



Production and Application of Chitin Deacetylase from *Bacillus licheniformis* HSA3-1a as Biotermiticide

Ischaidar ^{a)}, Hasnah Natsir ^{b)}, Seniwati Dali ^{b)}

^{a)} Laboratory of Biochemistry, Chemistry Departement of Hasanuddin University, Makassar 90245
*E-mail : edhar_taz02@yahoo.com

^{b)} Chemistry Departement of Hasanuddin University, Perintis Kemerdekaan Km. 10 Tamalanrea, Makassar, Indonesia 90245.

ABSTRACT

Chitin deacetylase (EC.3.5.1.41) is a class of hydrolase enzymes that can degrade chitin. The biochemical property characteristics of chitin degrading enzymes are very diverse, particularly chitin deacetylase enzymes, which really depends on the types of microbes that express it. *Bacillus licheniformis* HSA3-1a, a thermophilic microbial strain used in this study was obtained from the hot spring of Sulili Pinrang South Sulawesi. The mechanism of chitin deacetylase in the termite which hydrolyzes chitin is associated with the presence of chitin on the termites skin that can be utilized by the enzyme as a substrate. The purpose of this research is to produce and determine pathogenicity chitin deacetylase enzymes excreted by the bacteria *B. licheniformis* HSA3-1a as biotermiticide. Pathogenicity determination is done in two methods; feeding (*baiting*) and contact (*spraying*) which are further analyzed quantitatively by calculating termite mortality percentage during 2 weeks of the observation. Based on the research, it is resulted that chitin deacetylase from *B. licheniformis* HSA3-1a has a good level of pathogenicity in inhibiting the growth of termites. The percentage of mortality obtained, the method of spraying reached up to 100% on the 6th day of observation and the method of feeding reached up to 100% on the 11th day of observation. This suggests that the chitin deacetylase is effective to be used as termite, and to replace conventional termiticide.

Keywords: Chitin deacetylase, *Bacillus licheniformis*, biotermiticide, termite

INTRODUCTION

Indonesia is one country with a very large biodiversity due to the diversity of environments, which provide many sources of microbial isolates and economic value. Search of isolates can be used in industries such as isolates capable of producing commercial enzymes needs to be pursued. One approach that can be done to explore the metabolic capacity is by studying the characteristics of producing microbes and genes encoding enzymes involved in chemical processes (Dawes and Sutherland, 1992).

Many organisms such as bacteria, fungi, higher plants and animals produce chitin deacetylase that degrade chitin into monomers or oligomers. These organisms usually have a variety of chitin deacetylase genes whose expression is induced by the substrate chitin or its derivatives (Guswenrivo, 2008). Bacteria utilize chitin deacetylase for assimilation of chitin as a source of carbon and nitrogen. The

mechanism of action of chitin deacetylase in the termite chitin hydrolyzation is associated with the presence of chitin in the skin of termites that can be utilized by the enzyme as a substrate. Chitin is a linear polymer composed of monomers 2000-3000 N-acetyl-D-glucosamine linked by β -1,4-glycoside bonds. Some sources of chitin include shrimp shell, lobster, and crab shells, cell walls of fungi and bacteria and insects. Considering the amount of chitin is abundant in nature, therefore that chitin is a readily available substrate (Natsir, et al., 2002; Wang and Chang, 1997).

Termite control technology is still based on the use of anti-termite pesticide (termiticide) that is effective but toxic in nature and are not environmentally friendly, and the results do not permanently eradicate the termites. For that purpose there needs to be an alternative termites control that is more environmentally friendly and effective. Several alternative termite control termiticide other than that have been studied is

the use of biological agents such as nematodes, bacteria, viruses and fungi.

Based on the background of this research aims to produce and apply chitin deacetylase (CDA) of *B. licheniformis* HSA3-1a and determine pathogenicity CDA crude extract enzyme as biotermiticide that can replace the use of termiticide.

