Production of extracellular chitinase *Beauveria bassiana* under submerged fermentation conditions

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**Abstract.** Chitinase-producing microbes have attracted attention as one of the potential agents for the control of phytopathogenic fungi and insect pests. The fungus that potentially produces chitinase is *Beauveria bassiana*. This study aims to determine the growth curve and chitinase activities of *B. bassiana* isolated from *Helopeltis antonii* insects after application. Method of measuring growth curve was done by dry cell period method, while for measurement of enzyme activity done by measuring absorbance at spectrophotometer. The results showed optimum growth time of *B. bassiana* with the highest cell count of 0.031 g on day 4 which was log phase, while the highest enzyme activity was 0.585 U / mL on the 4th day for 7 days incubation. Based on these results when correlated growth with enzyme production, chitinase enzyme products are produced in log phase and categorized as primary metabolism.

**Keyword:** Chitinase production, *Beauveria bassiana*, Submerged Fermentation

1. **Introduction**

Chitinase-producing microbes have attracted attention as one of the potential agents for the control of phytopathogenic fungi and insect pests. Chitinolytic enzymes have been used for the isolation of fungal protoplasts, the manufacture of bioactive chitosan, oligosaccharides of shells waste reclamation and production of single cell proteins [1]. As well as several that have been applied in various fields, such as pharmaceuticals, cosmetics, medicine, food, water treatment, and fisheries [2], [3].

The fungus that potentially produces chitinase is *Beauveria bassiana*. Entomopathogenic fungi *B. bassiana* are generally isolated from dead insects in the environment [4]. *Beauveria bassiana* is known to produce extracellular chitinase through SmF (Submerged Fermentation) or submerged fermentation and under SSF (Solid Substrate Fermentation) or solid fermentation conditions in the presence of chitin colloids [1]. This research successfully isolated the chitinase from *B. bassiana* that have infected *Helopeltis antonii* in cocoa plants.

The production of extracellular enzymes is linked to the process of insect infection because, during multiplication within the insects, conidia produce different virulence factors, identified as extracellular enzymes, primarily chitinases. Penetration of the virulent factor in the cuticle that formed almost 70% protein, chitin, and lipids. Fungi growth gets the nutrients from the hemolymph process [4].

The many benefits of chitinase enzyme and chitin derivative compounds result in increased need for chitinase enzymes. Therefore, continuous research to obtain a cheap, high activity and stable chitinase are necessary [5]. The purpose of this study was to investigate the activity of chitin-derived enzyme during growth of *B. bassiana* isolated from *H. antonii* insects after application.
2. Materials and methods

2.1 Tools and materials
Materials used in this study were: B. bassiana fungi obtained from infected H. antonii insects after application in the field, Chitin, Potato Dextrose Agar (PDA), bacto agar, concentrated HCl, NaOH, NaCl, NH₄Cl, MgSO₄.7H₂O, K₂HPO₄, CaCl₂, Na₂HPO₄.2H₂O, (NH₄)₂SO₄, pH 3-5 citrate buffer, DNS, phosphate buffer pH 6-8, glycine-NaOH 9-10 buffer, yeast extract. While the equipment consists of thermometers, glass tools, magnetic stirrers, falcon tubes, eppendorf tubes, ose needles, micropipes, analytical scales, centrifuges, spectrophotometers, memmert incubators, Laminar Air Flow Cabinet, water baths, shaker incubators.

2.2 Media creation
Solid media for the isolation and rejuvenation of B. bassiana isolates used Potato Dextrose agar medium (PDA). Media growth and production of chitinase enzyme consisted of (g/100 mL distilled water): 0.3% colloid chitin, 0.1 g peptone, 0.5 g yeast extract, 0.65 g NaCl, 1.5 g KH₂PO₄, 0.12 g MgSO₄.7H₂O, 0.005 g CaCl₂.H₂O, pH set 7 then in autoclave.

2.3 Isolation and rejuvenation of B. bassiana
Isolation of the B. bassiana fungus on infected H. antonii was then grown on PDA (Potato Dextrose Agar) medium. Petri dishes containing PDA media are inoculated with a small portion of the area so that B. bassiana has grown from stock to tilt that is placed in the center of the Petri dish. Petri dishes were incubated at room temperature for 7 days.

2.4 Production of enzyme chitinase
Two cultures of B. bassiana fungus were inoculated in a liquid chitin medium containing 100 ml of production medium. The culture was incubated for 5 days in a swaying incubator (120 rpm) at room temperature. A total of 10 ml of B. bassiana mushroom culture (+5.000 spores/ml) was piped and inoculated into 100 ml of aqueous chitin medium by the aseptic technique. The culture incubated in the incubator wobbled at room temperature for 7 days [6]. Culture is centrifuged at 4000 rpm for 20 minutes. The supernatant formed was a crude extract of chitinase enzyme.

2.5 Measurement of growth curve
The growth curve was done by dry cell period method. Isolate B. bassiana which have been rejuvenated in the solid medium is taken with wire ose then put into the growth medium. Furthermore, incubation at room temperature with shaking at 120 rpm. Sampling was done every 24 hours for 7 days then were filtered and washed with aquadest then dried by oven until obtained by constant dry cell period [7].

