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PRODUCTION AND CHARACTERIZATION OF CHITINASE ENZYMES FROM SULILI HOT SPRING IN SOUTH SULAWESI, *Bacillus* sp. HSA,3-1a

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ABSTRACT

Chitinase is an extracellular enzyme which is capable in hydrolyzing insoluble chitin to its oligomeric and monomeric components. The enzyme produced by thermophilic bacteria was screened and isolated from Sulili hot spring in Pinrang, South Sulawesi, Indonesia. The gram positive spore forming rod shape bacteria was identified as Bacillus sp. HSA,3-1a through morphological and physiological analysis. The production of chitinase enzyme was conducted at various concentration of colloidal chitin at a pH of 7.0 and a temperature of 55 °C. The bacteria optimally was produced the enzyme at a colloidal chitin concentration of 0.5% after 72 h of incubation. The optimum temperature, pH and substrate concentration of chitinase were 60 °C, 7.0 and 0.3%, respectively. The enzyme was stable at a pH of 7.0 and a temperature of 60 °C after 2 h of incubation. The chitinase activities was increased by addition of 1 mM Mg^{2+}, Ca^{2+} and Mn^{2+} ions, whereas the activities were decreased by 1 mM Co^{2+}, Fe^{2+} and Zn^{2} ions. The molecular weight of the crude enzyme was determined using SDS-PAGE analysis. Five protein fractions were obtained from SDS-PAGE, with MWs of 79, 71, 48, 43 and 22 kDa.

Keywords: *colloidal chitin, thermophilic bacteria, chitinase*

INTRODUCTION

Chitinase (EC 3.2.1.14) is an enzyme which can hydrolyze chitin to its oligomeric, dimeric and monomeric component. The enzyme found in numerous bacteria, fungi, insect, plant and animal involves in natural protection mechanism. Many bacteria and fungi containing the chitinolitic enzyme convert chitin into carbon and nitrogen that can serve as energy source. Chitinolitic bacteria are typically detected and screened through the production of clearing zones on chitin containing agar as selective medium. Chitinases have many industrial and agricultural applications such as preparation of chitooligosasccharides, biocontrol of plant pathogenic fungi, and production of single-cell protein [1-3].

Chitin is highly insoluble β-(1,4)-linked polymer composed primarily of N-acetyl-glucosamine and some glucosamine residues, and is believed to be the second most abundant biomaterial after cellulose. It is widely distributed as a structural component of crustaceaes, insects, marine invertebrates, such as cuttlefish, crab, and lobster as well as a component of the cell walls fungi and algae, the annual production in marine water alone is estimated to be more than 100 billion tons [2,4-5].

A research on isolation of microbes producing chitinase has been successfully conducted. The researches included acidophilic chitinase isolated from kamojang Crater of West Java [6] and chitinase from thermophilic bacteria isolated from hot water source of Tamposo, North Sulawesi [7].

The previous research on isolation of thermophilic bacteria from the hot water source in South Sulawesi produced several isolates of chitinolitic bacteria, but the optimum condition to produce them and characteristic of biochemical properties of the isolates have not been known, yet. Therefore, a further research on the isolate is necessary. The HSA 3-1a isolate was selected because the isolate had the highest chitinolitic activity [8].

In this research, identification of the HAS,3-1a isolate, production optimalization and characterization of the crude enzyme were carried out. The research involved determination of optimum pH and temperature, pH and temperature stability, the metal ion which can be function as activator and inhibitor to the activity of chitinase as well as analysis of molecular mass of protein with SDS-Page (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis).

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EXPERIMENTAL SECTION

Materials

Thermophilic bacteria, HSA-3,1a isolate, colloidal chitin (chitin sigma, EC. 215-744) as the enzyme substrate, medium containing yeast extract, bacto pectone, bacto agar, $(NH_4)_2SO_4$, K_2HPO_4, NaCl, $MgSO_4$. 7 H_2O, several divalent cations, Bovine Serum Albumin (BSA) as the protein standard.

Instrumentation

Analytical balance (Ohaus), autoclave (Model 8000-DSE Napco), shaker water bath (Memmert), spectrophotometer, pH meter (Memmet), Centrifuge (Model 800), hotplate stirrer (Fisher Versamix TM).

