Characterization of crude chitinase produced by *Trichoderma virens* in solid state fermentation

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Abstract : The objective of this study to ensure the appearance of the enzymes produced by *Trichoderma virens* and to obtain information on the optimum conditions for the enzymes in its specified reaction. Crude chitinase produced by *Trichoderma virens* presented three types of chitinolytic enzymes: endochitinase, exochitinase and protease. The optimal temperature for crude chitinase at 60^0C and optimum pH at 3.0. Crude chitinase stability decreases as incubation temperature increases, however, crude chitinase was found to be stable over a range at pH acidic. *T. virens* growth in the solid substrate with shrimp waste as substrate produced crude chitinase with several chitinolytic enzymes based on its protein visualization on SDS-PAGE. This crude chitinase has endochitinase (50 and 42 kDa) exochitinase (33 and 25 kDa) and protease (18 kDa).
Keyword : Chitinase, *Trichoderma virens*, Solid state fermentation

INTRODUCTION

Chitin is the second most abundant natural polymer on earth after cellulose. It is a high molecular weight polymer of N-acetyl-D-glucosamine (NAG) units linked by β-D-(1-4) linkage with 1000-3000 units (Patil *et al*., 2013). The enzyme responsible for chitin degradation and modification is chitinases. Chitinolysis is performed by three separate enzymes : endochitinases, which produce multimers of N-acetyl-glucosamine (NAG), exochitinases, which release soluble low molecular weight dimers, and chitobiose which hydrolysis chitobiose to NAG (Patil *et al*., 2013).

Chitooligomers produced by enzymatic hydrolysis of chitin have been of interest in the past few decades due to their broad range of medical, agricultural and industrial applications. This potential is associated with its characteristic being reported as antibacterial, antifungal, hypocholesterolemic, and antihypertensive. (Yuli *et al*., 2004). The commercial interest in the utilization of chitin and its derivatives has led to the need for inexpensive, reliable sources of active and stable chitinase preparation. The current limitation on the use of industrial enzymes from natural resources, their stability and their activity within narrow temperature and pH ranges. A scientist has focused their attention to microorganisms capable of living under the extreme environment in the course of evolution.

Each of microorganisms will secrete unique and different characteristic of chitinase dependent on the type of substrate and fermentation conditions exposed to. Therefore, characterization should be done in order to ensure the appearance of the enzymes produced by one of the microorganisms and to obtain information on the optimum conditions for the enzymes in its specified reaction (Yuli *et al*., 2004)

Several studies on characterization of chitinase had been reported by several researches. Binod *et al* (2005) reported that *Penicillium aculeatum* is able to produce chitinase enzymes during 3 days of incubation in SSF. The optimum pH for these enzymes at pH 4.0 and the optimum temperature at 50^0C. Thermal stability of the enzymes

was appreciable up to 45^0 C and pH stability at pH 5.5. In a different experiment, Patil *et al* (2013) reported that chitinase produced by *Penicillium achrochlorum* was optimum temperature was 40^0C and stability up to 50^0C, optimally active in the pH range 6-7.

In this study, *Trichoderma virens* was used as a microorganism that can produce chitinase by degrading chitin from shrimp waste as the substrate using solid state fermentation. The objective of this study to ensure the appearance of the enzymes produced by *Trichoderma virens* and to obtain information on the optimum conditions for the enzymes in its specified reaction.

MATERIALS AND METHODS

Substrate and Pretreatment

Shrimp shell was used as the substrate in the production of chitinase by *T. virens*. Shrimp shell was provided by the local market of Makassar, Indonesia. This material was dried in the microwave for ten minutes until the weight was constant and moisture content was 10% (w/v). The dry shrimp shell was ground and stored in a dry place at room temperature. It was used as the solid substrate for SSF without demineralization or deproteinization.

