Characteristics of alkaline thermostable mannanase isolated from limestone microorganism

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Abstract. Mannanase is the main enzyme that catalyses depolymerisation of β-1,4 mannosidic linkages within the main chain of mannan releasing manno-oligosacharides. Since mannan is most soluble in alkaline solution and industrial enzyme applications prefer high thermostability, therefore mannanase with alkaline and high thermostability is more desired. The aims of this study were to isolate mannanase-producing microorganisms and to characterise the mannanase that obtained from partial purification. Nine mannolytic isolates were found and the chosen isolate was R311 with mannolytic index of 3.287. The purity of the enzyme from partial purification was 6.06-fold with the specific activity of 61.54 U/mg. Molecular mass of the mannanase analyzed by electrophoresis and zymogram was 32.39 kDa. The optimal temperatures and pH were 65°C and pH 8, respectively. The enzyme was stable at pH 5–8 and temperature up to 75°C. This property make mannanase R311 an attractive enzyme for biotechnological application for industries.

1. Introduction

Biotechnology plays an important role due to the environmental cost savings advantages in today’s production of industrial products. Among various processes, enzyme catalysis meets the requirements for performance in the large-scale production of specific products under mild conditions. Microbial enzymes are preferred for industrial application because of their easy and economical production as well as their novel properties such as activity in wide range of temperature and pH.

Among those enzymes, mannanase is an important enzyme for industrial applications. β-mannanase is a main enzyme that catalyse depolymerisation by randomly hydrolysis of β-1,4-mannosidic linkages within the main chain of mannan releasing manno-oligosachharides of various lengths. Mannanases have been isolated from animal, plants, fungi and bacteria. Bacteria with mannanase activity were reported as follows: Bacillus subtilis WY 34 [1], Bacillus circulans NT 6.7 [2], Paenibacillus Illinoisensis ZY-08 [3], and Paenibacillus sp. DZ3 [4]. These mannanases have been shown to belong to either glycosyl hydrolase family 5 or family 26 on the basis of their amino acid similarities.

In the enzymatic treatment of pulp bleaching, β-mannanase and its accessory enzymes are able to cleave the mannan component in the pulp selectively without affecting cellulose. Alkaline mannanases are stable in detergents and have found to be applicable in laundry segments as stain removal boosters. Mannanase can play important role as slime control agent in water purification system, vacuum sewer systems, waste water treatment and cooling water treatment systems [5]. Thus, industrial applications require alkaline and thermostable mannanases.
2. Materials and Method

2.1 Chemical and substrates
Bacteriological peptone and agar were obtained from Pronadisa Laboratory. Nutrient broth and nutrient agar were obtained from Oxoid Laboratory. 3,5-dinitrosalicylic acid (DNS), Acrylamide, Bis-acrylamide, β mercaptoetanol and Bovine Serum Albumine (BSA) were obtained from Sigma Chemicals. Dialysis membrane from Serva, Prestained Protein Marker Broad Range from Nacalai, USA. All other chemicals used in this study were reagents and analytical grades materials from Merck chemicals. The substrates, Amorphophallus muellerii Blume glucomanan was provided by PT Ambico, East Java, Indonesia.

2.2 Source of microorganisms
Samples of limestone that were collected randomly from Rengel limestone hill, Tuban and limestone mine in Plumpungredjo, Kademangan, Blitar, as well as dry soils from Situbondo, East Java, Indonesia were used as sources for screening of mannanase producing microorganisms.

2.3 Isolation of mannanase producing bacteria
The isolation of bacteria was carried out on enrichment media containing glucomannan 1%, KH₂PO₄ 0.1%, bacteriological peptone 0.1%, and MgSO₄ 0.027% using the collected samples. The pH of the medium was adjusted to 8.0. A tenfold dilution was made by mixing 1.0 g of the sample in 10 ml sterile distilled water and shaken vigorously for 5-10 min using a vortex [6]. Then 0.1 ml of the mixture was transferred on a modified carbon limited growth medium [3] which contain glucomannan 1%, KH₂PO₄ 0.1%, peptone 0.1%, MgSO₄ 0.027% and agar 1.5%. Spread plate was performed using an L-shaped glass rod. The inoculated plates were incubated at 55°C for 48-72 hours. The positive bacterial isolates were sub cultured to purity and examined for mannanase activity. Positive mannanase-producer isolates were detected based on the clear zone around the colony after 24 hours of incubation.

