Paper 3

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Partial biochemical characterization of crude extract extracellular chitinase enzyme from *Bacillus subtilis* B 298

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Abstract. Extraction and characterization of extracellular chitinase from *Bacillus subtilis* B 298 have been done. Growth curve determination of *B. subtilis* B 298, production curve determination of crude extract chitinase from *B. subtilis* B 298, and partial biochemical characterization of crude extract chitinase have been achieved in this study. Optimum growth of *B. subtilis* B 298 was achieved at logarithmic phase within 9 hours incubation time, so it was used as inoculum for enzyme production. According to production curve of the enzyme, it was known that incubation time which gave the highest chitinase activity of 15 hours with activity of 6.937 U/mL respectively. Effect of various temperatures on chitinase activity showed that optimum activity was achieved at 40°C with an activity of 5.764 U/mL respectively. Meanwhile, the optimum pH for chitinase activity was achieved at pH of 5.0 with an activity of 6.813 U/mL respectively. This enzyme was then classified as metalloenzyme due to the decline of the activity by EDTA addition. All divalent cations tested acted as inhibitors.

1. Introduction

Chitin (β-1,4-linked polymer of N-acetyl-D-glucosamine) is one of the most abundant polysaccharides in nature, after cellulose. It is a major cell wall constituent of higher fungi belonging to Chytridiomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes, insect exoskeletons and crustacean shells [1]. Approximately, 75% of total weight of shellfish such as shrimp, crabs, and krill are considered as waste and chitin comprises 20 to 58% of dry weight. Microbial degradation of insoluble macromolecules such as lignin, cellulose, and chitin depend on the production of extracellular enzymes that have the ability to degrade these components [2].

One kind of the enzyme that plays an important role in degrading these components is chitinase. Chitinase is the hydrolytic enzyme that spesifically degrade chitin. According to [1], chitinases are produced by several bacteria, actinomycetes, fungi and also by higher plants. Microorganisms produce the chitinase primarily for the assimilation of chitin as a carbon and nitrogen source. Chitinases have been isolated from a variety of bacteria including Bacillus spp., and some of them are reported to produce multiple forms of chitinases with different molecular masses [3]. Chitinase enzymes are used in various applications such as biological control of fungal pathogens [4], preparation of oligosaccharides and N-acetyl-D-glucosamine [5], and protoplast from filamentous fungi [6].

In this study, a chitinolytic bacterium *Bacillus subtilis* B 298 was investigated for the production and characterization of crude extract extracellular chitinase. The investigation included (i) bacteria growth curve determination, (ii) chitinase production curve determination, (iii) partial biochemical characterization of crude extract chitinase.

2. Materials and methods

2.1. Materials

B. subtilis B 298 isolate, Nutrient Agar (Merck), Nutrient Broth (Merck), Chitin powder (Sigma), DNS (Sigma), NAG standard solutions, NaOH solution 1% (w/v), Sodium acetate buffer 50 mM pH 4.0, Sodium acetate buffer 50 mM pH5.0, Sodium phosphate buffer 50 mM pH 6.0, Sodium phosphate buffer 50 mM pH 7.0,Tris-HCl buffer 50 mM pH 8.0, Tris-HCl buffer 50 mM pH 9.0, $CaCl_2$ 1.0 mM, $MgCl_2$ 1.0 mM, $CuCl_2$ 1.0 mM, $ZnCl_2$ 1.0 mM, $HgCl_2$, EDTA 1.0 mM.

2.2. Determination of bacterial growth curve

Fresh isolate from solid Nutrient Agar (NA) medium was transferred to 100 mL of liquid Nutrient Broth (NB) medium pH 7.0 and was incubated using shaker bath at room temperature. Sampling was done every hour, and the absorbance of the culture was measured using UV spectrophotometer at λ 600 nm. Growth curve was made by plotting the absorbance at Y axis and incubation time at X axis. Growth at logarithmic phase was used as inoculum for the production of chitinase enzyme.