METHODS

The microbes producing chitin deacetylase used in this study were *B. licheniformis* HSA3-1a. As the media used to grow bacteria yeast extract, NaCl, bacto agar, bacto peptone, K_2HPO_4 , $MgSO_4 \cdot 7H_2O$, $CaCl_2$, colloidal chitin as inducers, glycol chitin, phosphate buffer, acetic acid, sodium nitrite, ammonium sulfamic, HCl, indole, ethanol, distilled water, chlorpyrifos as a control. The instrument used was a shaker incubator, centrifuge, spektronik 20D +, hygrometers, and other tools commonly used in laboratory.

Stages in this study are consisted of rejuvenation of microbial, enzyme production, analysis of protein content and enzyme activity test of chitin deacetylase (CDA) and the determination of the pathogenicity of crude extract chitin deacetylase enzymes as biotermiticide.

Microbial rejuvenation

Prior to the rejuvenation of microbes (*B. licheniformis* isolates HSA3-1a) the preparation of solid medium composition had been done: yeast extract (0.05%), NaCl (0.1%), bacto agar (1.5%), bacto peptone (0.01%), K_2HPO_4 (0.01%), $MgSO_4 \cdot 7H_2O$ (0.01%), $CaCl_2$ (0.01%), colloidal chitin (0.5%) as enzyme inducers. Thermophilic microbes (*B. licheniformis* isolates HSA3-1a) were cultured in a solid medium for 24 hours under conditions of pH 7 with a temperature of 50°C. Furthermore, microbes that grow and have clear zones are used for further processing (Yuli, et al., 2004; Natsir, et al., 2010).

Chitin Degrading Enzyme Production

Bacteria *B. licheniformis* HSA3-1a that has been rejuvenated was carried on enzyme production process to determine the optimum time of the enzyme production of the enzyme by testing the activities of the enzyme. This process began with the creation of inoculum followed by a fermentation process (enzyme production), where the composition of the medium for this

process is $(NH_4)_2SO_4$ (0.7%), K_2HPO_4 (0.01%), NaCl(0.1%), $MgSO_4 \cdot 7H_2O$ (0.01%), bacto peptone (0.1%), yeast extract (0.1%) and colloidal chitin (1.0%). Active inoculum that was incubated for 18-24 hours at a temperature of 50°C, 180 rpm, was taken around by 10% to be inoculated into the production medium. Every 24 hours the sampling of measurement for bacterial growth, chitin deacetylase enzyme activity (CDA) and the enzyme protein content was done (Natsir, et al., 2010).

Test of Chitin Deacetylase Activity

The reaction mixture was 600 mL (100 mL glycol chitin 1% + 300 mL of phosphate buffer pH 7.0 + 200 mL enzyme solution), and was shaken and incubated at 50°C, for 30 minutes. Then 500 mL of 33% acetic acid, 500 mL of 5% sodium nitrite, and left for 10 minutes at room temperature. Then added 500 mL of ammonium sulfamic 12.5% and incubated room temperature for 30 minutes. Then added 2000 mL of 0.5% HCl and 200 mL 1% indole, then boiled for 5 minutes in a water bath, then cooled. Then add 96% ethanol, then measured the absorbance at the spectrophotometer with maximum wavelength (Tokuyasu, et. Al., 1996).

Measurement of protein content by Lowry method

Lowry reagent composition B; 2% Na_2CO_3 in 0.1 N NaOH: $CuSO_4$ 1%: Sodium-Potassium-Tartrate (100:1:1), Lowry reagent A; solution of phospho-tungstic acid-phospho-molybdic (foolin): distilled water (1:1). The protein content was measured using BSA (Bovine Serum Albumin) as standard and measured using a spectrophotometer at the maximum wavelength.

Determination of protein content is done by the method of Lowry, which included 1 mL of enzyme solution in a test tube and then added 8 mL of Lowry reagent B, average well shaken until mixed, then left at room temperature for \pm 10 minutes, then added 1 mL Lowry A. The mixture was shaken until blended, then left at room temperature for \pm 20 minutes, and then the absorbance of the solution was measured by UV-Vis spectrophotometer at maximum wavelength. The protein content of the sample (mg / mL) was determined by a curve calibration relationship between protein content and absorbance (Bollag, 1991).