Procedure

Microorganism Analysis

The culture stock of thermophilic bacteria, HAS,3-1a isolate, was cultivated for 18-20 h at the temperature of 55 °C in the medium of agar slant without colloidal chitin [6,9]. The morphological identification and the biochemical test were then conducted. The former involved bacterial shape, colonies color, cell shape, gram test (positive or negative) with or without spores, and mortality test; the latter included reactions: catalase, oxidase, indol, citrate, gelatin, methyl red and carbohydrate test [7].

Optimation of Chitinase production [6,9]

Thermophilic bacteria, *Bacillus sp.*, particularly the HSA-3,1a isolate was inoculated as many as 10% isolate into the fermentation medium with the same composition as the medium. The isolate was then shaken at 180 rpm, 55 °C for 1-6 days. Sampling was taken every 24 h for analysis of the optical density (OD), the chitinase activation and the protein content. After knowing the concentration of colloidal chitin and the fermentation optimum time, reproduction of chitinase in the large scale (2L) was conducted for enzyme characterization.

Characterization of Chitinase [6,9]

The chitinase enzyme produced at the optimum condition was put in the centrifuge at the speed of 3500 rpm for 30 min and 4 °C. The product was characterized by: determining optimum temperature and pH, temperature and pH stability, metal ions functioning as activator or inhibitor at the concentrations of 1.0 mM and 5.0 mM, with metal ions used of $MgCl_2$; $CaCl_2$; $MnCl_2$; $NiCl_2$; and $CuCl_2$. Molecular mass analysis of proteins by a method of SDS-PAGE.

Determination of Protein Content

Determination of protein content was conducted using the Lowry method [10]. Two solutions were used in the method; Lowry B and Lowry B. The former consisted of 2% Na_2CO_3 in a mixture of 0.1 N NaOH, 1% $CuSO_4$, and sodium potassium tartrate with the ratio of 100:1:1 and the latter was a solution of phospho-tungstic-phospho-molybdic acid (folin) in aquadest (1:1). The protein content was measured by a spectrophotometer at the maximum wavelength using BSA as the standard.

Determination of Chitinase Activity

The chitinase activity was determined by using the Ueda and Arai method [11] as follows: 2 mL of phosphate buffer solution (pH 7) was mixed with 1 mL of the enzyme solution (sample) and 1 mL of 0.3% colloidal chitin (substrate). The mixture was incubated at 55 °C for 60 min. 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.

Determination of Chitinase Molecular Mass [10]

A method used to determine the molecular mass of chitinase was the electrophoresis method using a LMW (Low Molecular Weight) marker: phosphorylase-b (M_r: 94,000); BSA (M_r: 67,000); ovalbumin (M_r: 43,000); carbonic anhydrate (M_r: 30,000); soybean trypsin inhibitor (M_r: 20,100), and silver stain colorization.

RESULT AND DISCUSSION

It was known from the previous work that the HSA,3-1a isolate of thermophilic bacteria had the chitinolitic activity [8]. Morphological and physiological identification results of the bacteria showed that the bacteria had properties as follows: they were positive gram bacteria with the shape of bacillus and with spores. Based on the identification and Bergey's Mannual of Systematic Bacteriology the HSA,3-1a isolate is in *Bacillus* genus. (Fig. 1b). Therefore, the isolate is named *Bacillus* sp. HSA-3,1a (Fig. 1a).

Results of chitinase production at various concentrations of colloidal chitin showed that the enzyme was optimally produced at the colloidal chitin concentration of 0.5% with the pH medium of 7.0; the incubation temperature of 55 °C (Fig. 2).

The optimum condition of chitinase production obtained from the research was similar to the one obtained from *Bacillus* MH-1 grown at 58 °C [5] and from *B. licheniformis* MB-2 using 0.5% colloidal chitin 0.5%

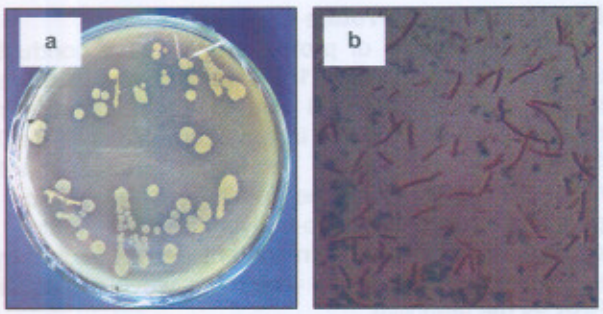

Fig 1. *Bacillus* sp. HSA-3,1a: a) the colony of bacteria grew at 55 °C, pH 7.0 for 3 days. b). cell shape and spore coloring.

