Production of the chitinase by Beauveria bassiana in infecting Tribolium castaneum

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Abstract. Beauveria bassiana is one of the microorganisms that produce the enzyme chitinase. Chitinase has a high economic value which is widely used as a biocontrol agent because it can degrade chitin into an environmentally friendly product. The aim of the study was to investigate the production of chitinase by B. bassiana isolate in the presence of the cuticle of T. castaneum. In this study, the isolates of B. bassiana were cultured into potato dextrose agar. Further isolation, purification, and determination of the activity of chitinase. The results show that chitinase can be obtained from B. bassiana isolate derived from T. castaneum by using chitin colloidal substrate. The highest average specific activity of chitinase originating from isolated B. bassiana was 1 Unit/mg. Protein test using standard BSA solution and Lowry method obtained reading results with a spectrophotometer that was r = 0.9925.

1. Introduction

Beauveria bassiana (Balsamo) Vuill (Deuteromycota; Hyphomycetes) is an entomopathogenic fungus that can control pests on a field scale [1]. For a long time, this type of fungus can potentially have potential as a biological control agent against insect pests. Their high ability caused this fungus to be widely developed as a biological agent in the field of agriculture [2]. Fungus B. bassiana produces the enzyme chitinase when it penetrates the insect's body. The enzyme chitinase can hydrolyze β-1,4-acetamido-2-deoxy-D-glycoside bonds to chitin and insect chitin oligomers [3]. The ability to hydrolyze chitin polymer compounds on insect cell walls causing fractures and broken of cell walls. The destruction of the cell wall is continued with the formation of mycelium, which will wrap around the host body [4]. The use of enzymes in hydrolyzing chitin polymer is more developed due to its more specific inability to produce products and having no side effects that can cause environmental pollution [3]. Chitinase obtained from fungal biological control agents such as Trichoderma sp. was previously conducted [5], but the enzyme produced by B. bassiana is still limited. This encourages researchers to conduct further research on the production of the chitinase enzyme by B. bassiana while degrading...
insect cuticles of *T. castaneum*. The purpose of this study was to evaluate the activity of the chitinase enzyme produced by *B. bassiana* during the degradation of insect cuticles.

2. Methods

2.1. Fungus *B. bassiana* isolates

Fungus *B. bassiana* isolates used as research samples come from *T. castaneum* warehouse insect pests. In the next stage, the isolated *B. bassiana* fungus isolates from *T. castaneum* were purified in a solid medium, potato dextrose agar (PDA), to further test enzyme activity.

2.2. The manufacture of chitin colloidal medium and agar chitin medium

The production of a colloidal chitin medium, which was as much as 10 grams of chitin from shrimp skin powder, dissolved in 200 mL of thick HCL, then sealed and incubated 24 hours at 4ºC (all stages of treatment was performed at cold temperatures)—further filtered using glass wool. The resulting filtrate was added to 100 mL of Aquadest and neutralized with NaOH 12 N. Then centrifuged at 8,000 rpm at 4ºC temperature for 20 minutes. The resulting pellet was added to 100 mL of Aquadest and then centrifuged at 8,000 rpm for 20 minutes at 4ºC temperature. Sediment in the form of pellets (colloidal chitin) was stored at cold temperatures [6].

Chitin agar medium was made from a colloidal mixture of chitin 0.3%, K₂HPO₄ 0.1 g, MgSO₄.7H₂O 0.01 g, yeast extract 0.05 g, peptone 0.1 g, NaCl 0.5 g, (NH₄)₂SO₄ 0.1 g, and 1 g agar into an Erlenmeyer containing Aquadest 100 mL. The solution was then homogenized with a magnetic stirrer and heated until dissolved, then sterilized in an autoclave at 121ºC for 15 minutes at a pressure of 2 atm [7].