2.4 Enzymes production
The positive isolates were grown in a liquid medium consisting of glucomannan 1%, KH₂PO₄ 0.1%, bacteriological peptone 0.1%, and MgSO₄ 0.027% [3]. The bacterial inoculum was added into 150 mL of medium in an Erlenmeyer flask and kept in a shaker water bath (Memmert) with an agitation rate of 120 rpm and incubated at 45°C for 36 hours. Optimization of growth rate was analyzed through bacterial growth by Total Plate Count (TPC) and spectrophotometer at 420 nm every 1 hour. Enzymes activity was assayed based on the released reducing sugar by DNS [7]. After growth procedure, the culture supernatant was separated from the bacterial cell by centrifugation at 10,000 rpm for 10 min and stored at 4°C for subsequent use as a source of β-mannanase activity.

2.5 Partial purification
The crude mannanase was partial purified to homogeneity by using combination of ammonium sulphate precipitation (40-80%) and membrane dialysis. All the purification steps were performed at 4°C. Precipitation was done by first excluding the undesired protein and then salting-out the protein containing desired mannanase. The precipitated proteins were collected by centrifugation (10,000 rpm, 30 min, 4°C), dissolved in 100 mM phosphate buffer pH 8.0 and dialyzed against the same buffer.

2.6 Enzyme assay
β-mannanase was assayed by using 10 g/L solution of glucomannan in 50 mM phosphate buffer pH 8.0 (0.5 mL) to which 0.5 ml of the appropriately diluted enzyme solution was added. The release of reducing sugars for 30 minutes at 65°C was measured as mannose equivalents by dinitrosalicylic acid method [7]. All enzymatic activities were expressed as micromole mannose formed per minute and per milliliter enzyme solution e.g., as international units per milliliter. The protein concentration was measured by method of Bradford [8] using bovine serum albumin as a standard.
2.7 Enzyme characterization
The determination of the optimum temperature of the mannanase was carried out in the temperature range of 35-85°C. The optimum pH of β-mannanase was determined by measuring the activity at 65°C for 30 minutes at various pH from 4.0 to 9.0 using 50 mM of citrate buffer (pH 4 to 5) phosphate buffer (pH 6 to 8) and Tris-HCl buffer (pH 9). The thermal stability was determined by assaying for residual β-mannanase activity after incubation of β-mannanase in 50 mM phosphate buffer pH 8.0 at various temperatures (35-85°C) for 1 hour. Following incubation, the remaining activity was determined under standard enzyme assay conditions. The pH stability was determined by incubating the enzymes solution at different pH values at 37°C for 1 hour. The activities were measured as described above.

2.8 Polyacrylamide gel electrophoresis (PAGE) and zymography
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12.5% (w/v) polyacrylamide gel. Protein bands were visualized by Coomassie brilliant blue R-250 staining. The molecular weight standard used was Prestained Protein Marker Broad Range (Nacalai, USA): myosin (200 kDa), β-galactosidase (120 kDa), Bovine Serum Albumin (91 kDa), Glutamate Dehydrogenase (62 kDa), Ovalbumin (46 kDa), Carbonic Anhydrase (38 kDa), Myoglobin (26 kDa), Lysozyme (20 kDa), Aprotinin (9 kDa). Zymograms were obtained by co-polymerizing 0.2% Glucomannan with 12.5% Polyacrylamide. After electrophoresis, the gel was soaked in casein renaturant buffer with gentle shaking to remove SDS and renature the proteins in the gel. The gel was then washed four times at 4°C in 100 mM phosphate buffer pH 8.0 for 30 minutes. After further incubation for 1 hour at 65°C, zymograms were stained for residual carbohydrates with Congo red solution (0.1%, w/v), and was distained with 1 M NaCl. The activity bands were observed as clear colorless band.

