Isolation, Purification and Characterization of Mannanase from Bacillus subtilis MAN-511

Dwi Retnaningtyas Utami
Student of Magister Program
Department of Food Science and Technology
Faculty of Agricultural Technology, Brawijaya University, Malang. East Java

Aji Sutrisno
Lecturer
Department of Food Science and Technology
Faculty of Agricultural Technology, Brawijaya University, Malang. East Java

Joni Kusnadi
Lecturer
Department of Food Science and Technology
Faculty of Agricultural Technology, Brawijaya University, Malang. East Java

Abstract

A bacterium, MAN-511 that produced extracellular mannanase, was isolated and identified as Bacillus subtilis on the basis of 16S rDNA phylogenetic analysis. The enzyme was purified to apparent homogeneity by precipitasi ammonium sulfat 70% and Sephadex G-75 chromatography procedures. The mannanase was purified 7.6 fold and specificity of 9.3 U/mg protein. SDS-PAGE of the purified enzyme showed a single protein band of molecular mass 45.08 kDa. The mannanase activity from this strain had optimum pH of 7.0, stable at a pH 6-7, optimum temperature at 30ºC and stable at 30-40ºC. In addition, crude mannanase preparation were successfully employed for the degradation of glucomanan which has been proved by reduce of viscosity.
Keywords: Bacillus subtilis sp., Glucomannan, Mananase, Purification, Porang (Amorphophallus muelleri Blume)

I. INTRODUCTION

β-Mannanase is an important enzyme for the depolymerization of these polymers. It hydrolyzes the β-1,4-linkages within the mannan backbone, releasing mannooligosaccharides of various lengths [1]. Over the years, there has been increasing interest in the potential application of β-1,4-mannanases in various industrial processes. Hemicellulases, including β-mannanase, play an important role in the bioconversion of lignocellulose material. The coconut residue is a source of highly concentrated mannan which can be hydrolyzed by mannan-degrading enzyme system to produce single-cell protein [2]. However, few above-mentioned bacteria were isolated from environment containing polysaccharides such glucomannan, which brings in some problem like the lower yield of the mannanases and higher production costs for industrial application [3]. To overcome these short comings, we screened predominant microorganisms in soil of Amorphophallus konjac field to investigate new bacteria with high mannanase activity. Furthermore, research indicated that this strain can produce extracellular enzyme to degrade glucomannan. This work describes the isolation, purification and characterization of the extracellular mannanase from a newly isolated Bacillus subtilis MAN-511.

II. MATERIAL AND METHODS

A. Chemicals and Media

Glucomannan, the media were consisted of 1% glucomannan, 0.5%pepton, 0.12%KH_2PO_4 and 0.02 % $MgSO_{4.7}H_2O$, 1.3% agar, aquades, alcohol 70%, etanol 95%, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, $MgCl_2$, $FeSO_4$, $CuSO_4$, NaCl, $ZnCl_2$, $BaCl_2$, Natrium acid, EDTA, Na_2CO_3, citrat acid, glicerol, phenol, Na-metabisulfit, HCL 0,1N, 3,5–dinitrosalisilat (DNS), congo red, buffer acetat pH 4-5, buffer phospat pH 6-7 and buffer Tris-HCL pH 8-9. Akrilamid (SIGMA), β-merkaptoetanol, Bis-akrilamid, TEMED, APS, Tris base, glicin, selofan, sephadex G-75.

B. Isolation of Microorganisms

Soil and glucomannan were put into Erlenmeyer and incubated at 37ºC, 48 hours. Plate containing glucomannan (1% glucomannan, 0.5%pepton, 0.12%KH_2PO_4 and 0.02 % $MgSO_{4.7}H_2O$, and 1.3% agar). After 24 h, the colony was picked out and stained with congo red. The colony with clear zone was selected for further experiment.

C. *Phylogenetic analysis by 16S rRNA of the MAN-511*

The PCR was performed to amplify the 16S rRNA coding region, using two oligonucleotide primers, 5' – GGTTACCTTGTTACGACTT - 3' and 5' – AGAGTTTGATCCTGGCTCAG - 3'. The PCR products were subcloned into Applied Biosystems GeneAmp PCR System 9700. Sequencing was performed with 96-well sequencer (Applied Biosystem 3130 Genetic Analyzer), in Sequencing Laboratory, Biologi Molecular Eijkman ABI Prism 3100 Genetic Analyzer Sequencing Chemistry Guide (ABI PN 4315831). Homology alignment was performed with the MEGA 6 program.

