Characterization of thermostable chitinase from 
*Bacillus licheniformis* B2

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Abstract. Chitinases is an enzyme capable of degrading chitin into oligomers to produce chitin derivatives products which are more useful. Thermostable-chitinase is of important in the relevant industrial application, since the degradation process oftently requires pretty high temperature. This research report a characterization of chitinase isolated from thermophilic microorganism. The chitinase was obtained from *Bacillus licheniformis* B2 isolated from Ijen hot spring, East Java. It has the best chitinolytic activity at pH 7 when colloidal chitin was used as substrate. The enzyme exhibited activity in broad temperature range, from 50 °C to 70 °C, optimally at 55 °C. It was stable at 50 °C up to 90 min, at 60 °C up to 60 min and at 70 °C up to 30 min. At neutral pH this enzyme has negative charge but further purification is needed to determine its pl. The $K_m$ and $V_{max}$ of this chitinase for colloidal chitin were 101.96 mg mL$^{-1}$ and 2.72 μmol (min mL)$^{-1}$, respectively. Addition of NaCl, KNO$_3$ and MgSO$_4$ decreased the activity of chitinase following mixed inhibitor mode. This enzyme should be a good candidate for applications in the recycling of chitin waste.

**Keywords**: *Bacillus licheniformis*, chitinase, shellfish waste, waste to food

1. Introduction

Chitinase (EC 3.2.1.14) is a group of enzyme capable of degrade chitin to low-molecular-weight products. The production of inexpensive chitinolytic enzymes is an important element in the utilization of shellfish wastes that not only solves environmental problems but also promotes the economic value of marine products [1]. Chitinase can be isolated from many kind of organism, such as fungi, bacteria [2], plant and animal. Thermostable-chitinase usually produce by thermophilic microorganism at geothermal environment [3] for instance volcano area and hot springs.

Enzyme work optimally in specific condition, such as temperature, pH, substrate concentration and the presence of inhibitor and activator [4]. Those aspect affect three dimension comformation and polypeptide folding of enzyme. Ion addition to enzymatic reaction can enhance enzyme activity and work as activator or inhibit enzyme and work as inhibitor [5]. In this experiment, NaCl, KNO$_3$ and MgSO$_4$ used because of its soluble property in phosphate buffer. Many study has been done to characterize thermostable chitinase produced by microorganism but the role of each ion in enzyme activity varies depending on the type chitinase produced and the producing bacteria species.

Previously, *Bacillus licheniformis* B2 that produced a particular high activity of thermostable chitinase was obtained when cultured in the medium containing colloidal chitin as the sole carbon
source. According to BLAST analysis, this microorganism have the highest similarity with B. licheniformis strain PR with 95% identification. Crude chitinase from this microorganism was successfully used to prepare low-molecular-weight chitin monomer and oligomers. This paper describes the characterization and some properties of chitinases from the culture medium of B. licheniformis B2.

2. Materials and methods

2.1. Materials

Bacillus licheniformis B2 isolated from Ijen hot spring, chitin extracted from shrimp shell, KNO₃ (Merck), MgSO₄ (Merck), HCl (J. T. Baker), NaOH (Merck), CaCl₂ (Merck), (NH₄)₂SO₄ (Merck), K₂HPO₄, NaCl (Merck), MgSO₄.7H₂O, yeast extract (Becton & Dickinson), bacto tryptone (Merck), H₂O, Na₂HPO₄(Merck), NaH₂PO₄, Na₂SO₄, CuSO₄, H₂SO₄, ammonium molybdat, NaHAsO₄.7H₂O, BSA, phosphoric acid 85% (Merck), acetic acid (Merck), CTAB, Bio-rex (Bio-Rad), DEAE cellulose (Sigma Aldrich), GlcNAc (Carbosynth).

2.2. Production of chitinase

Colony of B. licheniformis B2 inoculated to thermus broth without chitin and incubated at 50 °C with 180 rpm shaking for 12 h or until maximum density. Afterwards, to make starter add 7.5 mL of culture to 67.5 mL of thermus broth media and incubated for 16 h at 50 °C with 180 rpm shaking (1 rpm = 1/60 Hz). Total of 75 mL starter then added to fermentor with 675 mL thermus broth media. The production of chitinase is carried out with following condition, temperature 50 °C, pH 5, 200 rpm agitation and 2 L min⁻¹ aeration. After incubation, all media at fermentor centrifuged at 11 000 rpm for 5 min to separate chitin and cell debris (pellet) with chitinase crude extract (supernatant).

