Production of chitosanase from termophylic bacteria isolated from Bora Hotspring

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Abstract. The production of chitosanase enzyme was carried out by utilizing isolate of thermophilic bacteria from Bora hot spring in order to determine the optimum condition of its production process. B1211 bacterial isolates with the highest Chitinolytic Index values of 1.71 were used in crude chitosanase production. The crude chitosanase production was performed using a Completely Randomized Design (RAL) consisting of four independent variables, in the form of chitosan substrate concentration (0.25%, 0.50%, 0.75%, 1.00%), agitation speed (60, 90, 120, and 150 rpm), fermentation time (1, 3, 5, 7, and 9 days), and fermentation temperature (30, 40, 50, and 60°C). The activity of chitosanase as dependent variable was determined by Schales method, which referred to standard curve of D-glucosamine. The results showed that 1% chitosan substrate concentration yielded crude chitosanase with the highest activity of 0.408 U/mL and dissolved protein content of 9.887 mg/mL. Furthermore, the best agitation speed (90 rpm) resulted in 0.385 U/mL chitosanase activity and dissolved protein content of 23.74 mg/mL. In the fermentation time treatment, the 5-day fermentation time resulted in the highest chitosanase activity of 0.384 U/mL with dissolved protein content of 36.52 mg/mL. The fermentation temperature determination showed that the temperature of 60°C as the preferred treatment with chitosanase activity of 0.569 U/mL and dissolved protein content of 5.752 mg/mL.

1. Introduction

Chitosan, a derivative of chitin through the deacetylation process by using a strong base, having a large molecular weight that affect its solubility in water, requires hydrolysis to obtain a water soluble chlorine oligomer to widen its benefit [1]. Chitosan oligomers have antibacterial activity, can bind excessive fat, control blood pressure, lower blood sugar, increase the absorption of calcium in the body, prevent heart disease, lower uric acid levels, and act as antitumor and anticancer [2].

Chitosan oligomer can be obtained by chemical or enzymatic hydrolysis. The latter is much better due to its ability in producing chitosan ligomers with high specificity [3]. Specific enzymes that hydrolyze chitosan into oligomers or monomers are called chitosanases [4]. These enzymes can be obtained from plants [5], bacteria [6], and fungi [7]. However, chitosanases derived from bacteria receive a lot of special attention because they can maintain ecological and ecosystem balance [8]. One type of bacteria that is potentially exploited is a thermophilic chitinolytic since it has the ability to live at a temperature of 60-80°C which valuable in the industry. Bacillus sp. from hot springs in Korea [4]
and *Bacillus licheniformis* MB-2 bacterium isolates from the Tompaso lake hot spring, Manado [9] have been used to produce thermophyllic chitosanase enzymes.

Existing hot water sources can be a major opportunity to be empowered and applied in the development of science; one of them is Bora’s hot spring located in Sigi Regency of Central Sulawesi Province, Indonesia. The Bora hot spring is assumed to have great potential as a place to live of the thermophile bacteria similar to the Tompaso hot spring in Manado [9, 10]. The thermophilic bacteria utilized as chitosanase is a group of chitinolytic bacteria with certain Chitinolytic Index values. Chitosanase from chitinolytic and thermophilic bacterial isolates can be produced by fermentation by referring to several factors, including time, temperature, pH, and substrate concentration [11]. The study of the use of Bora hot water as a source of chitosanase-producing bacteria is needed from bacterial screening to optimizing enzyme production to support the development of science. In this research, we report that chitosanase isolated from B1211 is stable at 60°C and potential in industrial application.

2. Experiment procedure

2.1. Materials

*Culture media.* NB media, MSM media (0.08% K$_2$HPO$_4$, 1% NaCl, 0.04% (NH$_4$)$_2$SO$_4$, 1% yeast extract, with or without agar 1 %), colloidal chitin, standard glucosamine, Schales reagent, acetate buffer pH 6, phosphate buffer pH 6, and 1% chitosan solution.

2.2. Screening of thermophyllic bacteria

Sediment and liquid samples for screening were obtained from Bora Hotspring (pH 7, temperature of 55°C). Bacterial screening was carried out based on modified procedure by Chasanah et al. [12] which has been modified. Samples were produced using solid media Nutrient Agar (NA) and MSM media. Each medium contains colloidal chitin 2% and incubation at 37°C and 50°C for 7 days. The formation of clear zones that appeared in the surrounding colonies indicate the presence of chitinolytic enzymes produced. Isolates which had clear zones were isolated by quadrant scratch method on MSM media containing colloidal chitin and incubated at 37 ° C for 7 days.