Substrate Preparation for Solid State Fermentation (SSF)

Five grams of dry shrimp shell was supplemented with appropriate volume of basal medium containing 0.17% (w/v) $(NH_4)2SO_4$; 0.025% (w/v) $MgSO_4.7H_2O$; 0.028% (w/v) KH_2PO_4; 0.007% (w/v) $CaCl_2.2H_2O$. The initial pH substrate was adjusted to 5.5. The thoroughly mixed substrate was autoclaved at 121ºC, 1.03 bar for 20 minutes, and cooled to room temperature before inoculation.

Microorganism and Inoculum Preparation

T. virens was grown on medium Potato Dextrose Agar (PDA) for 7 days. 10 mL of 1.0% (v/v) Tween 80 was used to harvested the spores. Spore suspension was collected and centrifuged at 4,000 rpm for 30 minutes at 4ºC. The pellets resuspended in distilled water in order to obtain spore inoculum of 1×10^7 spores/mL.

Extraction of Chitinase

The fermented substrate (5 g) was diluted in an adequate amount of buffer to get a total extraction volume of 100 mL. The contents were thoroughly mixed by keeping the flask on a rotary shaker at 150 rotation per min (rpm) for 30 minutes. The mixture was centrifuged at 4,000 rpm for 30 minutes at 4ºC. The supernatant was collected and used for chitinase and protein assay.

Chitinase Assay

Chitinase activity was determined by a dinitrosalycylic acid (DNS) method [21]. It was defined based on the release of N-acetyl glucosamine (NAG) as the reaction product of enzyme degradation. The 2 mL reaction mixture contained 1 mL of 1.0% (w/v) colloidal chitin in citrate phosphate buffer (pH 4.0) and 1 mL crude enzyme extract. The well vortexed mixture was incubated at 50ºC for 1 h. The solution was then centrifuged at 4,000 rpm for 5 minutes. The reaction was terminated by adding 1 mL DNS reagent followed by heating at 100ºC for 5 minutes. The reaction was measured at 540 nm using UV spectrophotometer (UV-160A, Shimadzu, Japan). Colloidal chitin was prepared based on the modified method of Azaliza. One unit (U) of chitinase activity is defined as the amount of enzyme that is required to release 1 µmol of N-acetyl-β-D-glucosamine per minute under assay conditions.

Partial Characterization of Crude Chitinase

Identification of Optimum Temperature and Thermal Stability

For optimum temperature determination, the chitinase activities were assayed at temperatures from 30 to 80^0C in citrate phosphate buffer (pH 4.0, 0.2 M). For thermal stability assay, the crude chitinase was pre-incubate at different temperatures (30, 40, 50, 60, 70 and 80^0C) in the absence of substrate. The control was incubated at 40^0C for the same time. After incubation for different intervals (0-120 min), the chitinase activity was measured by assaying the residual chitinase activities and relative chitinase activity.

Identification of Optimum pH and Stability

The optimum pH of crude chitinase was determined at the optimum temperature of the respective enzyme by carrying out the enzyme assay at different pH using the following buffers : 0.2 M citrate phosphate (pH 2.0, 3.0, 4.0, 5.0), 0.2 M phosphate (pH 6.0, 7.0, 8.0), 0.2 M glycine-NaOH (pH 9.0 and 10.0). in determining the pH stability the crude enzymes were incubated at 60^0C (based on optimum temperature) by diluting the culture supernatant two-fold in the above buffers for specific intervals time between 0-120 min. After completion pH of the particular enzyme and the assay conducted at the optimum temperature for that assay.

Characterization of crude chitinase.

12% separating gel and 5% stacking gel were prepared inside the gel plate assembly. Load 20 µL of the sample into the wells. The reference, 10 µL molecular weight markers (Promega Broad Range Protein) was loaded into a separate well. The electrophoresis system was conducted at voltage 200 V for 45 minutes. After the run has been completed the gel was stained with silver staining.