D. *Enzyme Production*

The bacterial culture was first amplified at 37°C in liquid medium and then grown on a defined agar medium, using 1% (w/vl) glucomanan as carbon source. Colonies grown on this agar medium were first amplified by vigorous shaking at 37°C in 25 mL liquid medium. The overnight cultures were then further amplified under identical conditions in 250 mL of the same medium until growth had reached stationary phase (18h). After centrifugation at 8500 rpm for 15 min, the bacterial pellet was gently transferred into buffer citrate acid pH 6. Maximum hemicellulase activities occurred within 48 h and were monitored as described below.

E. *Purification of the Mannanase.*

1) Ammonium Sulfate [(NH4)2SO4] Fractionation and Dialysis
A volume of one litre of crude mannanase was taken initially and then the required quantity of ammonium sulfate was added slowly to obtain various saturation levels (40%-80%). Proteins were precipitated and then separated by centrifugation at 8500 rpm 4ºC for 15 min. The separated proteins were then dissolved in a minimum amount of 0.05 M citrat buffer (pH 6) and refrigerated for further analysis. Precipitated proteins were transferred into a dialysis tube using a micropipette and dialyzed against citrat buffer (0.05 M, pH 6) at 4ºC. Dialysis was conducted overnight and the buffer was changed several times to increase the efficiency of the dialysis.

2) Filtration Gel chromatography
The dialyzed enzyme was loaded onto a filtration gel column (1 × 60 cm) equilibrated with citrate buffer (0.05 M, pH 6) and the column was washed with the same buffer. The enzyme bound to sephadex G-75 was eluted with a buffer (50 mM, pH 6). Fractions were collected at a flow rate of 4 min/mL, and fractions (1 ml) were collected. All fractions were checked for protein (A280) and β-mannanase activities (A540).

3) Zymogram Analysis and Molecular Mass Determination by SDS-PAGE
Protein bands were stained with Coomassie Brilliant Blue. For zymogram analysis, a substrate gel was prepared by adding 1% agarose to a 1% glucomanan solution and heating until the agarose dissolved. After electrophoresis, the native gel was placed on the substrate gel. The two gels were then incubated at 50ºC for 3 h followed by staining of the substrate gel using 0.1% (w/v) Congo red solution for 20 min. The gel was destained using 2 M NaCl for 15 min. Electrophoresis was done on 12% gel and the separated protein band was detected by Coomassie blue staining. The separating gel consisted of 12% polyacrylamide and the stacking gel consisted of 30% polyacrylamide. The sample (25 µl) and 25 µl of sample buffer with 50 mM Tris HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, β-mercaptoethanol, and glycerol were loaded into the well. Molecular mass markers were purchased from Biosains, University of Brawijaya Malang-Indonesia.and were run parallel to the samples.

4) Determination of Protein Content
Protein content was determined by the method of Bradford (1976) [4]. Standard curve was plotted using Bovine serum albumin (Sigma, U.SA.).

5) Assay for Mannanase Activity
1% (w/v) glucomanan dissolved in 0.05 M citrate buffer at pH 6 was used as the substrate mixture. 1 mL of the substrate mixture was added to 1ml of the crude enzyme solution and incubated in a water bath at 30ºC for 30 minutes. Afterwards 1mL of dinitrosalicylic acid (DNS) was added to 0.5 mL of each enzyme – substrate mixture and boiled for 15 minutes. The absorbance of the mixture was thereafter measured at 540 nm in a spectrophotometer. The amount of mannose released was determined by the method of Miller, (1959). One unit of mannanase was defined as the amount of mannanase that released 1 micromole of mannose in one millimeter of the reaction mixture under the assay conditions.