2.3. Determination of chitinase production curve

Chitinase production curve was made by transferred inoculum to the 200 mL production medium (NB) fortified with 0.5% colloidal chitin (w/v) as an inducer, and incubated at room temperature. Sampling was done every 3 hours, and the culture was then measured the absorbance at λ 600 nm. After that the culture was centrifuged (5000 rpm, 4°C, 20 minutes). The supernatant is the crude extract enzyme and was determined the chitinase activity. Production curve was made by plotting the chitinase activity at Y axis and incubation time at X axis. The incubation time that gave the highest chitinase activity was used to produce the enzyme.

2.4. Production of crude extract chitinase

Production of crude extract chitinase was done according to the growth curve and chitinase production curve obtained. *B. subtilis* B 298 isolate was grown in instant Nutrient Agar medium and incubated for 48 hours at room temperature. After that, growing colonies were transferred to 200 mL inoculum medium (Nutrient Broth) in erlenmeyer flask and incubated for 9 hours at room temperature in a shaker bath. The inoculum (20% v/v) was then transferred into 1 L production medium (Nutrient Broth) in erlenmeyer flask, fortified with 0.5% (w/v) colloidal chitin as inducer, and allowed to grow at room temperature for the time of incubation that gave the highest chitinase activity from production curve, in shaker bath. The medium was then centrifuged (5000 rpm, 4°C, 20 minutes). The cell-free supernatant obtained was crude extract chitinase.

2.4.1. Assay of chitinase activity. Chitinase activity was determined spectrophotometrically by estimating the amount of free reducing groups formed after colloidal chitin hydrolysis. The reaction mixture was composed of 1 ml of 0.5% colloidal chitin suspended in 0.2 M sodium phosphate buffer pH 7.0 and 1 ml of the enzyme solution. After 30 minutes incubation at 37° C, the reaction mixture was stopped by adding 1 ml of 1% NaOH solution, and then boiled for 5 minutes. The mixture was then centrifuged (4000 rpm, 5-10 minutes). One ml of supernatant was added with 1 ml of 1% DNS solution, then incubated in boiled water for 5 minutes. The supernatant absorbance at 550 nm was measured. A standard curve was obtained using N-acetylglucosamine as a standard. One unit of chitinase activity was defined as the amount of the release of 1 μmol product (NAG) per minute.

2.5.Characterization of crude extract extracellular chitinase

2.5.1. Determination of various temperatures effect towards chitinase activity. Optimum temperature was determined over a temperature range of 30-50°C in Sodium phosphate buffer 50 mM pH 7.0. The activity was measured using the method described before.

2.5.2. Determination of various pH effect towards chitinase activity. Optimum pH was determined over pH values range from 3.0 to 9.0 at the temperature of 37°C. Sodium acetate buffer 50 mM was used for pH 3.0 to 5.0, Sodium phosphate buffer 50 mM for pH 6.0 to 7.0, and Tris-HCl buffer 50 mM for pH 8.0-9.0. The activity was measured using the method described before.

2.5.3. Determination the effect of EDTA and divalent cations towards chitinase activity. The effect of EDTA was examined by estimating activity in the presence of 1.0 mM EDTA solution. The effect of various divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}) was examined by estimating activity in the presence of 1.0 mM solution of chloride metal salts. 250 μL of enzyme solution was preincubated in the presence of 250 μL of EDTA or divalent cations for 30 minutes at 30°C, followed by measuring activity using colloidal chitin as substrate at pH 7.0 and temperature of 37°C as described before. Control was done by measuring the chitinase activity without addition of either EDTA or divalent cations.

3. Results and discussion

3.1. Determination of B. subtilis B298 growth curve

The bacterial growth curve was made to determine the time of incubation that reaches logarithmic phase. The result was shown at figure 1.

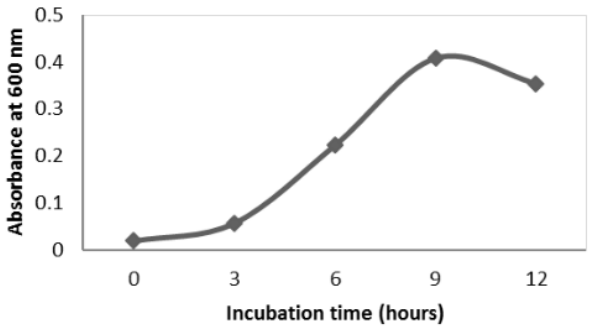

Figure 1. *B.subtilis* B 298 growth curve

According to the result shown at figure 1, the bacterial growth was reached optimum within 9 hours incubation with the absorbance value is 0.408. It's mean that the logarithmic phase of this bacterial growth was achieved at 9 hours incubation time. So, the inoculum that is used as the source for chitinase production is *B.subtilis* B 298 isolate that is incubated for 9 hours.