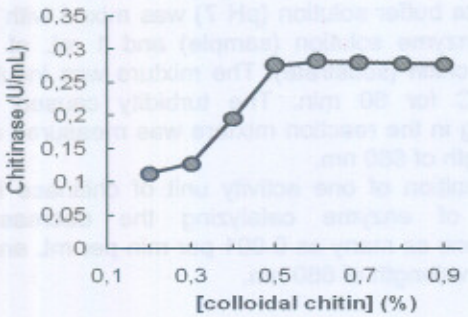

Fig 2. The effect of colloidal chitin concentrations on the extracellular chitinase production from *Bacillus* sp. HSA,3-1a.

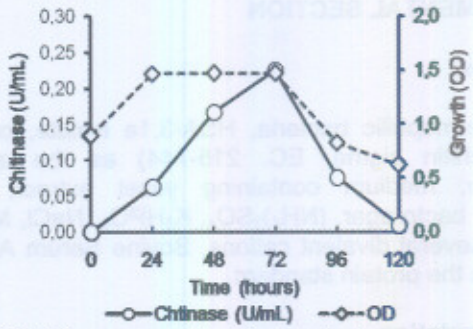

Fig 3. Optimum condition for producing chitinase by the *Bacillus* sp. HSA,3-1a at the substrate concentration of 0.5%, the medium pH of 7.0, and the temperature of 55 °C.

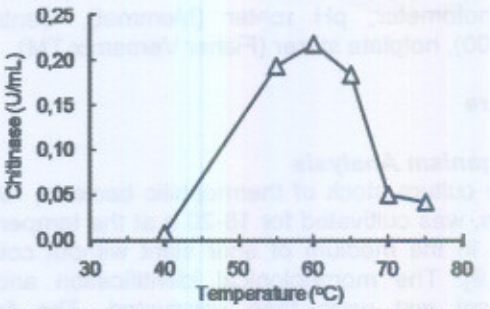

Fig 4. The chitinase activity of the *Bacillus* sp. HAS,3-1a as a function of the incubation temperatures with the chitin colloid concentration of 0.3% and the phospate buffer pH of 7.0.

at the cultivation temperature of 50 °C [12]. The optimal result was used in the chitinase production in order to find out the optimum time of chitinase production.

The chitinase production achieved the optimum condition at the end of stationary phase for the growth of *Bacillus* sp. HSA,3-1a, i.e. at hour-72 (Fig. 3). This may be caused by the decrease of nutrition in the medium. Therefore, the chitinase was secreted to the outside of cell in the large amount. The chitinase was used to degrade colloidal chitin acting as the source of carbon and nitrogen in the bacteria. Thus, the optimum condition to produce chitinase from *Bacillus* sp. HSA,3-1a was as follows: the colloidal chitin concentration of 0.5%; the medium pH of 7.0; temperature of 55 °C and the fermentation time of 72 h. The condition was used in producing chitinase for characterization.

Determination of Optimum Temperature and the Stability

Fig. 4 illustrates the effect of the incubation temperature on the chitinase activity. It is obvious that the HAS,3-1a isolate has the high activity in the temperature between 55 and 65 °C, but the optimum temperature is 60 °C (Fig. 4).

The chitinase produced from the isolate is categorized into a thermophilic enzyme. A stability test conducted at temperatures of 55 and 60 °C in the pre-incubation step showed that the enzyme was still stable for 2 h. This result indicates that the enzyme is thermostable. A different result was obtained when the *Bacillus licheniformis* MB-2 isolate was used. The chitinase enzyme produced by the isolate was stable for 3 h at the temperature of 60 °C [12].

Determination of Optimum pH and the Stability

Enzymes can behave as acid, base or neutral based on their amino acid constitution which can influence their active sites. The effect of the buffer pH on the chitinase activity is given in Fig. 5.