F. *Biochemical Characterization of β-Mannanase*

1) Determination of optimum pH and pH stability
The optimum pH of enzyme activity was examined at pH 4.0-9.0 under standard assay conditions. 0.05M of buffers were used citrate (pH 4.0-9.0). The enzyme reaction was incubated at 30ºC for 30 min in the presence of 1% glucomanan dissolved in the buffers. The effect of pH on enzyme stability was determined using the same buffer system in the pH range of 4.0-9.0. Then, the remaining enzyme activity was measured (A 540 nm).

2) Determination of Optimum Temperature and Temperature Stability
The effect of temperature on enzyme activity was performed at temperatures ranging from 30-60ºC in 0.05M citrat buffer at optimum pH for 30 min. Thermal stability of the enzyme was determined at various temperatures in 0.05M buffer at optimal pH for 30 min. Then, the remaining enzyme activity was measured (A 540 nm).

3) Viscosity Measurements

Viscosity of glucomanan liquid was measured with a viscometer. Each measurement was repeated three times. For the determination of the relationship between specific fluidity of glucomanan and the released reducing sugars during hydrolysis by crude enzyme mannanases of *Bacillus subtilis* MAN-511, a 1% (w/v) solution of glucomanan in 0.05M citrate buffer pH 6 was incubated with 1.8 U ml−1 mannanase activity at 30°C. The decrease in the viscosity was monitored. The released reducing sugars were determined using the DNS method (A 540 nm).

III. RESULTS AND DISCUSSION

A. Strain Properties and Identification

The strain used in the present study, Bacillus subtilis MAN-511 was isolated from soil enriched with glucomanan. This strain was a Gram-positive bacil shaped bacterium. The partial nucleotide sequence of the 16S rDNA gene, corresponding to the region from 1,500 bp. The phylogenetic tree based on the analysis of the 16S rDNA sequences are shown in Fig. 1. Therefore, the culture was used for further experimental studies.

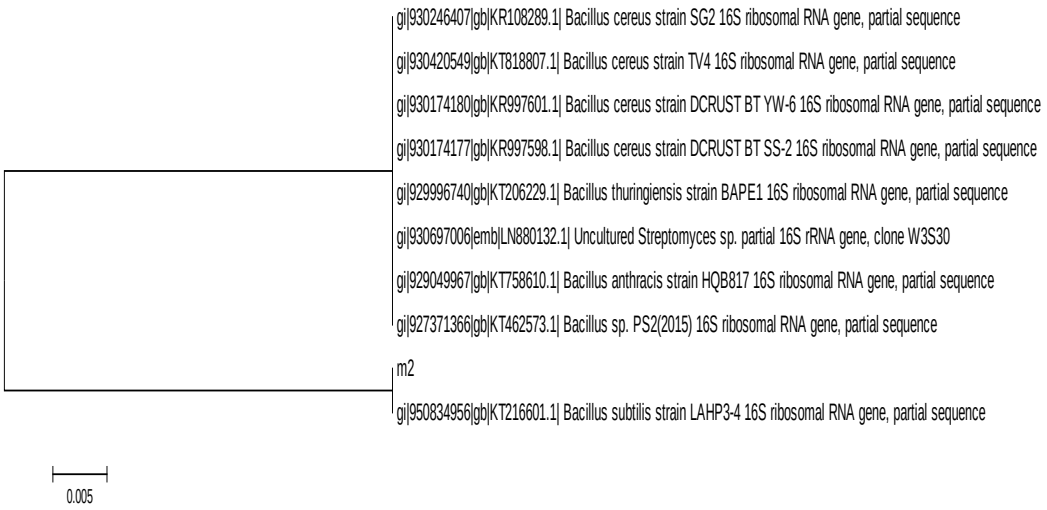

Fig. 1: Phylogenetic tree based on 16S rDNA sequences, showing the positions of strain MAN-511 and other Bacillus subtilis species.

B. Production of the Mannanase

The newly isolated Bacillus subtilis MAN-511 produced maximum level of extracellular mannanase during growth on 1% glucomannan at 37ºC. To the best of our knowledge, this is the report on the production of β-mannanase Bacillus subtilis WY34 [5]. Similarly, Bacillus subtilis NM-39 produced mannanase at 37ºC with locust bean gum as the substrate [6]. Other Bacillus subtilis strains isolated so far produced maximum mannanases at below 50ºC [7]. Some Bacillus subtilis strains can produce mannanases at up to 45ºC [8,9]. Five β-mannanases were secreted by Sclerotium rolfsii when cultivated in glucomannan [6]. Bacillus sp. KK01 produced four β-mannanases in the culture medium [4].