3.2. Determination of chitinase production curve

Chitinase production curve was made to determine the time of incubation that give the highest chitinase activity. The result was shown at figure 2.

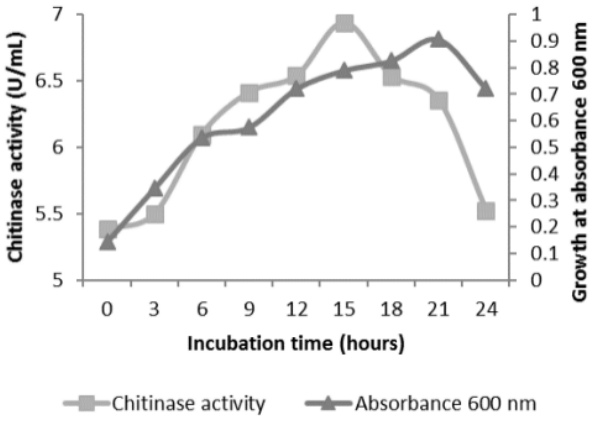

Figure 2. Chitinase production curve from *B. subtilis* B 298

Based on the result shown at figure 2, the chitinase enzyme from *B. subtilis* B 298 was produced maximumly within 15 hours incubation of the inoculum. That was indicated by the highest chitinase activity with its value is 6.937 U/mL. It's mean that for the production of crude extract chitinase, the inoculum should be incubated for 15 hours. Contrary, the chitinase enzyme from *B. licheniformis* MB-2 was produced maximumly within 72 hours incubation of the inoculum [7]. It's mean that *B. subtilis* B 298 produced this enzyme maximumly faster than *B. licheniformis* MB-2.

3.3. Production and characterization of crude extract chitinase

Crude extract chitinase was obtained by incubating *B.subtilis* B 298 inoculum in Nutrient Broth medium fortified with 0.5% (w/v) of colloidal chitin as the inducer for 15 hours that has been determined before. The addition of colloidal chitin in the medium induced the production of chitinase enzyme in bacteria cells. After incubating, the medium was centrifuged to get cell-free supernatant that is considered as crude extract chitinase. The crude extract was then used for further characterization including the effect of various temperatures and pHs towards chitinase activity as well as the effect of the addition of EDTA and various divalent cations.

3.4. Effect of various temperatures toward chitinase activity

Assay of various temperatures effect on chitinase activity was conducted to determine the optimum temperature that give the highest chitinase. The result was shown at figure 3. Based on the result shown at figure 3, the enzyme was active at the temperature range observed. The optimum temperature for chitinase activity from *B.subtilis* B 298 was achieved at 40°C with activity value is 5.764 U/ mL respectively, meanwhile the activity decreased slightly above 40°C. So, chitinase from *B.subtilis* B 298 is classified as the mesophilic enzyme. Similar study was described by other workers. Two chitinases isolated from *Pseudomonas aeruginosa* K-187 has the optimum temperature of 40°C and 50°C [8]. Some other workers obtained different results. Chitinase from *Bacillus amyloliquefaciens* SAHA 12.07 had optimum temperature 50°C, from *Serratia marcescens* KAHN 15.12 had optimum temperature 60°C [9], chitinase from *Bacillus cereus* 108 had optimum

temperature 65°C [1]. Increasing the incubation temperature of an enzyme could cause the increase of enzyme activity. This is due to the increased speed of thermal motion of enzyme molecules, increased the numbers of molecules that have enough energy to reach the transition state [10].

