducing sugar as glucosamine equivalents per minute under the conditions described above. Glucosamine levels in samples were calculated based on glucosamine standard curves with concentrations of 0, 20, 40, 60, 80, 100, 120, 140, and 160 µg/mL.

Determination of optimum pH and temperature

Determination of optimum pH was carried out by reacting enzymes on buffers with a variety of pH values ranging from 3 to pH 9. The buffer used in determining the optimum pH was citrate buffer (pH 3.0-4.0), citrate-phosphate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0), boric buffer (pH 8.0-9.0). The procedure for measuring enzyme activity at each pH treatment was carried out by the measurement standards described earlier.

The determination of the optimum temperature was done at the enzyme's optimum pH. The temperature variations used in this research were (30, 37, 50, 60, 70 and 80°C). Initially, the enzyme was mixed with the substrate and phosphate buffer solution in the microtubes. The homogeneous mixture solution was incubated for 30 minutes at the desired temperature. The measurement of enzyme activity at each temperature treatment was carried out similarly to the standard test described earlier.

Identification of isolates

Two loops of chitosanolytic bacteria were inoculated in nutrient broth (Merck) and incubated overnight at 120 rpm, 30°C. As much as 3 mL of the culture was centrifuged (13,000 rpm, 5 min) and the pellet was used for genome extraction following the method described by Ausubel *et al.* (1997).

Amplification of 16S ribosomal RNA gene sequence was done using primer pairs of 9F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1541R (5'-AAG GAG GTG ATC CAG CC-3'). The composition of 50 µL Kappa ready mix master kit for PCR consisted of 20 µL dH2O, 25 µL PCR master mix, 2 µL Primer 9F (20 pmol), 2 µL Primer 1541R (20 pmol) and 1 µL template DNA. PCR Thermal Cycler was run with the following programs for 30 cycles: denaturation (96°C, 30 s), annealing (55°C, 30 s), elongation (72°C,1 min) and extension (72°C, 7 min).

PCR products were electrophoresed on 1% agarose gel in TAE 1x buffer, 100 volts for 30 min and visualized using gel illuminator. After sequencing, the nucleotide sequences of 16S ribosomal RNA was analyzed using NCBI BLAST. The whole identification process was carried out in the Biotechnology Research Center, Indonesian Institute of Science (LIPI).

RESULTS AND DISCUSSION

Chitosan is a linear nontoxic polycationic polysaccharide consisting of d-glucosamine (GlcN) and N-acetyl-d-glucosamine (GlcNAc) units (Rydz *et al.*, 2018). The chitosanolytic index was calculated by comparing the diameter of the clear zone with the diameter of the colony. The clear zones were varied depending on the ability of bacterial isolates to produce enzyme and hydrolyze colloidal chitosan on agar medium. The magnitude of the clear zone produced depends on the number of monomers N - Glucosamine produced from the chitosan hydrolysis process by breaking down the β – 1,4 homopolymer N - Glucosamine bond. The greater the number of monomers produced, the greater the clear zones formed around the colonies. Every isolate collected in this research displayed varieties of the chitosanolytic index as presented in Table 1.

Table 1. Chitosanolytic index of isolates.

Isolates	Chitosanolytic Index
I1	1.33 ±0.11
I2	1.5 ± 0.57
I3	1.17 ±0.98

Isolate I2 produced the highest clear zone with the index value of 1.50 and then was used in the production of crude chitosanase. The clear zones around bacterial colonies were started to form after 48 hours of incubation. In another study conducted by Chasanah (2007) in marine bacteria associated with sponges, the incubation time required by the bacteria to obtain chitosanolytic index values was for 5 days (120 hours) with cchitosanolytic index values ranging from 1.05-5.40. According to Swiontek-Brzezinska *et al.* (2007), each bacterium has different chitosan degrading speed which depends on several factors such as temperature, substrate concentration, incubation time, and pH.

Chitosanase activity of the culture was determined by the measurement of glucosamine liberated from chitosan by the action of the enzyme under assay condition. The optimum incubation time for enzyme production and highest chitosanase activity was observed in 24, 48, 72, and 96 hours in terms of production of d-glucosamine (GlcN) which was indicative of the degradation of chitosan. The relation between the chitosanase activity of isolate I2 and cell turbidity which was measured as optical density at 660 nm is presented in Figure 1.

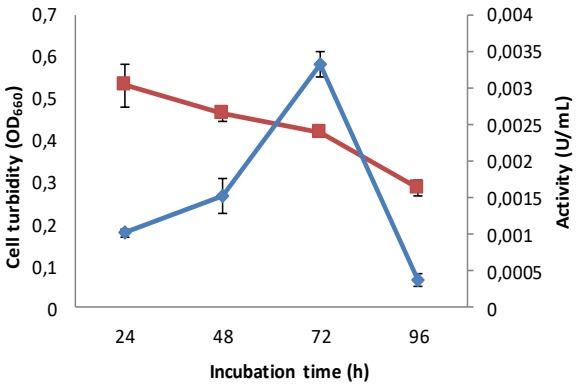

Figure 1. Relation between incubation time, cell turbidity (C1)and enzyme activity (C0) of I2 isolate.

The chitosanase activity of I2 isolate showed a slight increase at the first 48 h, but then rocketed and reached the maximum value of 0.00331 U/mL after 72 h incubation. However, the activity was then decreased to the lowest activity of 0.00036 U / mL in the 96th hour. As for the turbidity, cell density decreased steadily from 0.5315 at first 24 h to 0.2860 at the final observation hour. During their growth, bacteria utilize nutrients such as K□HPO4,

and MgSO4 which function as enzyme's cofactors and yeast extract as a source of nitrogen. As nutrients depleted, cell growth entered the decline phase. The lag and log growth phases in this research occurred at the time between 0 and 24 h. This result is in line with Chasanah (2007) who found that the log phase of chitosanolytic bacteria started from the 0 to 12 h, stationary phase observed at 16 to 24 h followed by declining phase at the 48 h. The optimum incubation time of 72 h with the highest chitosanolytic activity is by the results of Choi *et al.* (2004) which showed that *Bacillus* sp. KCTC 0377BP optimally produces enzymes within 72 hours of incubation.