C. Purification of the Mannanase

The purification results are summarized in Table 1. 7.6-fold purification of the mannanase were obtained (Fig. 2A). The specific activity determined using glucomannan as substrate was 15 U/mg protein. The enzyme was also confirmed to be mannanase by zymogram analysis by congo red staining (Fig. 2B). The apparent molecular mass of mannanase (45.08 kDa) from Bacillus subtilis MAN-511 is similar to that of mananase from Bacillus subtilis WY34 was 39,6 kDa [5], Bacillus subtilis KU -1 was 40 kDa [8].

Table – 1
Purification of mannanase from the isolated Bacillus subtilis MAN-511

Purification Step	*Total Activity (µmol/min)*	*Total Protein (mg)*	*Specific Activity (µmol/min/mg)*	*Yield (%)*	*Purification (fold)*
Crude extract	*370*	*188*	*1,97*	*100*	*1*
(NH4)2SO4 (70%)	*72,2*	*17,9*	*4,03*	*19,51*	*2,04*
Sephadex G-75	*15,3*	*1,02*	*15*	*4,13*	*7,61*

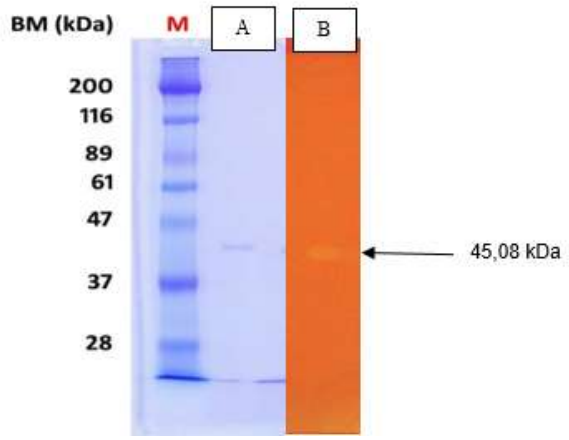

Fig. 2: SDS-PAGE and zymogram for mannanase from Baciluus subtilis MAN-511. Lane M, marker proteins; lane A, purified mannanase; lane B, zymogram of purified mannanase.

D. Temperature and pH optimal and stabilities

The optimal temperature for β-mannanase activity was 30ºC (Fig. 3A). The mannanase was optimally active at 30- 65ºC from Bacillus circulans NT 6.7 at 45ºC [13], Bacillus subtilis WY34 at 65 ºC [5], Bacillus stearothermophilus at 70 ºC [12] Bacillus subtilis KU -1 at 50 [8], Bacillus sp. MG-33 at 65 ºC [10]. The enzyme was stable up to 30-40ºC, but about activity was lost at 50ºC after 1 h of incubation (Fig. 3B). The optimal pH for β-mannanase activity was pH 7.0 (Fig. 4A). The enzyme showed stability within the pH range of 6.0-7.0 (Fig. 4B). This thermostability is comparable to those reported for several mannanases from the Bacillus subtilis WY34 [9], Paenibacillus illinoisensis ZY-08 [11], Bacillus sp.MG-33 [10], Bacillus subtilis KU-1 [8], Bacillus stearothermophilus [11], Bacillus licheniformis at pH 7 [12], Bacillus circulans NT6.7 at pH 6 [14], Bacillus stearothermophilus at pH 6.5 [12], Trichoderma reesei C-30 at pH 5 [15], Vibrio sp strain MA-138 at pH 6,5 [16].