Pathogenicity Determination of Crude Extract Enzyme Chitin Deacetylase as Biotermiticide

a. Spraying Methods

A total of 50 worker termites *Coptotermes* sp. Were sprayed with chitin deacetylase enzyme crude extract to wet the entire surface of the body of termites. Furthermore, the termites that have been sprayed with an the enzyme were transplanted into 5 cm diameter petri dishes that had been given a wet filter paper as the termites food. Then the petri dishes were stored in a dark place at room temperature with \pm 95% humidity for 14 days of observation. Observations of mortality termites were done do every day with spraying repetition 3-5 times (Guswenrivo, et al., 2008).

b. Baiting Methods

This method is done by feeding termites with bait that had been treated. In this study, eating bait used is paper discs that had been given chitin deacetylase enzyme crude extract. Furthermore, the paper disc has fed to termites. A total of 50 worker termites were fed with 5 mm diameter paper disc in a 5 cm diameter petri dish. Further experimental unit is stored in a dark place at room temperature and \pm 95% humidity for 14 days of observation. Observations of mortality in termites were done every day (Guswenrivo, et al., 2008).

Termite mortality was observed every day and observations can be calculated using the formula:

$$\text{Mortality (\%)} = \frac{N1}{N2} \times 100\%$$

Where = N1: number of dead termites after treatment

N2: number of initial termite

RESULT AND DISSCUSSION

Chitin deacetylase is isolated from thermophilic bacteria *B. licheniformis* HSA3-1a which was obtained from the screening soil samples and water from hot springs Sulili-Pinrang South Sulawesi. Natsir's research, et al., (2010), states that these isolates have optimum conditions 50°C with a pH of 7.0.

Bacteria *B. licheniformis* HSA3-1a (Figure1) further grown in fermentation medium to determine the optimum conditions of enzyme

production, because the growth of microbial fermentation in the medium and the resulting product can be monitored properly.



Figure 1. Colonies of *B. licheniformis* HSA3-1a in solid media

Chitin deacetylase is generally produced extracellular, which enzymes will be released by cell and accommodated in the medium, which was then centrifugated at a certain speed. So that the enzyme will be suspended in the filtrate or supernatant and separated from the cell. To avoid denaturation, the enzyme is separated from the cell in the cold at 3500 rpm centrifugation for 30 minutes and at a temperature of 4°C.

The growth of bacteria *B. licheniformis* HSA3-1a in the fermentation medium continued to increase up to 72 hours and begin to decline in the 78th hour to the 90th hour (Figure 2). This is likely due to the accumulation of toxic products or nutrients is reduced so that some cells start to die, and other cells to grow and divide

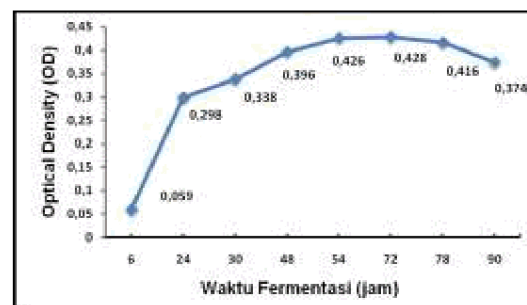


Figure 2. The growth of OD (*Optical Density*) compared to the time the fermentation of chitin deacetylase

The growth curve measurements was done at the same time with the enzyme activity measurements using glucosamine as a standard solution. Chitin deacetylase obtained from *B. licheniformis* HSA3-1a, were produced extracellular after the growth reached the stationary phase, the 72nd hour of fermentation time, wherein the phase of chitin deacetylase activity is optimum with 0,08322.10⁻³ U / mL activity value(Figure 3).

According to Natsir, et al., (2004), this occurs because the cell density is more dense and the availability of nutrients in the medium is diminishing thus chitin deacetylase enzymes was secreted out of the cell in large quantities to degrade the substrate (chitin) on the walls of microbes. The biodegradation result is used as an alternative source of nutrition.