2.3. Enzyme assay

Chitinas activity was assayed in a 0.5 mL reaction mixture containing 0.1 g mL⁻¹ colloidal chitin in 0.02 M phosphate buffer, pH 7, and 0.5 mL of enzyme solution. After incubation at 55 °C for 90 min, the reaction was stopped by centrifugation. For every 10 min, the tube was shaken to maximize enzyme-substrate contact. Reducing sugar produced was measured by Nelson-Somogyi method. Chitinase activity was defined using equation (1) [6].

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\text{Activity} \left( \frac{\mu\text{mol}}{\text{min mL}} \right) = \frac{\text{GlcNAc released per mL} \times \text{Total assay volume}}{\text{Mr GlcNAc} \times \text{Duration of incubation} \times \text{Volume of enzyme used}}
\]

Notes:
GlcNAc released per mL = GlcNAc mass per mL (μg mL⁻¹)
Mr GlcNAc = 221.21 (g mol⁻¹)

2.4. Optimum temperature and pH

The optimum temperature and pH were measured using colloidal chitin as a substrate. The enzyme activity was assayed for 90 min at temperatures 40 °C to 65 °C and pH 4.0 to 8.0 using 0.02 M phosphate buffer.

2.5. Thermal stability

The thermal stability was investigated by incubating the enzyme for 2 h at temperatures 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C in phosphate buffer pH 7.0. Aliquots were taken every 30 min, and residual activity of the enzyme was determined under standard assay condition.
2.6. *pI determination*

To determine enzyme’s *pI*, buffer pH 4 to 9 was used with addition of five drops 0.1 % CTAB (cationic wetting agent) for each mL of buffer. Buffer with CTAB was then added to 0.2 mL of enzyme with different pH at separate tube then turbidity of each tube was observed visually.

2.7. *Ion exchange chromatography*

Column resin was prepared corresponding to each protocol, i.e. bio-rex resin for cation exchange chromatography and DEAE cellulose resin for anion exchange chromatography. Resin was to column and flow rate was set to 1 mL min⁻¹. The amount of 2 mL enzyme was loaded then eluted using phosphate buffer pH 7 with gradient concentration of NaCl. Protein concentration was determined using Bradford method in every fraction obtained from chromatography.

2.8. *Molecular weight determination*

Separating gel (12.5 %) was prepared with the composition as follows: 3.125 mL acrylamide 30 % (29.2 g acrylamide and 0.8 g bis-acrylamide in 100 mL aquades); 2.75 mL Tris-HCl 1 M pH 8.8; 1.505 mL aquades; 75 μL SDS 10 %; 75 μL APS 10 % and 6.25 μL TEMED. Stacking gel was prepared on top of separating gel with the composition as follows: 0.45 mL bis-acrylamide 30 %; 0.38 mL Tris-HCl 1 M pH 6.8; 2.11 mL aquabidest; 30 μL SDS 10 %; 5 μL TEMED and 30 μL APS 10 %. Sample was prepared by adding sample buffer and boiled for 5 min. Total of 10 μL to 20 μL of sample was then loaded into the well. After electrophoresis run for 20 min to 30 min with 110 mA, the gel was stained with staining solution (1 g Coomassie Brilliant Blue G-250, 450 mL methanol, 450 mL aquades and 100 mL acetic acid) and then destained with destaining solution (100 mL methanol, 100 mL acetic acid and 800 mL aquades). Protein bands shown from sample were then compared to protein marker to determine enzyme molecular weight.

2.9. *Effect of salt addition*

To determine effect of salt addition, following variation was added to enzyme assay: 0.47 mM and 1.3 mM NaCl, 0.47 mM and 1.3 mM KNO₃, 0.47 mM and 0.238 mM MgSO₄. Enzyme activity was tested in 7 different substrate concentration (2.5 mg mL⁻¹ to 17.5 mg mL⁻¹ colloidal chitin) and compared to the activity without any salt addition.

3. Results

3.1. *Temperature and pH characterization*

The enzyme showed optimal activity at pH 7 (figure 1). At pH 7, the enzyme had the highest chitinolytic activity at 55 °C (figure 2). The half-life at 50 °C was about 90 min, whereas at 60 °C and 70 °C, no significant activity lost was observed within 60 min and 30 min, respectively (figure 3).

![Figure 1. Optimum pH of chitinase from *B. licheniformis* B2.](image-url)
Figure 2. Optimum temperature of Chitinase from *B. licheniformis* B2.

