The effect of different substrates on chitinase activity from 
Bacillus sp. WS4F

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Abstract. One of the roles of chitinase is as an antifungal which is widely used as a biocontrol agent for plant diseases caused by pathogenic fungi. Bacillus sp. WS4F has chitinase activity which can inhibit the growth of Ganoderma boninense, a fungus that attacks oil palm and causes basal stem rot (BSR). This study aims to investigate the effect of different substrates on the activity of the chitinase from Bacillus sp. WS4F. Two kinds of substrates i.e. chitin flakes and shrimp shells were used in this study. Enzyme activity of chitinase was analyzed after partial purification of enzyme was performed using ammonium sulfate precipitation followed by dialysis. The highest activity of chitinase was achieved by the substrate using shrimp shells. The ammonium sulfate precipitation (60-80% saturation) 0.0949 U/mL for activity enzyme and 0.2639 mg/mL for protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the enzyme showed a molecular weight of 64.389 kDa.

1. Introduction
Chitinases (E.C 3.2.2.14) are glycosyl hydrolases with the sizes ranging from 20 kDa to about 90 kDa, that are able to degrade chitin into its constituent monomers [1]. Chitinases have the ability to degrade chitin directly to low molecular weight chitooligosaccharides, which serve a broad range of industrial, agricultural, and medical functions such as elicitor action and anti-tumor activity [2]. Chitinases have been divided into 2 main groups: Endochitinases (E.C 3.2.1.14) and exo-chitinases. The endochitinases randomly split chitin at internal sites, forming the dimer di-cetylchitobiose and soluble low molecular mass multimers of GlcNAc. The exo-chitinases have been further divided into 2 subcategories: Chitobiosidases (E.C 3.2.1.29) which are involved in catalyzing the release of di-acetylchitobiose, and 1-4-β-glucosaminidases (E.C. 3.2.1.30), cleaving the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc [3]. Chitinases have been receiving an increased attention due to their role in the biocontrol of fungal phytopathogens and harmful insects [4].

One of the bacteria-producing chitinase that has potential as a biocontrol agent is Bacillus sp.(WS4F) from thermophilic bacteria in North Sumatra hot springs. It has known to have chitinase activity that can inhibit the growth of Ganoderma boninense[5]. Bacteria, fungi, plants, and animals are the main sources of chitinase. However, bacteria are the most efficient for chitinase production because of their rapid growth and easier to control during their growth. In the process of enzyme production, type of substrate has the important role to affect the activity of enzymes. The purpose of this study is to investigate the effect of different substrate on the chitinase activity of Bacillus sp. WS4F. Two different
sources of chitin (chitin flakes and shrimp shells) were used as substrate during the production of chitinase. Partial purification of chitinase were also performed by using ammonium sulfate precipitation followed by dialysis to increase the activity of chitinase.

2. Material and methods

2.1. Microorganism and growth conditions

Bacillus sp. WSFS was used as chitinase producer. The isolate was obtained from BPPT (Badan Pengkajian dan Penerapan Teknologi), Indonesia and it showed antifungal activity against Ganoderma boninense. The strain was maintained on Luria Bertani media (pepton 1% [Himedia], yeast extract 0.5% [Himedia], and NaCl 0.5% [Himedia] at 4 °C and 20% glycerol at -40 °C. The inoculum of Bacillus strain was prepared by inoculating 5 mL of sterilized Luria Bertani (LB) in 50 mL flask with loop full of pure Bacillus sp. WSFS and incubated overnight at 37 °C, 24 h. Media M9 was used for chitinase production, contained: Na₂HPO₄ (0.065 %) [Merck], KH₂PO₄ (0.15 %) [Merck], NaCl (0.025%) (Himedia), NH₄Cl (0.05%) [Merck], MgSO₄ (0.012%) [Merck], CaCl₂ (0.0005 %) [Merck] supplemented with 3% chitin flakes or shrimp shells.