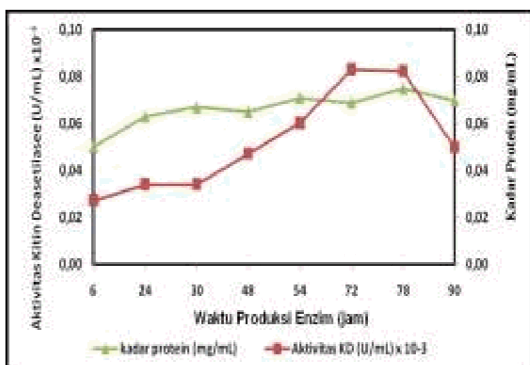


Figure 3. Activity of Chitin deacetylase and protein content againsts the production time

Chitin deacetylase used in this study is an crude extract enzyme enzyme which have not been purified thus chitin deacetylase activity was obtained that is $0,08322.10^{-3}$ U / ml. The U (units) of activity is defined as the amount of enzyme that liberates 1 μ mol glucosamine residues per minute with measurements at a maximum wavelength (Shimosaka, et al., 1995).

The results of measurements of chitin deacetylase activity against incubation time can be seen in Figure 3.

Once used chitin deacetylase enzymes are known, testing of the protein content was determined by Lowry method. Determination of protein content was using BSA (Bovine Serum Albumin) as a measured standard at a maximum wavelength of 640 nm. From this research as much as 0.069 mg / mL protein content of chitin deacetylase is obtained from *B. licheniformis* HSA3-1 isolates. Comparison of protein content, enzyme activity and specific activity of chitin deacetylase can be seen in Table 1.

Table 1. Protein Content, and Specific Chitin deacetylase activity on Fermentation Time 72 Hours

Sample	Protein Content (mg/mL)	Activity of Chitin Deacetylase (Unit/mL)	Specific Activity (Unit/mg)
Chitin deacetylase (crude)	0,069	$0,083 \times 10^{-3}$	$1,206 \times 10^{-3}$

Pathogenicity Determination of Crude Extract Chitin deacetylase As Biotermiticide

Determination of pathogenicity crude extract chitin deacetylase isolated from *B. licheniformis* HSA3 - 1a as biotermiticide was done through two methods, contact and feeding quantitatively by calculating the % mortality. Method of contact (spraying) is performed by directly spraying chitin deacetylase that has been conditioned at 50°C optimum temperature to termites body surface. Based on observations % mortality of termites on contact test (Figure 4) after 14 days of observation causes the chitin deacetylase % termite mortality increased 100 % up to 6th day observations . While on feeding trials % termite mortality reached 100 % on the 11th day of observation (Figure 4). According to Indrayani and Yusuf, (2009), that termites can survive within five weeks of the condition without food . This phenomenon may explain why the chitin deacetylase pathogenicity test contact method is better than the method of

feeding . In addition to the contact method , the enzyme is sprayed directly into the body so that the termites can directly degrade chitin existing on termite skin . This process causes hardening of the cuticle (sklerotisasi) is in complete so that cuticle functions as a conduit allowance (outside frame) for protection from water loss becomes disrupted, resulting in the death of termites over time .

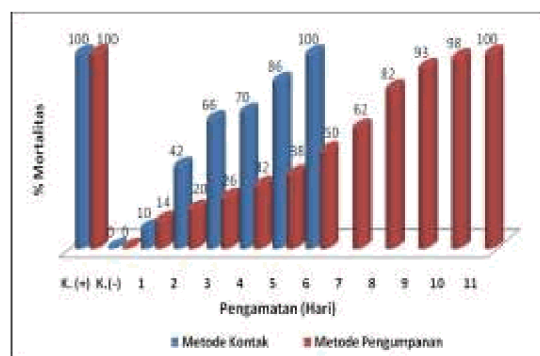


Figure 4. Pathogenicity test chitin deacetylase (CDA) against termites by contact and feeding method.

