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identified as the genus Bacillus. The bacteria was gram negative and has endospore.
Key words: Thermophilic bacteria, hot spring, mannanase activity
INTRODUCTION
Mannan is the predominant hemicellulosic polysaccharide in softwoods and hardwoods (Capoe et al., 2000), seeds of leguminous plants (Buckeridge et al., 2000) and beans (Chanzy et al., 2004). Hemicelluloses are linear or branched polysaccharides, which most them are in form of heteroglycans. Based on the primary sugar within the molecules hemicelluloses can be classified into mannan, xylan, arabinogalactan and arabinan. Mannan is known as anti-nutritional factor, since this compound can increase the viscosity of the diet due to its high absorbability of water. Morever, the mechanisms are elaborated by Dingle (1995) and Kumar et al. (1997) who state that due to the viscosity of gut contents, the speed of enzymes to reach their substrate and the rate of nutrient to reach the gut wall decreases and thus decrease nutrients absorption. Thereby, enzymes can break the cross linkages holding molecules together

in gell-like structure (Dingle, 1995). One way to enhance mannan utilization is

the use of mannanase. To obtain a good mannanase source, a variety

of organisms such as bacteria (Tamaru et al., 1995), fungi (Kurakake and Komaki, 2001), animal tissue and plant (McCleary, 1988) have been screened for enzyme. Recently, researches on thermophilic bacteria have extensively been carried out since these organisms offer many advantages either for development of basic sciences or for industrial applications (Akhmaloka et al., 2006). The most of mannanase such as gamanase and hemicel mannanase are available in the market is form of endo-B-mannanase. The world's sale of industrial enzymes consist of hemicellulases, cellulases and pectinases as feed additive were estimated approximately 20% of the more than one billion US dollars and continuing to rise (Bhat, 2000). In Indonesia, the mannanase has benn new research. The possibility of

using these mannanases in industry has not investigated. However, more

work needs to be done to obtain superior enzyme for industrial applications. It

includes screening for strain that produce high mannanase with better physicochemical properties, including high thermostability and suitable pH, along

with gen cloning. The objectives of this study were to isolate,
characterization production of mannanase enzyme from thermophilic bacteria from hot spring of West Sumatera in order to apply for degradation of mannan found in agricultural waste and its applications for feed. MATERIALS AND METHODS Material: Hot spring as source of thermophilic bacteria was obtained from South Solok, West Sumatera Indonesia at 75-95°C. Locust bean gum was obtained from Sigma Chemical Company. Other chemicals were analytical grade from Merck Industry. Culture medium: Agar plate A consisted of 2% pepton, 1% yeast extract, 1% NaCl and 2% agar at pH 7.0. this was used for isolation of thermophilic bacteria. Agar plate B contained 0.5% locust bean gum, 0.5% (NH4)2SO4, 0.754% Na2HPO4, 0.232% NaH2PO4, 0.02% Corresponding Author: Yetti Marlida, Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, Andalas University, Padang, Indonesia 360 MgSO4.7H2O, 0.001% FeSO4.7H2O, 0.005% CaCl2.2H2O, 2% agar at pH 7.0. This was used for sreening mannanase-producing bacteria. Isolation and sreening of thermophilic bacteria: One hundred microliters of hot spring water was poured and spread onto plate A. These plates were incubated at 60°C for 48 hours. The colonies that were found on the plates were inoculated onto agar plate B by first dipping a sterile tooth pick and incubated at 60°C for 48 hours. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl. To indicated the mannanase activity of bacteria, diameter of clear zone around colonies on agar plate containing locust bean gum were measured. Besides, a more quantitative assay method was used to determine the

mannanase activity of the selected bacterial isolate in liquid medium. The mannanase activity of each culture was measured by determining the amount of reducing sugars liberated by using Somogyi Nelson method (1944). A bacterial isolate with the highest mannanase activity was selected for optimization of crude mannanase production.