2.3. Selection of potential isolates

Selection of Chitinolytic bacteria was performed using needle and incubated for 7 days. Chitinolytic Index was determined by measuring the formed clear zone using following equation:

$$Chitinolytic\ Index\ (CI) = \frac{\text{diameter of clear zone (cm)}}{\text{diameter of colony (cm)}}$$  \hspace{1cm} (1)

With an assumption of the higher chitinolytic index the higher enzyme activity

2.4. Production of chitosanase extract

Isolate with highest CI were selected to produce Chitosanase [12] with several modifications. Inoculum was cultured in starter MSM medium (without chitosan) and incubated at 37°C, 60 rpm, 24 hours. Next day, one mL of cultured bacteria was moved into production MSM media (enriched with 0.25, 0.5, 0.75, and 1% chitosan) and incubated in various speed of 60, 90, 120, and 150 rpm and temperature of 30, 40, 50, and 60°C for 1, 3, 5, 7, and 9 days. Crude enzyme was harvested by collecting supernatant after centrifugation of 1000 rpm, 15 minutes. Enzyme activity and amount of dissolved protein were determined using Schales [13] and Warburg-Christian methods [14].

3. Results and discussion

We have screened and isolated thermophile bacterium producing Chitosanase. After screening and isolating in MSM medium containing 1% colloidal chitin and determining their Chitinolitic Index value, 18 isolates were obtained.
Figure 1. Isolates of bacteria.
(a) bacteria cultured in MSM medium,
(b) selected bacteria for Chitinolytic Index

The chitinolytic index value of several isolates was more than 1. For example, Isolate B1211 had the highest value of 1.71 (figure 2). Previously, it was reported that bacterial isolates associated with chitosanase-producing sea sponges had Chitinolytic Index value of up to 5.4 [12]. In our research, B1211 isolates were considered to have represented potential isolates so that the isolates were tested further to determine their production conditions.

Figure 2. Chitinolytic Index of isolated termophilic bacteria.

The use of chitosan substrate concentration variation of 0.2, 0.5, 0.75, and 1.0% produced chitosanase activity of 0.161 to 0.406 U/mL (figure 3A). Chitosanase activity increased with the highest susceptibility concentration and activity was found in chitosan 1.0% substrate concentration, which was 12.406 mg/mL. This is because with more and more substrates available, microbial growth increases so that the chitosanase enzymes produced by bacteria are also increasing. The dissolved protein content obtained in the treatment of 1% chitosan concentration was 9.887 mg / mL. Increased chitosan substrate concentration will trigger enzyme activity to degrade chitosan substrate to chitosan oligomer. This is explained by Michaelis-Menten about the occurrence of substrate enzyme complexes because of the contact between the active part of the enzyme and the substrate. So that at a fixed enzyme concentration, the reaction speed will increase with the increase in the substrate, because the higher the substrate
concentration, the more it reacts with the active side of the enzyme [15]. Previous studies reported that KBJ 12 SB isolates produced chitosanase with an activity of 0.0833 U / mL on the use of 1% chitosan substrate [12].

Another factor that influences enzyme production in addition to substrate concentration is aeration [16]. Agitation can provide aeration to maintain aerobic conditions during the fermentation process. In addition, agitation can help the substrate spread in the media, which acts as a substrate in chitosanase enzyme production is chitosan. The existence of agitation in the fermentation process can increase the production of enzymes so that their activities can increase [17]. Observations at the agitation speeds of 60, 90, 120 and 150 rpm in Figure 3B show that bacteria at certain agitation speeds tend to produce chitosanase enzymes with different enzyme activities. The agitation speed of 90 rpm showed the highest chitosanase enzyme activity and at the speed of 150 rpm agitation showed the lowest chitosanase enzyme activity. Like temperature, pH and substrate concentration, agitation also has an important role in enzyme production to enzyme activity [17].