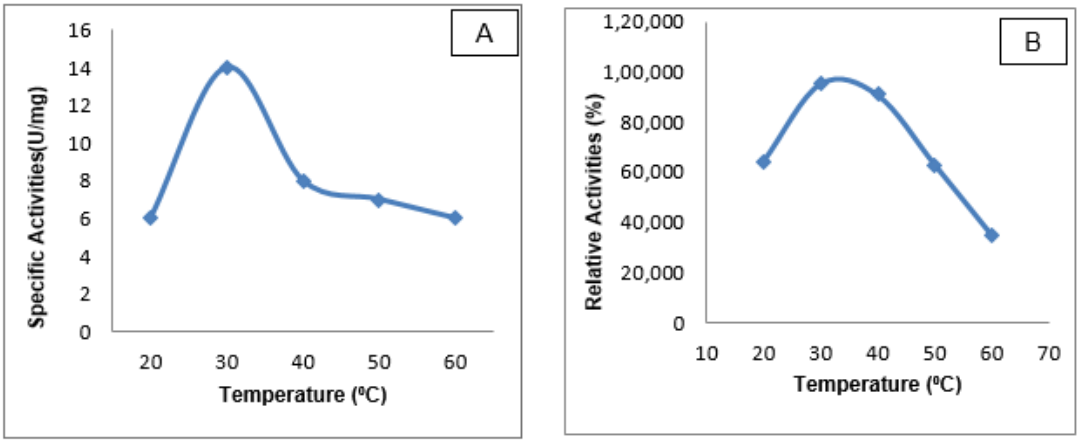

Fig. 3: Effect of temperature on mannanase activity (A) and stability (B). For determination of thermal stability.

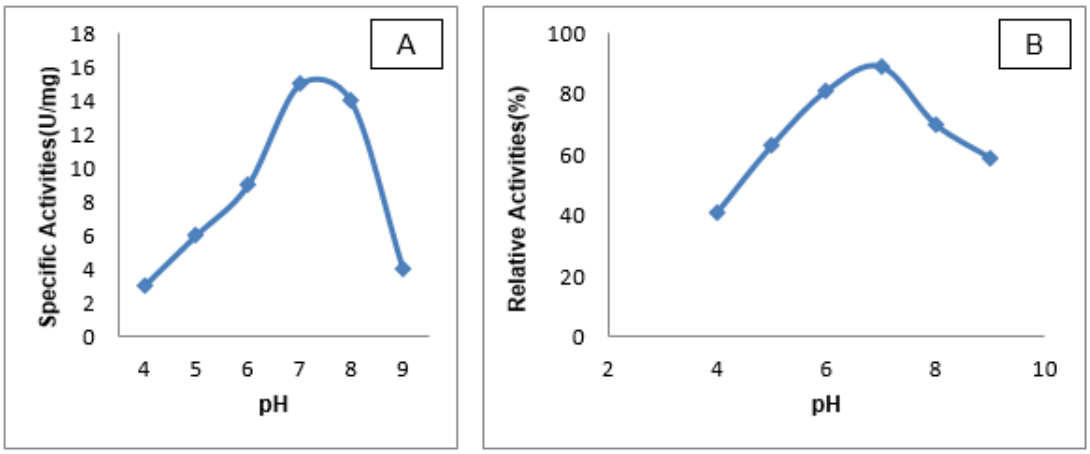

Fig. 4: Effects of pH on mannanase activity (A) and stability (B). The effect of pH on mannanase activity was monitored at 60ºC

E. Hydrolysis of Glucomanan by Mananase from Bacillus subtilis MAN-511

The rate of glucomanan hydrolysis by mananase was rapid and linier the periode to 30 min, viscosity of glucomanan liquid was decreased from 3.875 cpa.s to 510 cpa.s (86,8 %).Result from the hydrolysis study showed that this enzyme has a strong potential to be use to decrease high viscosity from glucomanan without causing any pollution as compared to the use of toxic inorganic acids.

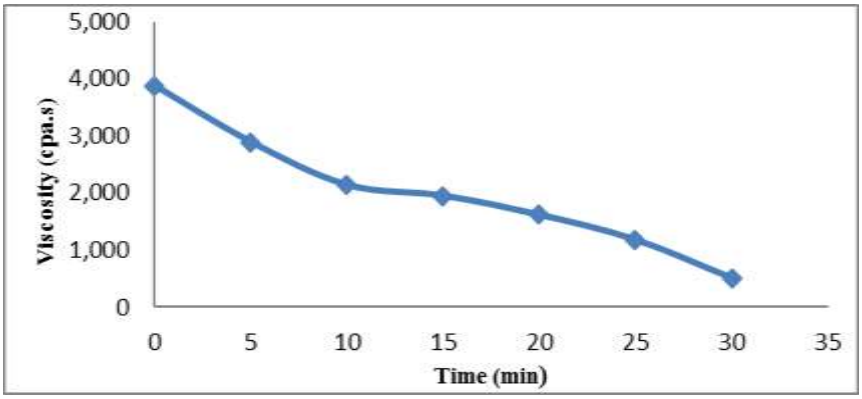

Fig. 5: Hydrolysis of Glucomanan by Mananase from Bacillus subtilis MAN-511at pH 7 and 30ºC