RESULT AND DISCUSSION

Optimum Temperature and Thermal Stability

Temperature has been identified as a strong factor in influencing the enzyme-substrate reaction. Each enzyme has an optimum temperature, where the enzyme can work well. The farther from the optimum temperature, the enzyme can not work well . The high temperature will be increasing the activity of the enzyme. Up to a certain limit of temperature enzyme activity decrease because of enzyme denaturation and drastically reduces the rate of reaction At low temperature, work or enzyme reaction rate will decrease. In the process of denaturation of enzymes will lose three-dimensional structure as a result of the termination of hydrogen bonding and non-covalent bonds. Denaturation of the enzyme causes changes in the composition of the active site amino acid, which causes the enzyme changes shape loses its catalytic ability (Tortora *et al.*, 2004).

The ascending of Figure 1.1 is known as temperature activation while the descending part is called temperature inactivation or thermal denaturation. At 70^0C chitinase retained about 90% activity and drastically reduce at 80^0C (33%) due to enzyme denaturation. The ability of crude chitinase from *T. virens* to retain its activity at high temperature gives an advantage of this enzyme to be used in the industrial application and recycling of chitin wastes.

The optimum temperature of chitinase enzyme was 60^0C. These results are in agreement with chitinase produced from *Microbiospora* sp which reported to exhibit highest activity at 60^0C (Nawani *et al*., 2002). High-temperature optima have been reported for chitinase Bacillus sp.13.26, which produces four chitinases with temperature optimal at 65^0C (Yuli *et al*., 2004) and chitinase from *Streptomyces thermoviolaceus* has been reported to have an optimum temperature of 80^0C. For a comparison, the *T.virens* crude chitinase optimum temperature of 60^0C obtained in this study is 10^0C higher then the optimum temperature of crude chitinase from *T.virens* UKM 1 strain growth in batch culture. (Azaliza, 2007).

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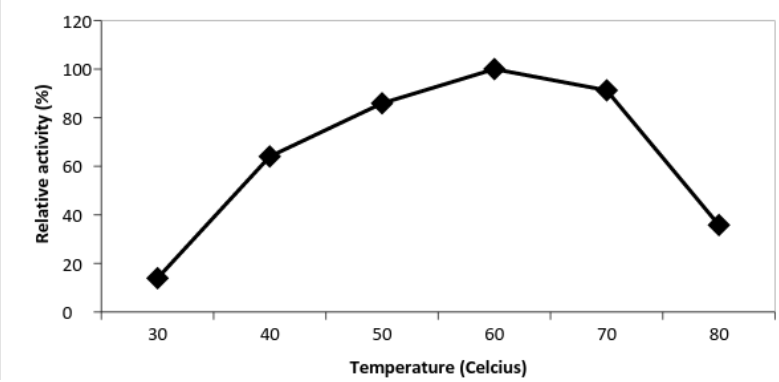


Figure 1.1 Relative activity of crude chitinase at different temperature

The thermal stability studies of the crude chitinase from *T. virens* were shown in Figure 1.2. From Figure 1.2 it can be observed that chitinase was stable at the first to 10 minutes incubation until 120 minutes incubation period within temperature 30^0C to 60^0C residual activity ranging from 100 – 56%. At temperature 70^0C chitinase was stable at 10 minutes incubation until 40 minutes with residual activity ranging from 87 – 72%, after 120 minutes residual activity was dropped to 14%, much lower compared ti incubate at 30^0C (66%), 40^0C (83%), 50^0C (69%), 60^0C (56%). As well as temperature 80^0C after 30 minutes residual activity was dropped to 6.2% after 120 minutes incubation period. This result can be explained by the increment of temperature can be the reduction of the enzyme stability, due to the hydrogen bonds interaction loss of function and ultimately the denaturation of enzymes (Buchholz *et al*., 2005). Based on a range of temperature stability, thermostable enzymes are classified into three groups : moderately thermostable (45-65^0C), thermostable (65-85^0C) and extremely thermostable (> 85^0C) (Yano and Poulus, 2003). The ability of crude chitinase from *T. virens* to retain residual activity as low as 56% at 60^0C makes the enzyme be classified in the range of moderately thermostable enzyme.