It is clear that the chitinase activity increases from the pH of 6.4 to 7.0 and then decreases at the pH of 7.2. The optimum pH is 7.0. The same optimum pH was obtained when the enzyme was produced from *Bacillus* sp. 13.26 [7]. However, the different optimum pH was given by the chitinase enzyme isolated from

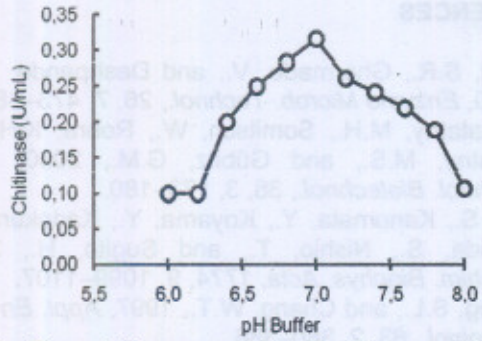

Fig 5. The chitinase activity *Bacillus* sp. HAS, 3-1a isolate as a function of the phospate buffer pH at the colloid chitin concentration of 0.3% and the temperature of 60 °C.

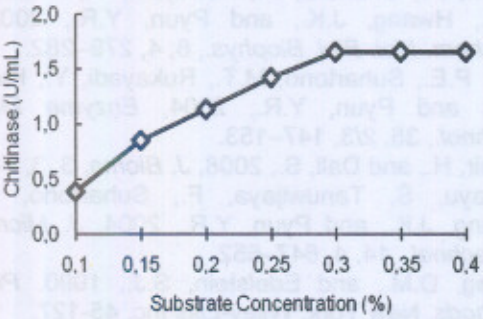

Fig 6. The effect of the substrate concentration on the chitinase activity from *Bacillus* sp. HAS, 3-1a at the temperature of 60°C and the phospate buffer pH of 7.0.

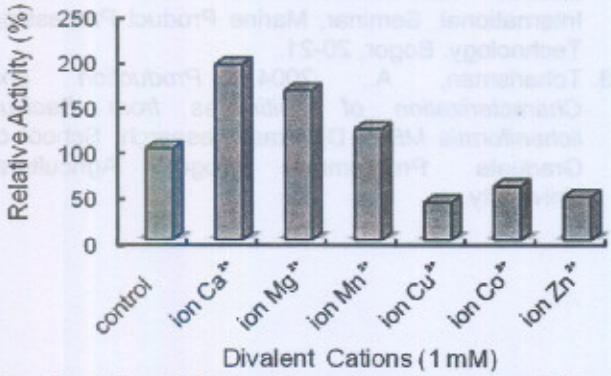

Fig 7. The Effect of metal ions on chitinase activities of *Bacillus* sp. HAS,3-1a at the colloidal chitin concentration of 0.3%, the temperature of 60 °C and the phospate buffer pH of 7.0.

different sources; the optimum pH of the enzyme from *B. licheniformis* MB-2 was 8.0 [12], and the one from *Bacillus* sp. K-22 was 5.0 which are stable for 2 h at the pre-incubation step [6].

Effect of Substrate Concentration on Chitinase Activity

Experiments with various substrate concentrations were conducted to know the optimum substrate concentration that can react with the enzyme. Fig. 6 shows that the chitinase activity for *Bacillus* sp. HSA,3-1a increases from the substrate concentrations of 0.1 to 0.3%.

This indicates that the activity of chitinase achieves the maximum at the substrate concentration of 0.3%. The condition is parallel with the chitinase activity from *Bacillus* sp. 13.26 and from *B. licheniformis* MB-2 which achieve the optimum at the substrate (colloidal chitin) concentration of 0.3% [7,12].

At the low substrate concentration, the active sites of enzyme bind only small amount of enzyme, but the higher the concentration of the substrate the larger amount of the substrate which can be bound by the active sites of enzyme. Therefore, the activity of enzyme will increase with the increase of the substrate concentration. However, at the certain substrate concentration, all active sites will be saturated by the substrate. At this condition, the increase of the substrate concentration will not increase the enzyme activity indicating that the substrate concentration has achieved the optimum condition.

Effect of Cofactor on Enzyme Activity

Generally, the enzyme needs non-protein compounds, such as, metal ions in doing its catalytic function. The compounds are required by the enzyme as components in the active side for activator or inhibitor in catalyzing the substrate at the certain concentration.

The pattern of activator or inhibitor effects of metal ions on the chitinase activity of *Bacillus* sp. HSA,3-1a was studied by using divalent ions. Results showed that chitinase was activated by 1 mM Ca^{2+}, Mg^{2+}, and Mn^{2+} ions. The result was similar to the one given by chitinase of *Bacillus* MH-1 [5] and of *B. Licheniformis* MB-2 [12] activated by 1 mM Ca^{2+} and Mg^{2+} ions. But the chitinase activity was inhibited by 1 mM Co^{2+}, Zn^{2+} and Cu^{2+} ions with competitive inhibition (Fig. 7).