3. Results and Discussion
3.1. Isolation of mannanase producing bacteria
Three limestone samples were used for primary screening of mannanase producing microorganisms. A total of nine bacterial isolates showed clear zone of mannanase activity in isolation medium using glucomannan 1% as substrate at 55°C. Isolate R311 showed the highest ratio of clear zone/colony in glucomannan of 3.287 (Table 1).

| Code | Source of sample | Colony Diameter (cm) | Diameter of Clear Zone (cm) | Activity index |
|------|------------------|----------------------|-----------------------------|---------------|
| R311 | Limestone Tuban  | 0.7                  | 2.3                         | 3.287         |
| S413 | Soil Situbondo   | 0.65                 | 1.5                         | 2.3           |
| S422 | Soil Situbondo   | 0.5                  | 1                           | 2             |
| S21  | Soil Situbondo   | 0.5                  | 1.5                         | 3             |
| S22  | Soil Situbondo   | 0.6                  | 1.6                         | 2.67          |
| B21  | Limestone Blitar | 0.5                  | 1.5                         | 3             |
| B22  | Limestone Blitar | 0.8                  | 1.7                         | 2.125         |
| K22  | Active Lime 1%   | 0.7                  | 1.5                         | 2.14          |
| K23  | Active Lime 1%   | 0.5                  | 1.4                         | 2.8           |

3.2. Partial purification and molecular mass
The results of purification procedure are summarised in Table 2. It was observed that after dialysis purification step, the enzyme was purified to 6.06-fold with a yield of 5.05%. The specific activity
determined using galactomannan as substrate was 61.54 U/mg protein. SDS-PAGE analysis of partial purified enzyme showed several bands as shown in Figure 1a with active band on zymogram of 32.39 kDa as shown in Figure 1b.

**Table 2.** Purification summary.

| Step            | Vol. (ml) | Activity (U/mL) | Total Activity (Unit) | Total Protein (mg/mL) | Specific Activity (U/mg) | Yield (%) | Purification Fold |
|-----------------|-----------|-----------------|-----------------------|-----------------------|--------------------------|-----------|-------------------|
| Supernatant     | 300       | 61.1            | 18333.33              | 6.02                  | 10.15                    | 100       | 1                 |
| Amonium Sulphate (70%) | 20          | 291.67       | 5833.33              | 10.68                 | 27.31                    | 31.81     | 2.69              |
| Dialysis        | 3         | 308.33         | 925                  | 5.01                  | 61.54                    | 5.05      | 6.06              |

**Figure 1.** SDS-PAGE (a) and zymograph (b) of ManR311. SDS-PAGE of partially purified mannanase and zymogram showed that the mannanase has molecular mass of 32.39 kDa

3.3. Effect of temperature and pH on the activity and stability of β-mannanase

The crude mannanase showed maximum activity at 65°C as shown in Figure 2a and more than 80% of the activity could be detected at temperatures between 45°C and 70°C (Figure 2b). The optimum pH for activity of ManR311 was found to be 8.0 and more than 80% activity was retained at pH 8.0 (Figure 2c). The ManR311 was highly stable between pH 5.0 to 8.0, retained >80% of the activity up to 1 h (Fig 2d). Thermostability profile of ManR311 shows that the enzyme was completely stable up to 1 h (75°C) (Figure 2b).
3.4. Discussion

The partial purification and characterization of an alkaline-thermostable β-mannanase from isolate R311 showed that the mannanase has molecular mass of 32.39 kDa on SDS-PAGE and zymogram. The molecular mass of the mannanase from isolate R311 was bigger than that of the mannanase from *Scopulariopsis candida* strain LMK008 28 kDa [9]. The size of the enzyme is smaller than the size of mannanase of *Paenibacillus sp.* DZ3 which has molecular mass of 39 kDa [4]. M-III mannanase from alkalophilic *Bacillus sp.* for 42 kDa [10] and *Bacillus nealsonii* PN-11 with a size of 50 kDa [5]. Low molecular mass of mannanase is suitable and desirable for pulp bleaching, in which they are able to penetrate more easily into the fiber resulting in an effective bleaching process.