Low agitation speed (60 rpm), does not show better results due to the speed of agitation media conditions are not homogeneous, so that the oxygen supply in the media is not met properly in the process of bacterial metabolism. The use of agitation that is too high can also reduce enzyme activity, this can be seen at an agitation speed of 120 rpm which starts to fall and gets lower at a speed of 150 rpm agitation. Increased agitation can increase dissolved oxygen in the medium of enzyme production. Shaking on the medium of enzyme production, which causes faster formation of foam, so that enzymes cannot be produced maximally [17]. Froth produced due to high agitation during the enzyme production process can reduce enzyme activity [18]. The foam froth can inhibit oxygen transfer so that the dissolution of oxygen in the medium decreases [19]. The optimal agitation speed for chitosanase enzyme production in Figure 3B was obtained at 90 rpm agitation with chitosanase enzyme activity of 0.385 U /mL. This is because in this condition nutrients and oxygen in the media can be fulfilled properly. A good agitation process can provide mixing, mass transfer, and sufficient heat transfer for the fermentation process [20]. The agitation speed of 100 rpm was used before to produce chitosanase enzyme from KBJ 12 SB bacterial isolates and produced an optimum activity of 0.0833 U / mL [12].

Meanwhile, research on the use of T5a1 chitinolytic bacteria isolated from shrimp paste using 100 rpm agitation speed was able to produce chitosanase enzyme with an activity of 0.52 U / mL [21]. Dissolved protein levels of agitation rate of 90 rpm with dissolved protein levels of 23.743 mg / mL. Protein levels of crude chitosanase enzymes have been carried out using bacteria from the marine environment to get dissolved protein levels of 28 mg / mL at 100 rpm agitation speed [2].

Fermentation time is another factor that affects chitosanase’s activity. The longer the fermentation time, the more products can be produced to reach the optimum point (Figure 3C). The highest activity of chitosanase from B1211 isolates was on the fifth day, which was 0.384 U / mL with soluble protein content of 36.520 mg/mL. Contact between the active side of the enzyme and the substrate so that the reaction speed increases with the duration of fermentation, the more it reacts with the active side of the enzyme [22]. On the third day, enzyme activity decreases before reaching the optimum point. The decrease in fermentation on the third day is assumed that the carbohydrates needed by microbes to divide cells from the production media begin to be limited so that the chitosanase produced is used by bacteria as a carbon source for cell development. This is consistent with the opinion that bacteria use chitosan as a single carbon source and chitosanase activity decreases if chitosan starts to decrease [23]. The seventh day of fermentation has decreased activity this is probably due to the depletion of nutrients available in the medium so that the bacteria are in the phase of death. End of the substrate in the production media during the fermentation process can reduce enzyme activity [24]. The results obtained have the same time (5 days) as the previous study about chitosanase production from KBJ 12 SB bacterial isolates with an activity value of 0.0833 U/mL [12].
The temperature of fermentation is the next factor that greatly influences activity [25]. The chitosanase which is isolated from the thermophilic bacteria will be affected by the temperature of the main fermentation at the thermophilic temperature interval. Fermentation temperature is assessed using 5 days fermentation time and 90 rpm agitation speed. The activity of chitosanase enzymes obtained will increase with the increasing temperature. The highest activity was obtained at a temperature of 60°C with an activity value of 0.569 U/mL and the lowest activity was shown at a temperature of 30°C with an activity value of 0.312 U/mL (3D images). The tendency of increasing activity is assumed to decrease at temperatures above 60°C because B1211 bacterial isolates are isolated from the environment which has a growth temperature of 55°C. The ability of chitosanase enzymes to work at high temperatures is likely to be influenced by environmental factors where the bacteria live, but at temperatures that are too high will cause enzyme proteins to be denatured faster [12]. Several studies have been conducted to produce chitosanase enzymes, such as chitosanase activity from Bacillus licheniformis MB-2 isolates 0.8 U/mL with a fermentation time of 7 days at 55 °C [9].

High chitosanase activity at 60 °C is not followed by dissolved protein levels from the enzyme. The protein content of chitosanase dissolved obtained was only 5.558 mg/mL or lower than the previous treatment. The cause is thought to be because measurable soluble proteins are not absolutely all enzyme proteins synthesized by microorganisms because in the media there is a soluble protein in the form of residual media or metabolized protein that has been secreted. However, soluble protein levels obtained were still higher than previous studies with other types of thermophilic bacteria, such as studies using

**Figure 3.** Correlation of chitosan concentration (A), agitation speed (B), fermentation time (C), and fermentation temperature (D) with enzyme activity.
Bacillus licheniformis MB-2 isolates and getting dissolved protein content of 0.759 mg / mL at 55°C fermentation temperature [25].

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