2.2. Colloidal chitin preparation

Five grams of chitin powder was added to 60 mL of concentrated HCl and stirred for 16 hours at 4ºC. The mixture was added into 2 L of ethanol 95% and stirred overnight at 4ºC. The mixture then was vacuum-separated. The precipitated were washed by RO water until pH reached 7.0. The obtained colloidal chitin (5%) was stored at 4 ºC [6].

2.3. Chitinase production

One dose of isolate Bacillus sp. WS4F from the stock was inoculated into 5 mL of liquid LB media (pH 7.0). Incubation was performed at 37 ºC, 150 rpm for 12 hours. Scale up of fermentation was performed by addition of 3% inoculum into 100 ml LB medium (pH of 7.0) incubated at 37 °C until OD600 nm reached 0.8. Then 3% of inoculum was added into 1000 mL production media, incubated at 37 °C, 150 rpm for 3 days. Sampling was performed every 24 h by centrifugation at 6000 rpm, at 4 ºC for 15 minutes for analysis of Optical Density (OD), enzyme activity and protein content. Media used can be seen in Figure 1.

![Figure 1. Different substrate for chitinase production.](image)

2.4. Partial purification of chitinase

Partial purification was performed using ammonium sulfate. Crude enzyme obtained was precipitated with various ammonium sulfates saturation (20, 40, 60, and 80%). The precipitation was carried out at 4 ºC, for 24 h and centrifuged at 3000 rpm for 10 min at 4°C. The obtained protein pellet was suspended into L-histidin buffer (0.02 M) at pH 5.8 with a ratio of 1:2. The precipitated enzyme was then further
purified by dialysis. Dialysis was performed by using dialysis membrane with 12.4 kDa MWCO. Cellulose tubing membranes used for dialysis were previously washed by RO water to remove contaminant such as glycerol. The enzyme was then incubated in L-histidin buffer (0.02 M) pH 5.8 for 12-16 hours at 4 °C [7].

2.5. Measurement of chitinase activity

The activity of chitinase was analyzed by determining reducing sugar [8]. Chitinase activity test was conducted by mixing 0.3 mL colloidal chitin (1%), pH 7 with 0.3 mL chitinase, then incubated at 37 °C for 30 minutes. Hydrolysis reactions was stopped and analyzed with the addition of 0.6 mL of DNS reagents. The mixture was then centrifuged at 14,000 rpm for 5 minutes. Supernatant was then taken and incubated in boiling water for 15 minutes.

Chitinase activity can be calculated by formula below:

\[
\text{Activities (U/mL)} = \frac{C \times 1000 \times FP}{\text{MW NAG} \times \text{Vol. Enzyme} \times \text{Incubation Time}}
\]

\[
C = \text{NAG concentration formed}
\]

\[
FP = \text{Dilution Factor}
\]

\[
\text{MW NAG} = 221.21
\]

The turbidity caused chitin remaining in the reaction mixture was measured at the wavelength of 660 nm. Definition of one activity unit of chitinase is the amount of enzyme catalyzing the decrease of absorbance as many as 0.001 per min per mL enzyme at the wavelength of 660 nm.

2.6. Protein concentration

Protein concentration was determined by Bradford method [9]. Twenty microliters of samples were added into 1000 μL of Bradford reagents. Mixtures were homogenized and incubated at room temperature for 15 minutes. Absorbance was measured at 595 nm. Protein content were then measured by using the protein standard curve equation. Bovine Serum Albumin (BSA) was used as a standard to make protein standard curve.

2.7. Molecular weight determination

SDS-PAGE consisted of 7.5% separating gel and 5% stacking gel was used to determine the molecular weight of protein. GangNam-STAIN Prestained Protein Ladder standard was used as protein standard. A total of 24 μL of enzyme samples was homogenized with 6 L of sample buffer. 20 μL of mixture was loaded into SDS-PAGE, then gel was run at 120V for 60-90 min. Then, the gel was stained with Page blue for 30 minutes and washed with RO water until protein bands were visible [10].