Each enzyme has the optimum pH and temperature where it displays its maximum activity. The pH condition of the enzymatic reaction affects the ability of the enzyme to hydrolyze the substrate. The use of correct buffers to maintain the pH of the reaction is important as an inappropriate buffer and pH values can lead to disruption of substrates-enzymes interactions. As enzymes are proteins composed of amino acids, the changes in pH are closely related to the acid-base properties of proteins. Each reaction that is catalyzed by enzymes occurs more quickly at a certain pH. If the medium is very acidic or highly alkaline the enzyme undergoes inactivation. The effects of pH on isolate I2 chitosanase activity is reported in Figure 2.

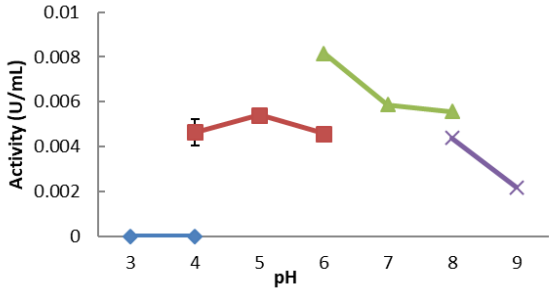

Figure 2. Chitosanase activity of I2 isolate as effected by pH and buffer types [citrate (C0) ; citrate-phosphate (C1) ; phosphate (C2) and boric (C3)].

The crude enzyme activity was tested in various pH using different buffers to determine the pH dependence of chitosanase activity. Citrate, citrate-phosphate, phosphate and boric buffers were used to create a range of pH values in the reaction mixtures. The highest chitosanase activity was obtained at pH 6, which was equal to 0.00816 U/ml. This result is similar to Pagnoncelli *et al.* (2010) who observed the highest chitosanase activity of *Paenibacillus ehimensis* at pH 6.

According to Muchtadi *et al.* (1996), enzymes are ampholytic, which means that they have associative constants in acid groups or their base groups. It is estimated that changes in enzyme activity were due to ionization in the ionic group which plays a role in maintaining the active side conformation in binding the substrate and in converting the substrate into a product. Ionization can also present in substrate or substrate enzyme complex, which also affects enzyme activity. Changing the pH of the solution changes the number of charged groups on the protein molecules

A rise in temperature to a certain extent in a reaction causes an increase in the speed of the enzyme reaction due to increased kinetic energy which speeds up the vibrational motion, translation and rotation of enzymes and substrates, thereby increasing the chances enzymes-substrates reaction. At temperatures greater than the reaction limit, the enzyme protein can experience a detrimental change in the three-dimensional arrangement of the polypeptide chain (Suhartono,1989). The effect of temperature on chitosanase activity is presented in Figure 3.

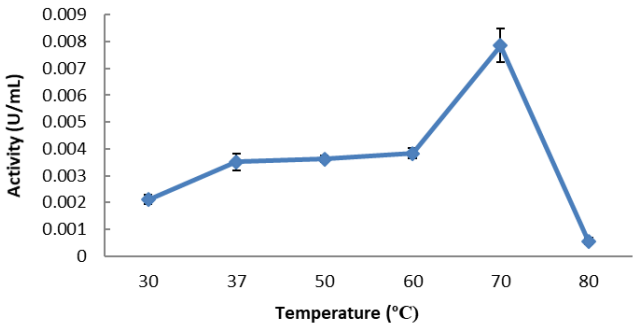

Figure 3. Effects of temperature on chitosanase activity of isolate I2.

Based on the data, the enzyme reached its highest activity at 70 °C with a value of 0.00785 U/ml. Increasing temperature to 80°C led to decrease inactivity. This shows that enzymes can work optimally at temperatures that are different from the growth temperature of the enzyme-producing bacteria. The optimum temperature of the I2 enzyme is similar to the previous study on chitosanase enzymes from *Bacillus coagulans* LH2838 (Haliza, 2003). However, this result is slightly different from extracellular chitosanase produced by *Bacillus subtilis* CH2 isolated from the intestine of scorpionfish (*Sebastiscus marmoratus*) which has optimum pH and temperature of 5.5 and 60 oC, respectively (Oh *et al.*, 2011).

Genetic identification of isolate I2 using 16s rRNA reveals that the isolate has a 95% similarity to *Bacillus cereus* Strain BFE 5400. The 1438 base pairs length nucleotide sequence was used as a query.

Other *Bacillus cereus* strains were also known as chitosanase producers. *Bacillus cereus* S1 studied by Kurakake *et al.* (2000) produced chitosanase with 45.000 Da molecular weight and exhibited optimum activity at pH 6 and temperature around 60°C. *Bacillus cereus* D-11 isolated from Taiwan soils was characterized by 41 kDa molecular weight with optimal pH and temperature were 6.0 and 60°C, respectively (Gao *et al.*, 2008).

CONCLUSIONS

The digestive tract of snakehead fish is a potential source of chitosanase-producing bacteria. *Bacillus cereus* Strain BFE5400 has been isolated from snakehead fish intestine and the enzyme has been partially characterized. Incubation time to produce a bacterial cell with the highest chitosanase activity was 72 h. Enzyme activity was optimum at pH 6 and temperature of 70°C. Further purification is needed to remove nonenzyme protein and other components that may interfere with enzyme activity.

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