2.3. Chitinolytic index method

The rejuvenated *B. bassiana* isolate was taken in two ose and put in a sterile Eppendorf tube containing 100 μl of sterile distilled water. The result of dilution as much as five μl was put on a petri dish in agar medium containing chitin colloid. Petri dishes were incubated for seven days, and then the colony diameter was measured. The clear zone formed was visualized by adding 0.1% congo red, then the plate was washed with distilled water and NaCl, then measured the diameter of the clear zone formed and documented. The chitinolytic index was obtained by comparing the apparent zone diameter and the colony diameter [7].

2.4. Chitinolytic fungal rejuvenation

The Chitinolytic fungus was derived from *B. bassiana* isolate. The fungus was rejuvenated by taking two ose isolates and grown on chitin media in a petri dish, then incubated for 24 hours [8].

2.5. Preparation of fungus suspension of the chitinase enzyme

Two ose colonies of chitinolytic fungus were suspended into a reaction tube containing 5 mL of liquid chitin medium, then incubated at 37ºC for 18-24 hours. The result of such treatment was called inoculum [8].

2.6. Production of the enzyme chitinase

Chitinase production was performed using a 10% active inoculum, by mixing 5 mL of inoculum with 45 mL of liquid chitin media. Then shaker at 37ºC at 180 rpm for 5 days [6].

2.7. Measurement of chitinase enzyme activity

Chitinase activity was tested according to methods of Veda and aria [9] namely with the colloidal substrate of chitin. Colloidal chitin 0.3% as much as 1 mL, Buffer acetate 0.2 M pH 5 2.0 mL, and Enzyme filtrate 1mL was put into the reaction tube and incubated at room temperature for a certain amount of time. Then the mixture was heated in boiling water for 20 minutes to stop the reaction enzyme
in the mixture and then cooled. The Enzyme activity was determined by spectrophotometry at λ maks = 660 nm. Chitinase activation was determined based on equation 1.

\[
\text{Activity unit} = \frac{x - y}{0.001 \times \text{incubation time (minutes)}} \times \frac{1}{x}
\]

Illuminance: 
\[
x = \text{Control Absorption}
y = \text{Sample Absorption}
\]

One unit of enzyme activity is measured as the number of enzymes resulting in a reduction in the absorbance of the reaction mixture by 0.001 per minute.

2.8. Determination of protein levels
As much as 2 mL protein sample was injected into the reaction tube, then added 2.75 mL of lowry B reaction, and hushed for 15 minutes. Then added 0.25 Lowry A reaction, then mixed and hushed for 30 minutes. Furthermore, measured its absorbers at a maximum wave of 660 nm.

Lowry A reagent was prepared by mixing the Reagen Folin-Ciocâlteu (FCR) with Aquadest (1:1). Lowry B reagent was prepared with Na₂CO₃ 2% in NaOH 0.1 N, CuSO₄ 1%, and Na-k-tartrate 1% a ratio 100:1:1. [10].

3. Results and discussion

3.1. Production of the chitinase enzyme
Rejuvenation of B. bassiana fungus isolates on agar media containing colloidal chitin 0.3% after incubation for seven days indicating the formation of clear zones around the colony. The clear zone indicates that the isolate was capable of degrading the chitin substrate contained in the chitin medium so that chitin in the media will stimulate B. bassiana isolates to produce chitinase to utilize chitin as a source of carbon. The clear zone formed as shown in figure 1.

![Figure 1. Chitinolithic index of B. bassiana (A) before congo red addition and (B) after congo red addition.](image)

The formation of clear zones around the colony of microorganisms indicates the production of extracellular enzymes [11]. Chitin substrate in the media will be hydrolyzed by chitinase, resulting in clear zones around the B. bassiana isolate colony. The size of the clear zone formed around the colony depends on the large number of monomers produced. The research results of [2] reveal that the size of the clear zone produced depends on the number of N-acetylglucosamine monomers produced from the process of chitin hydrolysis by severing β-1.4 homopolymer N-acetylglucosamine. The clear zone will become more apparent after adding congo red (C₂₃H₂₂N₆O₅S₂Na₂) solution associated with the polymer chitin substrate bond β-1.4 in the medium so that it turns red. Rinsing with distilled water and NaCl will dissolve congo red, especially in the area around the colony that contains reducing sugars so that a clear zone will appear [12, 13].
3.2. Protein content measurement

The protein content in each fraction was measured using the method of [9]. Quantitative analysis in this method is carried out in 3 stages. First, determine the maximum wavelength of the BSA, which in this study obtained 660 nm as the maximum wavelength. Second, making BSA standard curves to determine the concentration and absorbance of standard proteins, the absorbance results obtained are calculated by substituting the standard curve equation \( Y = ax + b \) (figure 2). The third is the stage of measuring chitinase protein levels (table 1).