Mannanase enzymes with low molecular mass are generally included in the glycoprotein group or carbohydrate-containing proteins. Bilbech et al. [11] characterised mananases with a low molecular mass of 18 kDa from *Penicillium occitanis* Pol6 and included in glycoproteins. Other glycoprotein mannanase was obtained from *Bacillus subtilis* WY34 with a molecular mass of 39.6 kDa [1] and *Vibrio sp.* MA-138 strains with a molecular mass of 49 kDa [12]. The enzyme mannanase from R311 isolate which has a molecular mass of 32.39 kDa is possible to be included in glycoprotein in accordance with glycoprotein mannanase which has been characterised which is between 18-49 kDa.

Optimum temperature and pH of ManR311 were 65°C and pH 8, which make this enzyme belonging to thermophiles and alkaline mannanases. ManR311 was highly stable at temperatures of 45-75 °C and pH 5.0 to 8.0 which makes this enzyme suitable for industrial applications. The enzyme mannanase produced by isolate R311 has the same optimal temperature as *Bacillus nealsonii* PN-11 [5] and *Bacillus subtilis* WY 34 [1]. The optimum temperature of R311 mannanase is higher than that of *Cellulosimicrobium sp.* strain HY-13 which has an optimal temperature of 50°C [13] and *Klebsiella oxytoca* KUB-CW2-3 which has an optimal temperature of 40°C [14]. *Fusarium oxysporum* isolate reported by Sumardi [15] had a higher optimal temperature at 70°C as well as *Bacillus pumilus* DYP 2 with an optimum temperature of 80°C [6].

Mannanase enzymes that have been characterised generally have a high stability against rising temperatures. Mannanase from R311 isolate has a stability that is almost identical to *Bacillus subtilis*.
WY34 which is stable at temperatures 30-65°C [1]. In another study by Lee [3] the stability of the enzyme produced by *Paenibacillus illinoisensis* ZY-08 was in the temperature range of 30-70°C.

The pH stability of mannanase from isolate R311 is in accordance with that of *Geobacillus stearothermophilus* [15] and *Streptacidiphilus luteoalbus* at pH between pH 4-8 [16]. The pH stability range of R311 mannanase is broader than that of *Scopulariopsis candida* strain LMK004 which is stable at pH 5-6.5 and LMK008 at pH 6-7 [9] as well as against *Aspergillus niger* GR which has stability at pH 3-7 [17]. However, *Bacillus sp.* JAMB-750 strains have a more alkaline pH stability compared to R311 mannanase at pH 6.5-10 [18]. The pH stability range of enzyme mannanase R311 is suitable for industrial applications that are carried out under alkaline conditions such as pulp and paper, detergents and wastewater treatment plants.

4. Conclusions

Isolate R311 with mannolytic index of 3.287 produce a single mannanase ManR311. The purity of the enzyme ManR311 from partial purification was 6.06-fold with the specific activity 61.54 U/mg. Molecular mass of the mannanase ManR311 analyzed by electrophoresis and zymogram was 32.39 kDa. The optimal temperature and pH were 65°C and pH 8, respectively. The enzyme was stable at pH 5 – 8 and at temperatures up to 75°C. These properties make ManR311 an attractive enzyme for industrial applications.

References

[1] Jiang Z, Yun W, Li D, Li L, Chai P, Kusakabe I 2006 High-level production, purification and characterization of a thermostable β-mannanase from the newly isolated *Bacillus subtilis* WY34 *Carbohydr. Polym.* 66 88-96.

[2] Phothichitto K 2006 Isolation and characterization of mannanase producing microorganism Master Thesis Kasetsart University Thailand.