Enzymatic characterization studies: The effect of the pH on the activity of

mannanase was examined from pH 3 .0- 10.0 in 50 mM buffer. The buffers

used were as follows: pH 2 .0- 4.0 Gly-HCl; pH 4.0-6.0 citrate phosphate; 6.0-8.0 kalium phosphate; pH 8.0-9.0 Tris- HCl; pH 10.0 Gly-NaOH. Temperature and time incubation versus enzyme activity were measured over a range of 27-95°C and 6-

48 hours. Enzyme activity assay: Mannanase activity was determined by

monitoring the release of reducing sugars. The reaction mixture

containing 1 ml of 5% locust bean gum dissolved in 50 mM phosphate buffer pH 7.0 and 1 ml enzyme solution, was incubated in shaker water bath at 80°C for 20 minutes. 1 ml of this mixture was removed and added to 1 ml Nelson reagent. This mixture was boiled for 10 minutes and then the absorbance was measured at 575 nm. The reducing sugar content was determined by Somogyi- Nelson method. One unit of enzyme was defined as the amount of enzyme producing 1 µmol of mannose per menit under the given assay condition. Specific activity was expressed as U/mg of protein. Controls were routinely included in which enzyme preparation or substrate was omitted. Protein determination: The amount of protein was determined according to Coomassie Blue method described by Bradford (1976) using Bovin serum albumin as a protein concentration standard. RESULTS AND DISCUSSION Identification of isolates: A total of eleven thermophilic bacterial isolates were screened for their ability to

produce extracellular mannanase. When applied the Congo red test, only 4 isolates forming clear peripheral zones on selected medium agar plate containing locust bean gum, were isolated from hot spring samples. Although the Congo red test was sensitive enough for primary isolation and sreening of mannolytic bacteria but the clear zone width was not implied the amount of mannanase activity. Upon further quantitative determination of mannan degrading enzyme, all 4 isolates were grown in liquid medium containing 0.5% locust bean gum for 48 hours and their activities were determined. The mannanase activities of all 4 strains were ranging 9.69-16.47 U/mg) with the highest enzyme activity lied on isolate SM-1.4 (Fig. 1). Thermophilic bacteria mannanase was isolated from hot spring samples and identified as Bacillus sp. was identified for further study. Fig. 1. Shown how the activities of four isolates, screened as high mannanase producers in the premilinary screening. It can be noted that isolate SM-1.4 exhibited the highest mannanase activity (16.47 Umg-1) which was obtained at the 48 hours of incubation and was followed by SM-1.3 with 12, 88 U mg-1. The isolates with the highest activities in the initial quantification were selected as a potential isolate for the present study. Many others thermostable mannanase producing strains such as, Paenibacillus sp DZ3 (Chandra et al., 2011); Geobacillus strearothermophilus L-07 (Sumardi et al., 2006); Thermotoga neopolitana 5068 (Duffaud et al., 1997). Paenibacillus sp DZ3 (2011), Bacillus sp. MG- 33 (Meenakshi et al., 2010). It may be concluded that only mannan induced this enzyme. Enzyme induction is due to physiological change in a whole microbial population and involves an accelerated rate renewed formation of enzyme in respon to a relatively specific nutritional stimulus (Mendoza et al., 1994). The mannanase was induced early phase of cultivation. It seems that the mannoses are released from mannan. According to the

results of morphological observation isolates SM-1.4 was classified as Bacillus sp. A microscopic examination of the isolate revealed that it was a gram negative bacterium with a terminal shape spore, rod shape cell (Fig. 2). The changes of

mannanase activity in fermentation were shown in Fig. 3. Enzymatic characteristics: Mannanase production in culture of Bacillus sp. SM-1.4 by submerged fermentation

reached its stationary growth phase after cultivation for 24 hours. During

exponential growth phase, its produced small amounts of mannanase. Mannanase production occurred in late stage of the exponential growth phase and

activity mannanase Fig. 1: Degradation of four isolates on locust bean gum as mannan source after 48 hours incubation Fig. 3a: pH versus activity profiles of mannanase from Bacillus sp. SM-1 Fig. 2a: Morphology of the gram-negative rod-shape cells-like bacteria Bacillus sp Fig. 3b: Temperature versus activity profiles of mannanase from Bacillus sp. SM-1.4 Fig. 2b: Cells produced endospore in terminal position after 48 hours incubation increased gradually with increasing incubation