Activator compounds until the certain amount can enhance the rate of enzyme catalyzed reaction, but the excess amount of the activator can cause the competition of the free activator and the activator-substrate complex to enzyme. The excess amount of the free activator causes the competitive inhibition.

Molecular Mass Determination of Chitinase

One method which is very efficient in separation and resolution of protein components in the sample is SDS-PAGE. Results of SDS-PAGE analysis from the

chitinase crude of *Bacillus sp.* HSA,3-1a showed that there were 5 protein bands observed with the estimated molecular masses of 79, 71, 48, 43 and 22 kDa. The protein sizes of chitinase are almost the same as that of other chitinases produced from different isolates. The molecular mass of chitinase from *Bacillus* MH-1 was 71, 62, and 53 kDa [5]. Two chitinase with different molecular mass (30 and 60 kDa) are isolated from *P.aeuroginosa* K.187 [4]. The one of chitinase from *Bacillus* sp. K-29, was 67 kDa [9]. The result showed that the molecular masses of protein/enzyme varied based on their source.

CONCLUSION

Based on the research results, it can be concluded that the optimum condition of chitinase production from thermophilic bacteria, *Bacillus* sp. HSA,3-1a was as follows: temperature of 55°C; medium pH of 7,0; colloidal chitin concentration of 0.5%; fermentation time of 72 h with the chitinase activity of 0.225 Unit/mL and the protein content of 0.154 mg/ mL.

Chitinase crude from *Bacillus sp.* HSA,3-1a worked optimally at the temperature of 60 °C, the phosphate buffer pH of 7,0 and the substrate concentration of 0.3%. The enzyme was activated by 1 mM Mg^{2+}, Ca^{2+} and Mn^{2+} ions and inhibited by 1 mM Co^{2+}, Zn^{2+} and Cu^{2+} ions. The estimated of molecular masses of the five proteins obtained from the SDS-PAGE analysis were 79, 71, 48, 43 and 22 kDa.

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REFERENCES

1. Patil, S.R., Ghormade, V., and Deshpande, M.V., 2000, *Enzyme Microb. Technol.*, 26, 7, 473–483.
2. El-Katatny, M.H., Somitsch, W., Robra, K.H., El-Katatny, M.S., and Gübitz, G.M., 2000, *Food Technol. Biotechnol.*, 38, 3, 173–180.
3. Itoi, S., Kanomata, Y., Koyama, Y., Kadokura, K., Uchida, S., Nishio, T., and Sugito, H., 2007, *Biochim. Biophys. Acta*, 1774, 9, 1099–1107.
4. Wang, S.L., and Chang, W.T., 1997, *Appl. Environ. Microbiol.*, 63, 2, 380–386.
5. Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., and Moriguchi, M., 1998, *Appl. Environ. Microbiol.*, 64, 9, 3397–3402.
6. Natsir, H., Chandra, D., Rukayadi, Y., Suhartono, M.T., Hwang, J.K., and Pyun, Y.R., 2002, *J. Biochem. Mol. Biol. Biophys.*, 6, 4, 279–282.
7. Yuli, P.E., Suhartono, M.T., Rukayadi, Y., Hwang, J.K., and Pyun, Y.R., 2004, *Enzyme Microb. Technol.*, 35, 2/3, 147–153.
8. Natsir, H., and Dali, S., 2008, *J. Bioma*, 3, 3, 7–15.
9. Rahayu, S, Tanuwijaya, F., Suhartono, M.T., Hwang, J.K., and Pyun, Y.R., 2004, *J. Microbiol. Biotechnol.*, 14, 4, 647–652.
10. Bollag, D.M., and Edelstein, S.J., 1990. *Protein Methods*. New York: Wiley-Liss Inc. 45-127.
11. Ueda, M., and Arai, M., 1992, *Biosci. Biotechnol., Biochem.*, 56, 3, 460–463.
12. Toharisman, A., Suhartono, M.T., Hwang, J.K., and Pyung, Y.R., 2002, *Thermostable Chitinase from Bacillus licheniformis MB-2*. The 5th JSPS International, Seminar, Marine Product Processing Technology. Bogor, 20-21.
13. Toharisman, A., 2004. *Production and Characterization of Chitinases from Bacillus licheniformis MB-2*, Disertasi Research, School of Graduate Programme, Bogor Agricultural University.