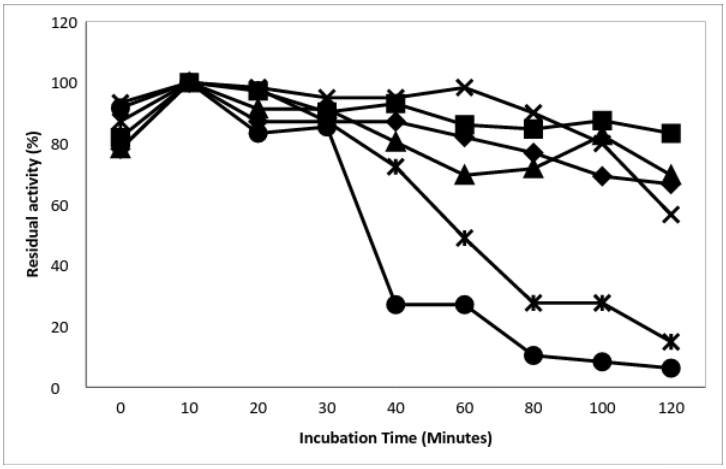


Figure 1.2 Thermal stability of crude chitinase at different temperatures ; 30^0C (♦), 40^0C (■), 50^0C (▲), 60^0C (x), 70^0C (*) and 80^0C (●)

Optimum pH and pH stability

The effect of the pH on the chitinase activities of the crude chitinase from *T. virens* was examined at various pH ranging from pH 2.0 to pH 10.0 as shown in Figure 1.3. Chitinase has optimal pH was pH 3.0. The optimal pH 3.0 obtained from this study was in agreement with Nawani *et al* (2002) who reported that chitinase from *Microbiospora* sp. V2 presented optimally at pH 3.0. Chitinase activity of crude chitinase was able to full relative activity (100%) under pH 3.0 and started decline under pH 4.0 until pH 10.0. Based on this result, low relative activity at pH 2.0 (44.5 %), it is increase until 100% at pH 3.0 and the activity decrease at pH 4.0 (59.4 %), pH 5.0 (44.5 %), pH 6.0 (24.75 %), pH 7 (20.79 %), pH 8 (19.8 %), pH 9 (12.8 %) and pH 10 (9.9%). Meanwhile, chitinase activity of the enzyme decreased rapidly below and above pH 3.0.
From the result, it was clearly showed that the crude chitinase from *T. virens* required acidic environment to be active where te ionic groups on the enzyme's active sites were in a suitable form to function (Shuler and Kargi, 2002). The present result also showed that crude chitinase from *T. virens* was not active at alkaline pH. Alkaline pH cause the ionic form of the active site of the crude chitinase changes and may also alter the three – dimensional shape of the enzyme and hence, can cause complete enzyme denaturation (Shuler and Kargi, 2002).

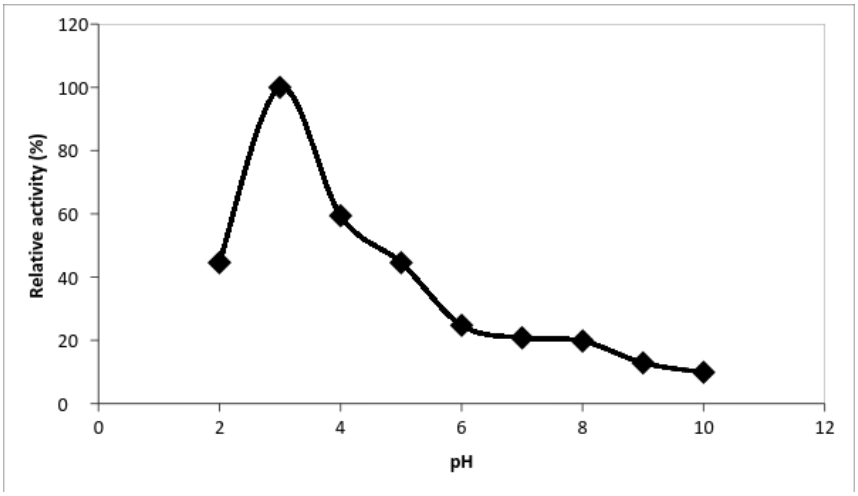


Figure **1.3** Relative activity of crude chitinase at different pH for 60 minutes incubation