time. Enzyme activity increased abruptly and reached the maximal value of

19.55 U/mg respectively. Temperature: The bacterial mannanase displayed maximum activity were at temperature of 60°C for 48 Fig. 3c: Time incubation versus activity profiles of mannanase from Bacillus sp. SM-1.4 hours (Fig. 3). At the lower or higher pHs and temperature almost all enzymatic activity of bacteria were reduced. The isolated Bacillus sp. SM-1.4 produced maximum level of extracellular mannanase during growth on 0.5% locust bean gum, at 60°C. To the best our knowledge, this is the first report on the production of mannanase from Bacillus sp. SM-1.4 which produces mannanase at 60°C which could make it interesting for industrial application like feed-pelleting. Similarly, Geobacillus stearothermophilus L-07 (Sumardi et al., 2006). This is higher than that reported other Bacillus subtilis strains isolated so far produced maximum mannanases at 50°C (Jiang et al., 2006). Some Bacillus subtilis starins can produced mannanases at up to 45°C (Khanongnuch et al., 1998). This mannanase reached levels of commercial acceptability. At present, a mayor drawback in using of mannanases in animal feed is the stability of added feed enzyme to the pelleting process, since pelleting can be associated with negative effects such as

inactivation of this enzyme during the feed- pelleting or expansion processes

(Eeckhout et al., 1995). pH profiles: The mannanase activity of Bacillus sp. SM - 1.4 reached the highest point at pH 7.0 for 48 hours. At the lower or higher pHs and temperatures almost all enzymatic activity of bacteria were reduced. The Mannanase has a broad pH optimum range (4.0-7.5) with a rapid drop in activity

at pH value above 7.5. Bacteria mannanase has pH optimal range 5.0-7.0. The mannanase from thermophilic Bacillus sp SM-1.4 has optimum pH 7.0 and stable

at pH range 4.0-9.0 was compatible with the internal environment of monogastric

animal's stomach such a in poultry. Similarly, Geobacillus stearothermophilus L-07 (Sumardi et al., 2006), Aspergillus niger (Ademark et al., 1998) and Vibrio sp MA-138 (Araki et al., 1996) but higher than that reported for mannanase production from Bacillus circulan NT67 (Phothichitto et al., 2006), Sclerotium rolfsii (Gubitz et al., 1996), Geobacillus sp MKK (Abdolvahab et al., 2010). Bacillus sp. MG-33 (Meenakshi et al., 2010). This mannanase is worthy of further research as strains activity over a wide range of pH values characteristic of digestive tract and could conceivably be more suited to increasingly higher feed processing temperatures currently employed in the animal feed industry. Conclusion: The results found 11 isolated where among them, 4 isolates has produced liquid medium using locust bean gum as inducer. After screening, isolate SM 1.4 was the highest of manannase activity, the isolate SM1.4 identified as Bacillus sp. usin 16 rRNA. The optimum manannase production were at pH 7.0, temperature 60°C and 24 hr of