Figure 3. The thermal stability of chitinase from *B. licheniformis* B2.

3.2. Protein charge determination

The result of pl determination using CTAB can be observed at figure 4. All solution gave almost same turbidity in pH 4 to pH 10 and enzyme pl can’t be determined. As shown at the graph (figure 5 and figure 6), enzyme can’t attach to column with negative charge and can be detect at early fraction beside at column with positive charge, high concentration of salt is needed to unattached enzyme from column so enzyme present in later fraction.

Figure 4. Enzyme at buffer with 0.1 % CTAB, from left to right: pH 4, 5, 6, 7, 8, 9, and 10.
3.3. **Ion exchange chromatography**

![Cation Exchange Chromatography](image)

**Figure 5.** Protein concentration in each fraction after IEC (1 mL per fraction)

![Anion Exchange Chromatography](image)

**Figure 6.** Protein concentration in each fraction after IEC (0.5 mL per fraction)

3.4. **Molecular weight determination**

Enzyme in crude extract visualized using SDS PAGE method with CBBG R250 staining. Visualization done to determine enzyme molecular weight. As shown in figure 7, in crude extract from *B. licheniformis* there were several protein bands shown. Proteins contained in crude extract sized between 50 kDa and 75 kDa, between 25 kDa and 35 kDa, between 15 kDa and 25 kDa, and also between 10 kDa and 15 kDa. It remains unclear which protein has chitinolytic activity.
3.5. Enzyme kinetic

The kinetic of the enzyme was studied on colloidal chitin. The kinetics of chitinase from *B. licheniformis* B2 followed the classical Michaelis–Menten kinetics. The $K_m$ and $V_{max}$ value calculated from Lineweaver–Burk plots was 101.96 mg mL$^{-1}$ and 2.72 µmol (min mL$^{-1}$), respectively (table 1). Addition of NaCl (figure 7), KNO$_3$ (figure 8), and MgSO$_4$ (figure 9) reduced both $K_m$ and $V_{max}$. Increasing salt concentration also increasing $K_m$ and $V_{max}$ impairment.

![Figure 7. Protein in crude extract (CE) visualization using SDS PAGE method.](image)

![Figure 7. Lineweaver-Burk Plot of Chitinase with NaCl addition](image)
Table 1. Km and Vmax value with salt addition.

| Without salt | Km (mg mL⁻¹) | Vmax (µmol (min mL)⁻¹) |
|--------------|--------------|------------------------|
|              | 101.96       | 2.72                   |
| 0.47 mM NaCl | 46.87        | 0.69                   |
| 1.3 mM NaCl  | 23.68        | 0.31                   |
| 0.47 mM KNO₃| 63.02        | 0.48                   |
| 1.3 mM KNO₃ | 23.76        | 0.28                   |
| 0.47 mM MgSO₄| 35.76        | 0.21                   |
| 0.238 mM MgSO₄| 23.63     | 0.24                   |

Figure 8. Lineweaver-Burk Plot of Chitinase with KNO₃ addition.

Figure 9. Lineweaver-Burk Plot of Chitinase with MgSO₄ addition.
4. Discussion

*B. licheniformis* B2 was isolated from Ijen hot spring, East Java, Indonesia. Temperature and pH characterization of chitinase obtained from this microorganism were carried out to know the best condition when using chitinase to degrade shrimp shell waste. Variation of pH 5 to pH 8 was chosen based on sampling environment pH, which is 6. Variation of temperature was also taken based on sampling environment temperature, which is 50 °C. The enzyme showed optimal activity at pH 7 with average activity $6.69 \times 10^{-2}$ µmol (min mL$^{-1}$) (figure 1). Activity at pH 7 was the highest and significantly different compared to activity at other pH when analyzed with One Way ANOVA (data not shown). At pH 7, the enzyme had the highest chitinolytic activity at 55 °C with average activity $9.95 \times 10^{-2}$ µmol (min mL$^{-1}$) (figure 2). Activity at 55 °C also significantly different compared to activity at other pH when analyzed with One Way ANOVA (data not shown).

The thermostability of enzyme was observed based on residual activity when enzyme was heated at certain temperature or enzyme half-time. According to the result, at 50 °C the residual activity remained 63.24 % after 90 min and decreased to 33.98 % after 120 min, at 60 °C the residual activity remained 56.34 % after 60 min and 31.99 % after 90 min, at 70 °C after 30 min the residual activity remained 65.72 % after 30 min and continued to decline afterwards. The enzyme is called stable when its residual activity is greater than 50 %. It was stable at 50 °C up to 90 min, at 60 °C up to 60 min and at 70 °C up to 30 min. While at 80 °C and 90 °C the enzyme was no longer stable.