CONCLUSION

Chitin deacetylase can be isolated from microbial isolates of *B. licheniformis* HSA3-1a with colloidal chitin substrate. The production of optimum chitin deacetylase at incubation time of 72 hours with a protein content of 0.069 mg / mL, the enzyme activity of 0.083 x10⁻³ U / mL and the specific activity of 1.206 x10⁻³ U / mg. Application of chitin deacetylase as biotermicide by using the contact method is better than the method of feeding, in which contact method percentage mortality reached 100% on 6th day, while the method of feeding on 11th day. This suggests that chitin deacetylase can be used as an alternative biotermicide safety and environmentally friendly.

ACKNOWLEDGEMENTS

The authors wish to thank you profusely for Mrs. Dr. Hj. Hasnah Natsir, M.Si and Mrs. Dr. Hj. Seniwati Dali, M.Si above guidance, time, funding, and ideas for this research.

REFERENCES

- Artiningsih, A., Noor, A., dan Natsir, H. 2003. *Usaha Biokonversi Kitin Asal Kepiting Rajungan Menjadi Khitosan. Marina Chimica Acta*, 5 (1), 10-13.
- Bollag, D.M. dan Edelstein, S.J., 1991, *Protein Methods*, New York, Wiley-Less.
- Dawes, I.W.; I.W. Sutherland. 1992. *Microbial Psychology*, Second Edition, London, Blackwell Scientific Publication.
- Guswenrivo, dkk. 2008. *Pemanfaatan Ekstrak Enzim Jamur Humicola Sp. Sebagai Biotermisida*, UPT Balai Litbang Biomaterial, Bogor.
- Indrayani Y., Yusuf S., 2009, *Isolasi dan Identifikasi Jamur Kelas Hypomycetes Sebagai Bio-Kontrol Untuk menghambat Aktifitas Rayap Terhadap Kayu*, Jurnal Penelitian Universitas Tanjungpura Volume XIV No.2 ,86.
- Natsir, H., Chandra, D., Rukayadi, Y., Suhartono, M.T., Hwang, J.K. and Pyun, Y.R., 2002, *Biochemical Characteristics of Chitinase Enzyme from Bacillus sp. of Kamojang Creater. Indonesian, J. of Biochem, Molecular Biology and Biophysics*. 6 (4): 279–282.
- Natsir, H., Noor, A., dan Asfari, N., 2004, *Konversi Khitin dari Kulit Kepiting (Scylla serrata) menjadi Khitosan dengan Enzim Kitin Deasetilase, Marine Chimica Acta*, 6 (1), 6-9.
- Natsir, H., Patong, A.R., Suhartono, M.T., dan Ahmad A., 2010, *Production and Characterization of Chitinase Enzymes From Sulili Hot Spring In South Sulawesi, Bacillus sp. HSA3-1a*, Indo J. Chem, 10 (2), 256-260.
- Shimosaka, M., Nogawa, M., Wang, X., Kumehara, M., Okazaki, M., 1995, *Produksi Dua Kitionase dari suatu Bakteri Pengasimilasi Kitosan Acinetobacter sp. Strain CHB101, Applied and Enviromental Microbiology*, 438-442.
- Tokuyasu, K., Onishi-Kameyama, M., dan Hayashi, K., *Purification and Characterization of Extracellular Chitin Deacetylase from Colletotrichum lindemuthianum*, 1996, *Biosci. Biotech. Biochem.* 60 (10), 1598 -1603.
- Wang, S.L. and Chang, W.T., 1997, *Purification and Characterization of Two Bifungsional Chitinases/Lysozymes Extracellularly Produced by Pseudomonas aeruginosa K-187 in a Shrimp and Crab Shell Powder Medium*, *J. Appl. and Environmental Microbiology*. 63 (2): 380.
- Yuli, E.P., Suhartono, M.T., Rukayadi, Y., Hwang, J.K. and Pyun, Y., 2004, *Characteristic of Thermostable Chitinase Enzymes from the Bacillus sp.*, *Enzyme and Microbial Technology*. 13–26.