IV. CONCLUSION

The mannanase activity from this strain had optimum pH of 7.0, stable at a pH 6-7, optimum temperature at 30ºC and stable at 30-40ºC, the purified enzyme showed of molecular mass was 45.08 kDa.

REFERENCES

[1] Franco, P. F., H. M. Ferreira, and E. X. F. Filho. (2004). Production and characterization of hemicellulase activities from Trichoderma harzianum strain T4. Biotechnol. Appl. Biochem. 40: 255-259.
[2] Hossain MZ, Abe J, and Hizukuri S. (1996) Multiple forms of β-mannanase from Bacillus sp. KK01. Enzyme Microb Technol 18, 95-98.
[3] Schiradi C and De rose M (2002) The production of biocatalysts and biomolecules from extremophiles. Trends Biotechnol 20, 515-521.
[4] Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254.
[5] Jiang Z, Wei Y, Li D, Li L, Chai P, and Kusakabe I (2006) High-level production, purification and characterization of a thermostable β-mannanase from the newly isolated Bacillus subtilis WY34. Carbohydrate Polymers 66, 88-96.
[6] Mendoza NS, Arai M, Kawaguchi T, Cubol FS, Panerio EG, and Yoshida T (1994) Isolation of mannan-utilizing bacteria and the culture conditions for mannanase production. World J Microbiol Biotechnol 10, 51-54.
[7] Khanongnuch C, Asada K, Tsuruga H, Ooi T, Kinoshita S, and Lumyong S (1998) β-Mannanase and xylanase of Bacillus subtilis 5H active for bleaching of crude pulp. J Ferment Bioeng 86, 461-466.
[8] Zakaria MM, Yamamoto S, and Yagi T (1998) Purification and characterization of an endo-1,4-β-mannanase from Bacillus subtilis KU-1. FEMS Microbiol Lett 158, 25-31.
[9] Stalbrand H, Siika-aho M, Tenkanen M, and Viikari L (1993) Purification and characterization of two β-mannanases from Trichoderma reesei. J Biotechnol 29, 229-242.
[10] Meenakshi, Singh G, Bhalla A, Hoondal GS (2010) Solid state fermentation and characterization of partially purified thermostable mannanase from Bacillus sp. MG-33. Bioresources 5(3):1689–1701
[11] Lee, Y.S., Y. Zhou, Park, I.H., Chandra, M.R.G.S., Ahn, S.C, dan Choi Y.L. (2010). Isolation and Purification of Thermostable β-Mannanase from Paenibacillus illinoisensis ZY-08. J. Korean Soc. Appl. Biol. Chem. 53(1), 1-7
[12] Talbot G and Sygusch J (1990) Purification and characterization of thermostable β-mannanase and α-galactosidase from Bacillus stearothermophilus. Appl Environ Microbiol 56, 3505-3510.
[13] Zhang J, He Z, Hu K. (2000). Purification and characterization of ß-mannanase from Bacillus licheniformis for industrial use. Biotechnology Letters 22:1375-1378.
[14] Phothichitto, K. (2006). Isolation and Characterization of Mannanase Producing Microorganism. Graduate School, Kasetsart University. Thailand
[15] Atac IA, Hodist R, Kristufek D, Kubicek CP. (1993). Purification and characterization of a -Mannanase of Trichorderma reesei C-30. Appl Microbiol Biotechnol 39:58-62.
[16] Tamaru, Y., T. Araki, H. Mori., H. Amagoi and T. Morishita, (1995). Purification and characterization of an extracellular 1-4-mannanase from a marine bacterium, Vibrio sp. Strain MA-138. Applied Environ. Microbiol., 61: 4454-4458.