2.6 Measurement of chitinase activity
Determination of enzyme activity was performed by 1 mL 0.3% colloidal chitin solution plus 2 mL 0.2 M phosphate buffer pH 7 and 1 mL of chitinase supernatant were inserted into the test tube and incubated at 37°C for 1 hour. As the control mixture was then heated to a temperature of 100°C for 10 minutes. Chitinase activity was determined by turbidimetry using a spectrophotometer at λ 420 nm. A unit of chitinase activity is defined as the amount of chitinase that causes an absorbance reduction of 0.001 reaction mixture per minute [8]. The chitinase activity unit is formulated as follows:

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\text{Unit of activity (U/mL) = Absorbance control} - \frac{\text{Absorbance sample}}{0.001\text{reaction mixture per minute}}
\]
3. Results and Discussion

3.1 Cultivation and growth of entomopathogenic fungi B. bassiana

The results of morphological observations of B. bassiana are presented in Figure 1.

![Figure 1. Growth of B. bassiana on PDA medium](image)

B. bassiana cultures in PDA medium have mycelia and white conidia [9]. Isolates that have been rejuvenated are used to make the starter to produce more active cells commonly referred to as inoculum. The inoculum is made by knowing the optimal growth of mold so that made the growth curve. In this study used the calculation of dry cell mass for making growth curve. The graph of the relationship between the incubation time and the dry cell period is shown in Figure 2.

![Figure 2. Growth curve of B. bassiana for 7 days incubation in liquid fermentation](image)

Figure 2 shown that the growth of B. bassiana grows without going through the lag phase (adaptation). This is evidenced by the increase in the weight of the dry cell mass. Based on research that has been done, log phase (exponential) starting on day 0 to day 4 without followed by phase lag (adaptation), then after that day followed by the stationary phase. This is due to the starter given to the growth medium. Wijanarka et al. [10] suggesting that a starter in growth media can negate the lag phase and growth will enter the exponential phase immediately. Karthik et al. [11] also mentioned that
inoculum administration may accelerate the production of chitinase in the medium without the adaptation phase.

Day 0 to day 3 is a log phase (exponential). In this phase, the microbial cells divide rapidly and constantly following the logarithmic curve. At the 4th day incubation period the peak but has entered the stationary phase until the 5th day incubation time indicates a decrease. In this phase, the number of cells fixed because the number of cells that grow equal to the number of dead cells. The size of the cells in this phase becomes smaller as the cells keep splitting even though the nutrients are depleted. Due to nutritional deficiencies, cells may have different compositions with cells that grow in the logarithmic phase [11]. On the 6th day until the 7th day is the phase of death. Based on the growth curve it is found that the optimal inoculation time of *B. bassiana* is the 4th day.

3.2 Measurement of enzyme activity

Chitinase enzyme is an extracellular enzyme secreted by the mold to its medium. This enzyme is obtained by separating the cell from its growth medium by centrifugation at a rate of 4000 rpm at room temperature until the colony is formed separately from the supernatant. The resulting supernatant is a crude extract of chitinase enzyme. Chitinase activity was measured using a spectrophotometer. The productivity of chitinase enzyme for 7 days incubation can be seen in Figure 3.

![Figure 3. Chitinase enzyme productivity profile of *B. bassiana*](image)

Microorganisms take a long time to decipher chitin and produce chitinase because of its higher molecular weight [12]. Chitinase activity is a measure of the amount of product produced from a breakdown of the chitin substrate [13]. The activity of chitinase of *B. bassiana* with colloidal chitin as its substrate had the highest activity value on day 4 with a value of 0.585 U/mL. Day 0 to 3 continues to increase and reach the optimum value on day 4, then decrease.

To obtain the maximum amount of enzyme, we need to know the activity of *B. bassiana* in producing chitinase enzyme. This research is doing activity test which is sampled every 24 hours once to get optimal fermentation time so that can be known when enzyme can be harvested. The graph of the relationship between growth and production of chitinase enzyme during 7 days incubation is shown in Figure 4.
Figure 4. Graph of the relationship between the growth of *B. bassiana* and the production of chitinase enzymes

Chitinase productivity continues to increase with increasing fermentation time to reach the optimum point of 0.585 U/mL on day 4. When the production of this enzyme is in the log phase. According to Wijanarka et al. [10] that the phase of growth will reach the maximum point so that there will be logarithmic growth or trophophase. After reaching the optimum point, chitinase enzyme activity gradually decreases. This is because many cells have died so that the decrease in the enzyme production.

When correlated with growth, chitinase enzyme products are generated at log phase and categorized as primary metabolism. According to Maria et al. [14], high enzyme activity is generated in the log phase. This enzyme is classified as a primary metabolite usually formed in the logarithmic growth phase [15]. After entering the 4th day until the 7th day, chitinase activity has a tendency to decrease. This is because on that day the cell biomass growth has entered the stationary phase (idiophase), so the chitinase enzyme is no longer in production by cells or cells still produce enzymes but the numbers are few or decreased.

Enzymes are classified as primary metabolites that are usually formed in the logarithmic (exponential) growth phase and are not produced in the stationary phase [15]. Furthermore, Madigan et al. [16] state that the mechanism of regulating enzyme synthesis in cells is based on the process of induction and repression. The mechanism of the process of induction and repression occurs at the stage of transcription. Enzyme repression is caused by a repressor that is a special protein attached to the region of the operator on the DNA chain. Since the area of the operator has been repressed, the RNA polymerase will not stick to the operator region, as a result the enzyme synthesis will not occur or if there is but very little.

4. Conclusion

The optimum incubation time for *B. bassiana* growth was on the 4th day with dry cell weight 0.31 g. While the optimum incubation time in the production of chitinase enzyme from *B. bassiana* was the 4th day with enzyme activity yielded 0.585 (U/mL).

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