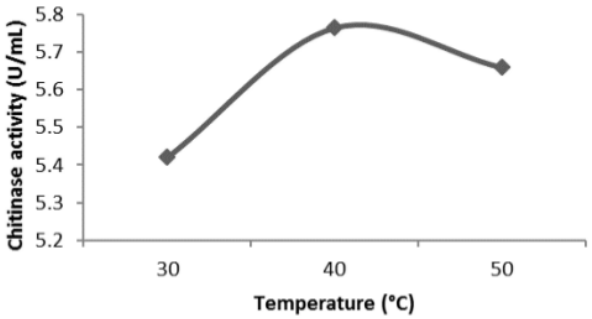

Figure 3. Effect of various temperatures on chitinase activity

3.5. Effect of various pH toward chitinase activity

Effect of various pH is examined to determine the optimum pH for chitinase activity. The result was shown at figure 4.

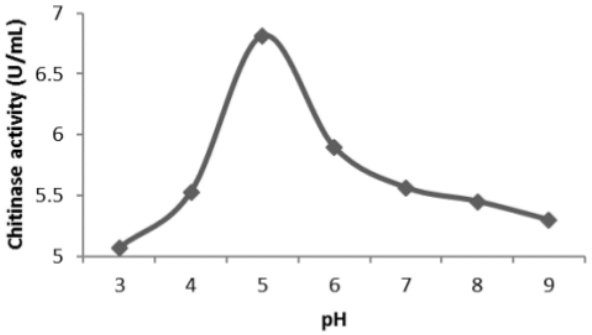

Figure 4. Effect of various pH on chitinase activity

Based on the result exhibited at figure 4, the enzyme was active in the pH range of 3.0 to 9.0 with optimum pH for chitinase activity from *B.subtilis* B 298 was achieved at pH 5.0, with its activity is 6.813 U/mL. The activity decreased sharply above the pH of 5.0. Similar study has been reported by other researchers. Crude extract chitinase from *Bacillus amyloliquefaciens* SAHA 12.07 had optimum activity at pH 5.0 with its activity is 1.158 U/mL [9]. Meanwhile, chitinase from *B. amyloliquefaciens*

V656 had optimum activity at pH 6.0 and 7.0 [11], chitinase from *S. marcescens* GEI and *S. marcescens* MO 1 had optimum activity at pH 7.0 [12, 13]. Each enzyme has an optimum pH that causes maximum activity. If the pH of the reaction is below or above the maximum pH, the enzyme activity decreases [14]. Bacterial chitinases generally have a pH range from acidic to alkaline [9].

3.6. Effect of EDTA and divalent cations addition towards chitinase activity

Several enzymes are known to require certain metal ions for their catalytic activity. Metal ions are required as a component of the active site of the enzyme to maintain its stability. Test of the addition of EDTA and divalent cations is conducted to determine whether the enzyme includes metalloenzyme or not and what kind of cations that are required for enzyme activity. The result was displayed at table 1.

Table 1. Effect of EDTA and divalent cations addition on chitinase activity

Treatment	Relative activity (%)
Control	100
EDTA	51.225
$CaCl_2$	53.088
$MgCl_2$	82.118
$ZnCl_2$	54.584
$CuCl_2$	63.826
$HgCl_2$	86.695

Based on table 1, the relative activity of enzyme decreased with the addition of 1.0 mM EDTA compared to control. The decline in activity reached about 51%. This means that chitinase from *B. subtilis* 298 bacteria is a metalloenzyme. According to [15], metalloenzyme is an enzyme that requires metal ions in the active site for catalytic ability. If addition of EDTA decreased enzyme activity compared to control, the enzyme including metalloenzyme. EDTA inhibits enzyme activity by chelating metal in the active site to alter the tertiary structure of enzyme and enzyme lose it's catalyzing ability.

Addition of 1.0 mM Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+} cations showed the decline of chitinase activity. This means that all the divalent cations tested were inhibited the chitinase activity of *B.subtilis* B 298. The largest decline in activity occured in the addition of Ca^{2+} cation that is equal to 53%.

4. Conclusion

Crude extract chitinase extracellular from *B.subtilis* B 298 bacteria has been produced and partially characterized the biochemical properties. Production of crude extract enzyme was conducted using inoculum within 8 hours incubation and production time within 15 hours incubation. The crude extract chitinase has the optimum temperature at 40°C and optimum pH at 5.0. This enzyme was categorized as metalloenzyme.

Acknowledgements

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