incubation. The activity optimum were 119, 44 U/ml and specific activity of 19, 55 U/mg. ACKNOWLEDGMENTS This research was supported by PhD Grant from Directorate General Higher Education of Indonesia (Dikti). The authors also are grateful to the undergraduate student for supporting the experiment in the Laboratory of Feed and Technology Industry in Faculty of Animal Science andalas University. REFERENCES Abdolvahab, M.H., F. Jamsid, G. Sara and S. Majid, 2010. Growth condition optimization of the Iranian Thermophilic Geobacillus sp MKK with the aim of characterizing the DNA Polymerase I enzyme and its applications in PCR. World Applied Sci. J., II: 354-361. Ademark, P., A. Varga, V. Harjunpaa, T. Drakenberg, F. Tjerneld, and H. Stalbrand, 1998. Sofwood hemicellulose-degrading enzymefrom A. Niger; purification and properties of a $-mannanase. J. Biotechnol., 63: 199-210. Araki, K., K. Oh. Fujiyama, and T. Yoshida, 1996. Charactristic of the oligosaccharide structure of jack bean alpha-manosidase with some lectins. J. Ferment Bioengin., 78: 225-258 Akhmaloka, A., S. Suharto, I.N. Nurbaiti and F.M. Warganegara, 2006. Ribotyping Identification of Thermophilic Bacterium from Papandayan Crater. PROC. ITB Eng. Sci., 38: 1-10. Bhat, M.K., 2000. Cellulases and related enzymes in biotchnology. Biotechnol. Adv., 18: 355-383. Bicho, P.A., T.A. Clark, K. Mackie, H.W. Morgan and R.M. Daniel, 1991. The characterization of a thermostable endo-$-mannanase cloned from Caldocellum saccharolyticum. Applied Microbial. Biotechnol., 36: 337-343. Bradford, M.M., 1976. A rapid and sensitivemethod for the quantitation og microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., 72: 248-254. Buckeridge, M.S., H. Pessoa dos Santos and M.A.S. Tine, 2000. Mobilisation of storage cell wall polysaccharides in seeds. Plant Physiol. Biochem., 38: 141-156. Capoe, P., M. Kubackova and J. Alfoldi, 2000. Galactomannan from the secondary cell wall of Picea abies. L. Krast. Carbohydr. Res., 329: 635- 645. Chandra, M.R.S., Y.S. Lee, I.H. Park, Y. Zhou, K.K. Kim and Y.L. Choi, 2011. Isolation, purification and characterization of a thermostable a-$-mannanase from Paenibacillus sp. DZ3. J. Korean Soc. Applied Biol. Chem., 54: 325-331. Chanzy, H., M. Dube and R.H. Marchessault, 2004. Single crystals and oriented crystallization of ivory nut mannan. Biopoly, 18: 887-898. Dingle, J.G., 1995. The use of enzymes for better performance of poultry. In: Queendsland Poultry Science symposium. The University of Queensdland. Gatton. Duffaud, G.D., C.M. McCutchen, P. Leduc, K.N. Parker and R.M. Kelly, 1997. Purification and characterization of extremely thermostable $- mannanase and "-galactosidase from the hyperthermophilic Eubacterium Thermatoga neopolitana 5068. Applied Environ. Microbiol., p: 169-177. Eeckhout, M., M. DeSchrijver and E. Vanderbeke, 1995. The influence of process parameters on stability of feed enzymes during steam pelleting. In: Proceedings of the 2nd Eurpean Symposium on Feed Enzymes. Noordwijkerhout, The Nederlands, pp: 163-169. Gubitz, G.M., M. Hayn, G. Urbanz and W. Steiner, 1996. Purification and properties of an acid $-mannanase from Sclerotium rolfsii. J. Biotechnol., 45: 165-172. Jiang, Z.Q., Y. Wei, D. Li, L. Li, P. Chai and I. Kusakake, 2006. High-level production, purification and characterization of a thermostable $-mannanase from the newly isolated Bacillus subtilis WY34. Carhyd. Poly., 66: 88-96. Khanongnuch, C., K. Asada, H. Tsuruga, T. Ooi, S. Kinoshita and S. Lumyong, 1998. $-mannanase and xylanase of Bacillus subtulis 5H active for bleaching of crude pulp. J. Ferment. Boeing, 5: 461- 466. Kumar, A., J.G. Dingle, K. Wiryawan and D.C. Creswell, 1997. Enzymes for improved nutritional value of layer diets. In: Queendsland Poultry Science symposium. The University of Queensdland, Gatton. Kurakake, M. and T. Komaki, 2001. Production of beta mannanase from Aspergillus awamori K4 and their properties. Curr. Microbiol., 42: 377-380. McCleary, B.V., 1988. Synthesis of beta-D- mannopyranosidases for the assay of beta-D- mannosidase and exo-$-D-mannanase. Methods Enzymol., 160: 515-518. Meenakshi, G. Singh, A. Bhalla and G.S. Hoondal, 2010. Solid state fermentation and characterization of partially purified thermostable mannanase from Bacillus sp. MG-33. Bioresources. Com. Mendoza, N.S., M. Arai, T. Kawaguchi, F.S. Cubol, E.G. Panerio and T. Yoshida, 1994. Isolation of mannan- utilizing bacteria and the culture conditions for mannanase production. World J. Microbiol. Biotechnol., 10: 51-54. Phothichitto, K., S. Nitisinprasert and S. Keawsompong, 2006. Isolation, screening and identification of mannanase producing. Kasetsart. J. Nat. Sci., 40: 26-38. Somogyi, N., 1944. Notes on sugar determination. J. Biol. Chem., 19-23. Sumardi, S. Antonius, T.S. Maggy and P. Tresnawati, 2006. Purification and characterization of extracellular ß-mannanase from

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