Isolation, Characterization and Production of Mannanase from Thermophilic Bacteria to Increase the Feed Quality

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Abstract: Eleven isolates of thermophilic bacteria were isolated from hot spring and collected from West Sumatera of Indonesia. The bacteri isolated were grown on medium agar containing locust bean gum at 60°C for 48 hours to examine their mannanase production by using Congo red test. It was found that four isolates showed positive results with clear zone around the cultures. All isolates were evaluated their mannanase activity by growing in liquid medium supplemented with 0.5% locust bean gum as mannan source. It was found that isolate SM-1.4 displayed that highest enzyme activity of 119.44 U/ml and specific activity of 19.55 U/mg protein. The optimal growth conditions were at 60°C, pH 7 and 24 hours of incubation. According to the morphological studies, the isolate SM-1.4 was primarily 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, arabinoalactan 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. Moreover, 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, 1986) have been screened for enzyme.

Recently, research on thermophilic bacteria have extensively been carried out since these organisms offer many advantages either for development of basic sciences or for industrial applications (Akmaloka 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 hemicelulloses, 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 been 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, as 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% NaH₂PO₄, 0.232% NaH₃PO₄, 0.02%
MgSO₄.7H₂O, 0.001% FeSO₄.7H₂O, 0.005% CaCl₂·2H₂O, 2% agar at pH 7.0. This was used for screening mannanase-producing bacteria.

**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 screening of mannotlytic 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 preliminary screening. It can be noted that isolate SM-1.4 exhibited the highest mannanase activity (16.47 U/mg) 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.

**Enzymatic characterization studies:** The effect of the pH on the activity of mannanase was examined from pH 3.0-10.0 in 10 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 minute 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.
Fig. 1: Degradation of four isolates on locust bean gum as mannan source after 48 hours incubation

Fig. 2a: Morphology of the gram-negative rod-shape cells-like bacteria *Bacillus* sp

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 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 of 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., 2008). Some Bacillus subtilis stains 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 circulans 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 mannanase activity, the isolate SM1.4 identified as Bacillus sp. usin 16 rRNA. The optimum mannanase 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.

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REFERENCES