An trial experiment was also done to determine pl using ionic detergent, i.e. CTAB. In buffer with same pH as enzyme pl, enzyme should have neutral charge and cause high turbidity. As shown in figure 4, buffer with pH 4 to pH 10 gave almost the same turbidity so pl enzyme could not be determined. This phenomenon can be happen because enzyme crude extract contained more than one protein with different pl and further purification is needed to determine the exact pl of this chitinase. Enzyme showed different characteristics when separated in positively charge column compared to that in negatively charge column. As shown in figure 5, chitinase was obtained at early fraction. This result proved that the enzyme has similar charge with column used (negative) or on its net-charge at pH 7. In figure 6, protein presented at early and later fraction. This result shown that high concentration salt needed to unattached this protein from the column and chitinase has different charge with column used (positive). Combined these two results, it was concluded that chitinase from *B. licheniformis* B2 had negative charge at pH 7.

Addition of 0.47 mM and 1.3 mM NaCl caused depletion of activity up to 46 % and 63 % respectively. Addition of KNO$_3$ with the same concentration also inhibited enzyme activity. Addition of 0.47 mM KNO$_3$ decrease 60 % of enzyme activity and addition of 1.3 mM KNO$_3$ decrease 71.7 % of enzyme activity. Addition of 0.47 mM and 0.238 mM of MgSO$_4$ also caused inhibition of enzyme. This result obtained because addition of salt increased the amount of ions in solution. Those ions can interact with enzyme or substrat-enzyme complex and decreasing product-making reaction. From these result we can conclude that NaCl, KNO$_3$ and MgSO$_4$ act as inhibitor for chitinase from *B. licheniformis* B2. The result was similar to characterization of chitinase from T5a1 [7], addition of 1 mM Mg$^{2+}$, Na$^+$ and K$^+$ caused enzyme activity depletion. Different result obtained at other species, NaCl can act as activator for chitinase from *Enteroabacter* sp. G-1 [8].

Enzyme Km and Vmax was obtained by enzyme assay with various substrate concentrations. The reaction was held for 90 min at 55 °C. The enzyme followed the classical Michaelis–Menten kinetics so the amount of Km and Vmax can be determined using Lineweaver-Burk plot. Chitinase from *B. licheniformis* B2 showed Km and Vmax value 101.96 mg mL$^{-1}$ and 2.72 µmol (min mL$^{-1}$), respectively. Different enzyme will give different Km and Vmax, Km and Vmax chitinase from *B. licheniformis* B2 were 0.321 µg mL$^{-1}$ and 71.429 µg, while Km and Vmax chitinase from *Enterobacter* sp. NRG4 were 1.41 mg mL$^{-1}$ and 74.07 µM (µg h$^{-1}$), respectively [9]. Km and Vmax chitinase from *Serratia marcescens* B4A was 8.3 mg mL$^{-1}$ and 2.4 mmol min$^{-1}$[10]. Km and Vmax chitinase from *B. cereus* 11 UJ were 29.71 µg mL$^{-1}$ and 1.035 × 10$^{-4}$ µg (mLs)$^{-1}$, respectively [11]. The lower the Km, the higher the enzyme activity towards colloidal chitin as substrat. Vmax or maximum velocity of this enzyme was 2.72 µmol (min mL$^{-1}$) which mean at optimum condition,
enzyme can convert colloidal chitin to 2.72 µmol mL\(^{-1}\) GlcNAc each minute. If both Vmax and Km value decreased and slope at Lineweaver Burk plot changed after addition of inhibitor, that inhibitor inhibits enzyme with mixed type. Mixed type inhibitor is inhibitor that binds to allosteric site of enzyme and not the active site of enzyme. Mixed inhibition can caused increment of enzyme affinity toward substrate but did not increase product formation [12].

5. Conclusion
Chitinase as a good candidate for applications in the recycling of chitin waste was obtained from \textit{B. licheniformis} B2 isolated from Ijen hot spring, East Java. It showed best chitinolytic activity at pH 7 and exhibited activity in broad temperature range, from 50 °C to 70 °C, optimally at 55 °C. The Km and Vmax of this chitinase for colloidal chitin were 101.96 mg mL\(^{-1}\) and 2.72 µmol (min mL\(^{-1}\))\(^{-1}\), respectively.

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