3. Results and discussion

3.1. The effect of different substrates on chitinase activity

Based on the data, effect of different substrates on chitinase activity (Table 1), the best result for chitinase activity was higher chitinase production using 3% shrimp shells as substrate than chitin flakes. Because shrimp shells had higher content of protein than chitin flakes which was highly processed during its preparation.
Table 1. Effect of different substrates on chitinase activity.

| Fraction                  | M9 + Shrimp shells 3% | M9 + Chitin flakes 3% |
|---------------------------|-----------------------|-----------------------|
|                           | Activity (U/mL)       | Total activity (U)    |
| Crude enzyme              | 0.0819                | 81.8712               |
| Amm. Sulfate 0-20%        | 0.1320                | 13.1961               |
| Amm. Sulfate 20-40%       | 0.1156                | 11.5620               |
| Amm. Sulfate 40-60%       | 0.1070                | 10.7005               |
| Amm. Sulfate 60-80%       | 0.0949                | 9.4882                |
| Dialysis                  | 0.0957                | 4.7863                |
|                           |                       |                       |
|                           | Volume (mL)           | Total activity (U)    |
| Crude enzyme              | 1000                  | 37.2195               |
| Amm. Sulfate 0-20%        | 100                   | 7.1999                |
| Amm. Sulfate 20-40%       | 100                   | 15.3335               |
| Amm. Sulfate 40-60%       | 100                   | 2.8889                |
| Amm. Sulfate 60-80%       | 100                   | 1.3000                |
| Dialysis                  | 50                    | 0.5000                |

3.2. The effect of different substrates on protein concentration

The effect of different substrates on protein concentration is provided in Table 2. Based on the data (Table 2), it was found that protein content was inversely proportional to the enzyme purity. The highest protein content was produced by crude enzyme (low purity), while the lowest protein content was produced by a fraction of enzyme precipitated by 60-80% of ammonium sulfate (high purity). Protein amounts can occur due to the deposition of proteins and impurities at each fraction level [11]. The gradual increase in the concentration of ammonium sulfate causes enzyme proteins and impurities to settle. The amount of protein and impurities calculated as the levels of protein and the total protein deposited decreases due to gradual precipitation at the previous concentration of ammonium sulfate. So the purity of enzymes increased due to protein content decreased [12].

Table 2. Effect of differences substrates on protein concentration.

| Fraction       | M9 + Shrimp shells 3% | M9 + Chitin flakes 3% |
|----------------|-----------------------|-----------------------|
|                | Protein (mg/mL)       | Total protein (mg)    |
| Crude enzyme   | 0.4770                | 1431.0000            |
| Amm. Sulfate 0-20% | 0.4512                | 45.1221              |
| Amm. Sulfate 20-40% | 0.3661                | 36.6115              |
| Amm. Sulfate 40-60% | 0.3193                | 31.9333              |
| Amm. Sulfate 60-80% | 0.2639                | 26.3999              |
| Dialysis       | 0.2610                | 13.0555              |
|                | Volume (mL)           | Total protein (mg)    |
| Crude enzyme   | 1000                  | 712.5333             |
| Amm. Sulfate 0-20% | 100                  | 20.2443              |
| Amm. Sulfate 20-40% | 100                  | 15.3335              |
| Amm. Sulfate 40-60% | 100                  | 12.0000              |
| Amm. Sulfate 60-80% | 100                  | 10.0888              |
| Dialysis       | 50                    | 4.3888               |

3.3. Molecular weight test with SDS page

The molecular weight of chitinase using SDS-PAGE on different fractionation is provided in Figure 2.

Figure 2. Molecular weight identification of chitinase by SDS-PAGE.
(1. Crude enzyme, 2. Sulfate 0-20%, 3. Sulfate 20-40%, 4. Sulfate 40-60%, 5. Sulfate 60-80%, 6. Dialysis)
Based on the Figure 2, it is known that the molecular weight of chitinase from *Bacillus sp. WS4F* has a molecular weight of 64.389 kDa by using Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE).

4. Conclusions

The results showed that the highest chitinase activity and protein content were obtained from the media using shrimp shells as substrate, 0.0949 U/mL for activity enzyme and 0.2639 mg/mL for protein, respectively. Enzyme purification was performed by using ammonium sulfate with 60% - 80% (w/v) saturation, followed by dialysis. The molecular weight of chitinase was 64.389 kDa.

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