The protein in chitinase will react with Cu in an alkaline solution forming a copper ion complex with amide bonds through this method. The blue darker after reacting with Folin Ciocalteau Reagent reduces the yellow phosphotungstate and phosphorolytic by tyrosine and tryptophan present in the protein into blue molybdenum and blue tungsten [10].

Based on the results of research shows that the greater concentration was, the more excellent absorbency. We obtained a standard curve between the absorption of the protein solution and its concentration. Curves formed in a linear straight line because the protein solution used was a dilute solution with a minor concentration. Bee Law deviations apply if the protein solution used is of high concentration, meaning the protein concentration is large, then the linear line will turn.

![Figure 2. Standard curve.](image)

The equation obtained in this study was \( Y = 3.5206x + 0.0581 \) with a value of \( R^2 = 0.9925 \) (figure 2). The value of the R2 price in the study is in line with the results of [14] research, namely the results obtained by the equation \( y = mx + c \) with a price of \( R^2 = 0.9927 \). In the lowry protein test, one thing to pay attention to is lowry reagents in new conditions because they are easily damaged by oxidation [14].

| Sample code | Absorbance | FP | Measured protein (mg/mL) |
|-------------|------------|----|-------------------------|
| Simplo      | 0.090      | 10 | 0.09                    |
| Duplo       | 0.099      | 10 | 0.12                    |
| Triplo      | 0.095      | 10 | 0.10                    |

| Average protein: | 0.10 |

The results in table 1 show that the measured protein from the chitinase enzyme in *B. bassiana* was the highest at an absorbance of 0.099, namely 0.12 mg/mL. The mean protein measured was 0.10 mg/mL. Protein content data is needed to determine the specific activity of an enzyme.

3.3. Measurement of chitinase specific activity

The specific activity of chitinase can be calculated from units of enzyme activity per mg protein. Fractions with the highest specified value of activity were fractions with the highest number of enzymes
than those of others. Thus, a specific enzyme activity describes the level of purity of an enzyme (table 2).

Table 2. Chitinase enzyme activity of each fraction of purification results.

| Sample code | Absorbance | Enzyme activity (U/mL) | Protein content (mg/mL) | Specific activities (U/mg protein) |
|-------------|------------|------------------------|-------------------------|-----------------------------------|
| Simplo      | 0.061      | 1.017                  | 0.100                   | 10.17                             |
| Duplo       | 0.060      | 1.000                  | 0.100                   | 10.00                             |
| Triplo      | 0.059      | 0.983                  | 0.100                   | 9.83                              |
| average     | 0.060      | 1.000                  | 0.100                   | 10.00                             |

Based on the results of testing on the enzyme chitinase activity, the average enzyme activity was 1 U/mL with a protein content of 1 mg/mL. In contrast, the specific activity of the chitinase enzyme was 10 U/mg Protein indicates that B. bassiana to degrade chitin in T. castaneum was high compared to research conducted by [15], which was the enzyme activity by B. bassiana from Enrekang isolate the highest of 7.15 U/mL due to several factors, including pH, temperature, substrate concentration, and medium components corresponding to the type of enzyme chitinase produced by B. bassiana in this study.

4. Conclusions
We conclude that chitinase enzyme can be obtained from B. bassiana fungus isolate from T. castaneum cadaver using chitin colloidal substrate. The average activity of specific chitinase origin from B. bassiana isolate was 1 Unit/mg. Protein test with BSA standard solution and Lowry method obtained as a result of reading with Spectrophotometer was R2=0.9925.

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