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## 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 bacteria 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 of 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. 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, 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 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, 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%  $(\text{NH}_4)_2\text{SO}_4$ , 0.754%  $\text{Na}_2\text{HPO}_4$ , 0.232%  $\text{NaH}_2\text{PO}_4$ , 0.02%

MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.001% FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.005% CaCl<sub>2</sub>·2H<sub>2</sub>O, 2% agar at pH 7.0. This was used for screening mannanase-producing bacteria.

**Isolation and screening 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 indicate 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 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.

## 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 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 preliminary screening. It can be noted that isolate SM-1.4 exhibited the highest mannanase activity (16.47 U/mg<sup>1</sup>) which was obtained at the 48 hours of incubation and was followed by SM-1.3 with 12, 88 U mg<sup>-1</sup>. 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 stearothermophilus* 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

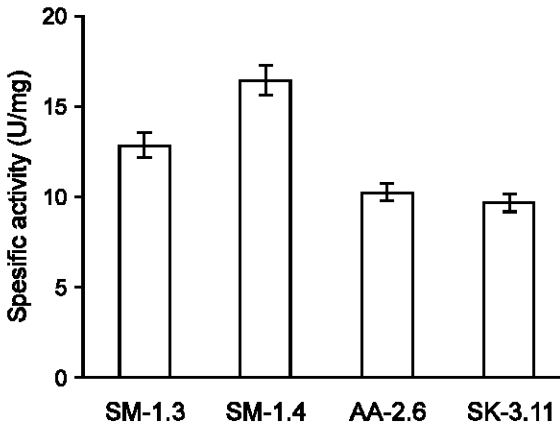


Fig. 1: Degradation of four isolates on locust bean gum as mannan source after 48 hours incubation

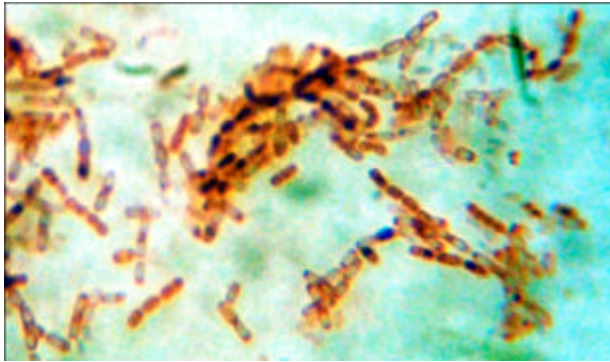


Fig. 2a: Morphology of the gram-negative rod-shape cells-like bacteria *Bacillus* sp

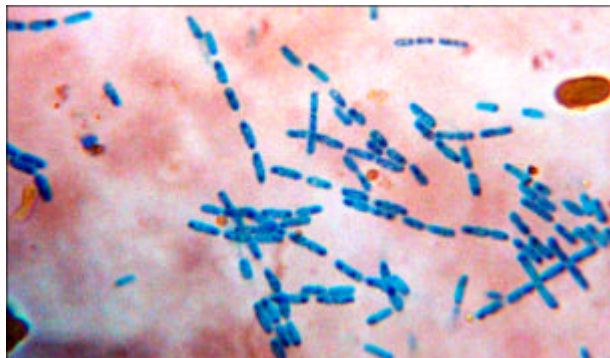


Fig. 2b: Cells produced endospore in terminal position after 48 hours incubation

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

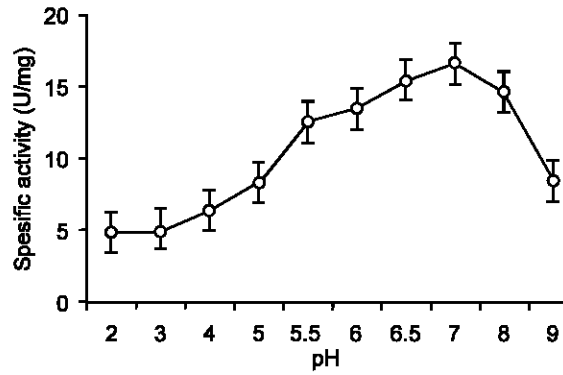


Fig. 3a: pH versus activity profiles of mannanase from *Bacillus* sp. SM-1

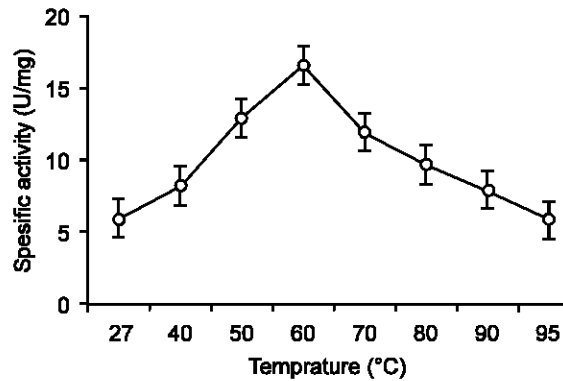


Fig. 3b: Temperature versus activity profiles of mannanase from *Bacillus* sp. SM-1.4

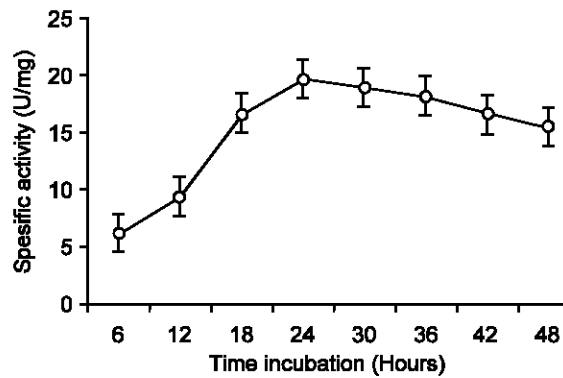


Fig. 3c: Time incubation versus activity profiles of mannanase from *Bacillus* sp. SM-1.4

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* strains 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 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.

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