The pH stability of chitinase at 60^0C was also examined at various pH ranging from pH 2.0 to pH 10.0 over a period of 120 minutes as show in Figure 1.4. The enzyme was found to be stable at range pH 3.0 – pH 6.0, the most stable at pH 3.0 and pH 4.0. The enzyme showed similar stability profiles in all the pH tested where it was comparatively stable during 10 until 60 minutes of incubation before the residual activity declined drastically afterward.

The more alkaline pH, more unstable chitinase activity as can be seen at the pH ranging from pH 7.0 – pH 10.0 with the lowest residual activity 10% was noted at pH 8.0 during 120 minutes incubation. This result is in agreement with the findings by Wang *et al* (2010) who reported that chitinase from *Pseudomonas* sp TKU015 showed the reduction in its stability when incubated in alkaline pH (7.0, 8.0, 9.0, 10.0, 11.0). on the other hand, Binod *et al* (2005) reported that chitinase production of *Penicillium aculeatum* NRRL 2129 relative activity was lower in pH 6.0 and pH 7.0 compared to the chitinase in acidic pH. At pH 2.0 chitinase activity declines sharply after 10 minutes incubation with residual activity 2.1% after 120 minutes. Nawani *et al* (2002) also reported chitinase from *Microbiospora* sp. V2 has relative activity < 40% at pH 2.0 after 1 hour incubation.

Crude chitinase from *T. virens* showed instability profiles where residual activity at pH 2.0 and pH 7.0 to pH 10.0. Crude chitinase showed maximal activity at pH 3.0 which was stable in acidic pH range (3.0 – 6.0). Increasing pH to more than 6.0 would change the ionic condition for the chitinase's active site and affect the

catalytic activity, thus reduced chitinase activity. For this observation, it can be concluded that crude chitinase from *T. virens* can be classified as an acidic tolerant microorganism (acidophiles) (Tortora *et al*., 2004).

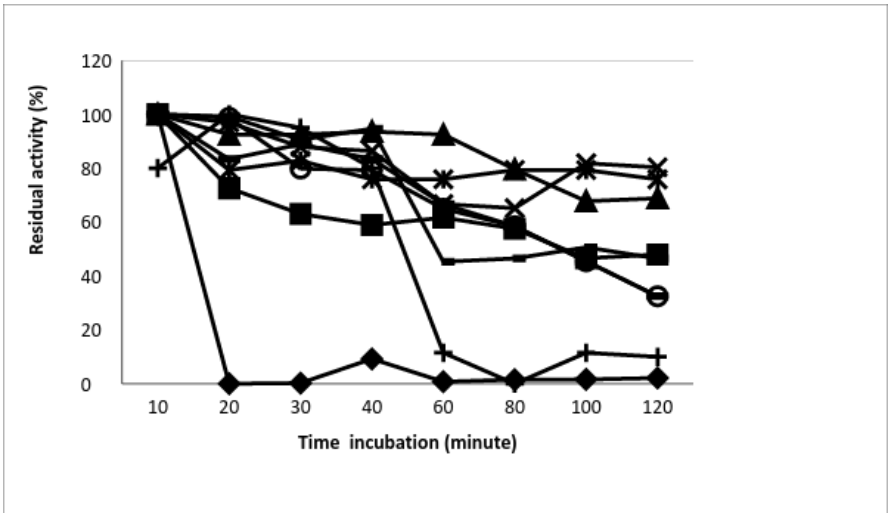
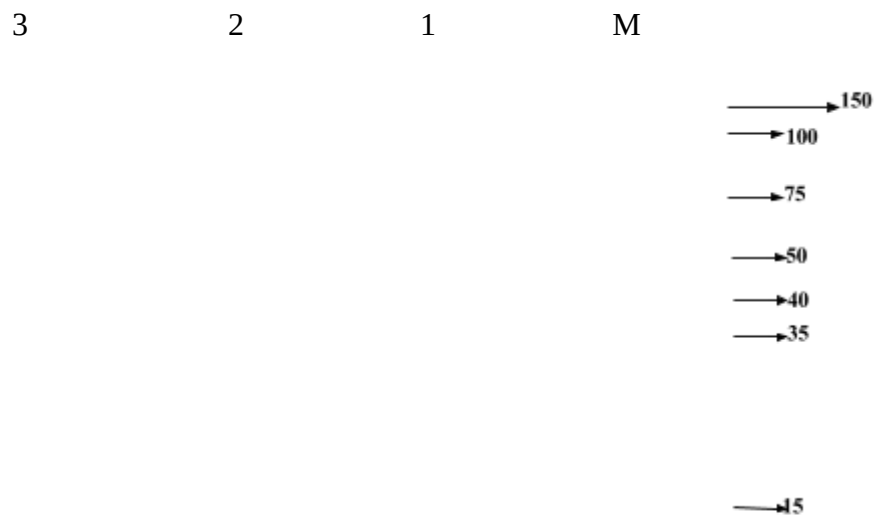