[3] Lee Y S, Zhou Y, Park I H, Chandra M R G S, Ahn S C, 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.

[4] Chandra M R S, Lee Y S, Park I H, Zhou Y, Kim K K, Choi Y L 2011 Isolation, purification and characterization of a thermostable β-mannanase from *Paenibacillus sp.* DZ3 *J. Korean Soc. Appl. Biol. Chem.* 54 3 325-331.

[5] Chauhan P S, Prince S, Neena P, Naveen G 2012 Mannanase: microbial sources, production, properties and potential biotechnological applications *Appl. Microbial Biotechnol.* 93 1817-1830.

[6] Aurora D D 2003 Isolasi dan karakterisasi enzim mannanase *Bacillus pumilus* DYP 2 (Isolation and characterisation of mannanase of *Bacillus pumilus* DYP 2) Undergraduate Thesis Institut Pertanian Bogor Bogor [In Indonesian]

[7] Miller G L 1959 Use of dinitrosalicylic acid reagent for determination of reducing sugar *Anal. Chem.* 31 416-429.

[8] Bradford M M 1976 A rapid and sensitive method for quantification of microorganisms quantities of protein utilizing the principle of protein-dye binding *Anal. Biochem.* 72 248–254.

[9] Mduaw M M, Setati M E 2006 Screening and identification of endomannanase-producing microfungi from hypersaline environments *Curr. Microbiol.* 52 477-481.

[10] Akino T, Nobuyuki N, Koki H 1988 Characterization of three β-mannanase of an alkalophilic *Bacillus sp.* Agric. Biol. Chem. 52 3 773-779.

[11] Belghith H, Ellouz-Chaabouni S, Gargouri A 2001 Biostoning of denims by *Penicillium occitanis* (Pol6) cellulases *J. Biotechnol.* Aug. 23 89 257-262.

[12] Tamaru Y, Toshiyoshi A, Hiroki A, Hisanori M, Tatsuo M 1995 Purification and characterization of an extracellular β-1,4-mannanase from a marine bacterium *Vibrio sp.* strain MA-138. *Appl. Environ. Microbiol.* 61 12 4454-4458.
[13] Kim D Y, Han M K, Lee J S, Oh H W, Park D S, Shin D H, Bae K S, Son H, Park K H H Y 2009 Isolation and characterization of a cellulase-free endo-β-1,4-xylanase produced by an invertebrate-symbiotic bacterium, Cellulosimicrobium sp. HY13 Process Biochem. 44 1055-1059.

[14] Chantorn S T, Nawapan P, Suttipan K, Arunee I, Dietmar H, Sunee N 2013 Characterization of mannanase S1 from Klebsiella oxytoca KUB-CW2-3 and its application in copra mannan hydrolysis Sci. Asia 39 236-245.

[15] Sumardi 2005 Isolasi, karaktersasi, dan produksi β-mannanase ekstraseluler dari Geobacillus stearothermophilus L-07 (Isolation, characterisation and production of β-mannanase extracellular from Geobacillus stearothermophilus L-07) Master Thesis Institut Pertanian Bogor Bogor [In Indonesian]

[16] Seftiono H 2008 Pemurnian dan Karakterisasi mannanase dari Streptacidiphilus luteoalbus (Purification and characterisation of mannanase from Streptacidiphilus luteoalbus) Undergraduate Thesis Institut Pertanian Bogor Bogor [In Indonesian]

[17] Naganagouda K, Salimath P V, and Mulimani V H 2009 Purification and characterization of endo-β-1,4 mannanase from Aspergillus niger for application in food processing industry J. Microbiol. Biotechnol. 19 10 1184-1190.

[18] Takeda N, Kazumichi H, Kohsuke U, Yuichi N, Yuji H, Ron U, Yasuhiko Y, William D G, Susumu I, and Koki H 2004 Purification and enzymatic properties of a highly alkaline mannanase from alkaliphillic Bacillus sp. strain JAMB-750 J. Biol. Macromol. 4 2 67-74.