Figure 1.4 The pH stability of crude chitinase at different pH; pH 2.0 (♦), pH 3.0 (■), pH 4.0 (▲), pH 5.0 (x), pH 6.0 (*), pH 7.0 (○), pH 8.0 (+), pH 9.0 (-), pH 10.0 (●)

Crude chitinase molecular weight determination



Lane 1 : Medium
Lane 2 : Crude chitinase from *T. virens* (0.467 U/gds, 0.53 mg/mL)
Lane 3 : Crude chitinase from *T. virens* (TCA precipitation)
M : Marker

SDS gel electrophoresis of the crude chitinase and sample concentrate with TCA precipitation process showed a similar number of protein with similar molecular weight and exhibit five protein bands (line 3 and 4). Protein band was described by Kubicek *et al* (2001) as N-acetyl-β-D-glucosaminidase (73 and 64 kDa), endochitinase (46 and 37 kDa), exochitinase (28 kDa) and protease (18.8 kDa). In this study, five protein bands of

molecular weights, 50, 42, 33, 25 and 18 kDa could be visualized by SDS-PAGE analysis. This protein can be described as endochitinase (50 and 42 kDa), exochitinase (33 and 25 kDa) and protease (18 kDa). The absence of banned in control lane (line 2) proved that the bands strictly appear from chitinase produced by *T. virens* and not from the production medium. This result is similar to what has been reported by Azaliza (2007) and Sazwani (2007) which reported that endochitinase enzyme from Trichoderma virens UKM-1 separated in SDS-PAGE gel showed endochitinase exhibited at 46 and 37 kDa; 42 and 32 kDa respectively. On the other hand, the various molecular mass of chitinase enzyme from Trichoderma sp has been reported by several literatures. These include 46 kDA (Lima *et al*., 1997), 40 kDa (Ulhoa and Paberdy, 1992), 52 kDa (Inbar and Chet, 1995) and 42 kDa (Cruzz *et al*., 1995; Haran *et al*., 1995).

CONCLUTION

Crude chitinase produced by *Trichoderma virens* presented an optimum temperature at 60^0C and optimum pH at 3.0. Crude chitinase stability decreases as incubation temperature increases, however crude chitinase was found to be stable over a range at pH acidic. *T. virens* growth in solid substrate with shrimp waste as substrate produced crude chitinase with several chitinolytic enzyme based on its protein visualization on SDS-PAGE. This crude chitinase has endochitinase (50 and 42 kDa), exochitinase (33 and 25 kDa) and protease (18 kDa). The presence these protein would allow a complete degradation of chitin.

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