Isolation, purification and characterization of mannan degrading enzyme from a new isolate Acinetobacter baumannii

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Abstract. A mannan-degrading microbe was isolated from rotting porang tubers (Amorphophallus muelleri Blume). Molecular identification using 16S-rRNA sequence analysis revealed that the isolate showed 99.67% similarity with Acinetobacter baumannii. A crude enzyme from ammonium sulphate precipitation was used for preliminary characterization. The characterization results showed that the enzyme activity is optimum at 45 °C, and stable at 35-50 °C, while the optimum pH is 7, and stable at pH 5-7. The substrate with the highest relative activity was found in guar gum which was 137.512%. The enzyme activity was inhibited by Ca, Na, K ions, and increased by Mn²⁺ ions.

1. Introduction
Mannanase is an enzyme that can randomly hydrolyze 1,4-β-D-mannosidic bonds in the main chains of mannan and heteromannans [1], into mannose, glucose, and galactose [2]. Mannanases participate in various biotechnological applications. In recent years, mannanase are receiving a lot of interest for mannoooligosacharides (MOS) production. MOS is a prebiotic that can not be digested by the mammalian digestive system but assimilated by a group of beneficial microbes in the gut. MOS plays some important role in the prevention of infectious diseases, immune stimulation, intestinal disorder and is found to decrease post prandial blood glucose and serum lipids[3].

Porang (Amorphophallus muelleri Blume) is an Indonesian local plant that grows a lot in Indonesian forests. Farmers in Indonesia have exported large quantities of chips of the tubers to China and Japan [4]. The tuber is rich in glucomanann which has benefits as a food ingredient, as well as a functional food. The high content of glucomannan in fresh porang tubers which is around 3.58% can induce mannan degradation to grow microbes in the medium [5].

Several bacterial species have been reported to be capable of producing mannancnase [6]. The utilization of bacteria is very beneficial because the enzymes produced can be reproduced along with the amount of growth, this is because single-celled organisms have a short life cycle, causing bacteria to be able to produce products in large enough quantities, in this case, the β-manannase enzyme [7]. Microbial isolation in decaying porang is expected to be able to find new species of bacteria capable of producing the mannanase enzyme. The utilization of this enzyme is still low, and the commercial price
is relatively expensive, therefore this research was conducted in order to obtain bacteria capable of producing the **mannanase** enzyme with high activity and knowing the specific characteristics of the enzymes produced.

### 2. Materials and methods

#### 2.1. Raw materials

The **porang** tubers used in this study were 2 years old and were obtained from the Pandalan market in the city of Pasuruan, East Java, Indonesia. The tubers were used as samples of the mannan-degrading microbial growth media on moist soil surfaces. Local **porang** flour is obtained from Ambico, Pasuruan, Indonesia. Ammonium sulfate is used for protein precipitation. All other chemicals are pro analysis standards.

#### 2.2. Isolation, screening and identification of manolytic bacteria

**2.2.1. Bacterial isolation**

Bacterial isolation was carried out by diluting a sample of rotten **porang** in broth media that was incubated at temperature 37 °C for 24 hours. 10^{-1} to 10^{-8} dilutions using 10 m peptone solution, the last 4 dilutions were taken. From the results of each dilution, the **porang** agar was inoculated into a petri dish using the spread plate method and put in an incubator at 37 °C, for 24 hours. The results of the isolation using the streak plate method obtained pure colonies to be inoculated 1 colony each in 1 petri dish containing mixed media and tested with Congo red 1% to confirm its mananolytic activity.

**2.2.2. Identification of bacteria**

A molecular method using the 16S rRNA was used to identify the isolate. This identification was carried out in Jakarta by PT Genetics Science. The first step in this method is the extraction of genomic DNA from isolates that are completely pure, then extracted using the Quick-DNATM Fungal/Bacterial Miniprep Kit produced by Zymo Research. During the DNA extraction process, cells were lysed using the lysis buffer provided in the kit, after which the DNA was purified using the DNA Clean & Concentrator kit. The DNA concentration obtained was measured using the ND-1000 software on a computer device connected to the Nanodrop machine. Molecular identification is carried out to produce DNA sequences from the sample tested. The sequences are compared to the NCBI database by tracing Basic Local Alignment Search Tool (BLAST). The search results from NCBI will be used to determine the level relationship of isolates.

#### 2.3. Enzyme purification

The bacterial growth curve determination was carried out first by inoculating the isolate into 10 ml of media broth with a composition of 0.9% peptone, 0.1% yeast extract, 0.1% KH_{2}PO_{4}, 0.05% MgSO_{4}, 1% **porang** powder, and aquades, incubated in an incubator at a temperature of 37 °C for 24 hours, 2 ml isolate taken and inoculated into a 250 ml Erlenmeyer flask as much as 150 ml, incubated using a shaker water bath at 90 rpm at 37 °C, and every 2 hours the growth curve and enzyme activity were measured until the death phase was reached. The absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 540 nm.

The enzyme purification was carried out by precipitating the crude extract enzyme using ammonium sulfate with a concentration of 50-70% in a beaker glass and stirring for 24 hours in cold conditions (4 °C). Put it into a falcon tube, and centrifuged at a temperature of 4 °C, 5000 rpm, for 15 minutes. The precipitated pellet obtained was resuspended with citrate buffer pH 6 at a concentration of 0.05 M. Then mannanase activity was determined using the DNS method of Miller, (1959) [8], and Protein content was determined by the method of Biuret [9]. The standard curve was plotted using Bovine serum albumin (Sigma-Aldrich).
The result of ammonium sulfate precipitation was dialyzed using a cellophane bag to remove the ammonium sulfate. The dialysis process was carried out for 24 hours, in cold conditions (4 °C), and every 5 hours the old buffer was replaced with a new one. To determine the ammonium sulphate presence in the dialysis buffer, 3 drops of 1 M BaCl$_2$ were added. The dialysis process finished when there was no white precipitate formed.

2.4. **Enzyme assay**

Determination of enzyme assay was carried out using glucomannan as substrate. One unit of mannanase enzyme activity is defined as the amount of enzyme that can produce 1 mol of mannose in 1 minute. The total activity of mannanase is the total of enzymes that can produce mannose per minute. The formula for calculating total mannanase activity is to use the formula used by [2]

$$\text{Total Mannanase Activity (U)} = \frac{(X_s-SK) \times 2 \times fp \times 1000}{ml \times t \times BM \text{ manosa}}$$

$$X_s : \text{mannose content of the sample}$$

$$X_k : \text{control mannose levels}$$

$$t : \text{incubation time (minutes)}$$

$$fp : \text{dilution factor}$$

$$BM : \text{molecular weight of mannose (180 g/mol)}$$

Determination of the specific activity of the enzyme is determined after the activity of the enzyme, and the protein content of the enzyme is known. The enzyme activity test method used is a modification of the enzyme activity test conducted by Seftiono [2]. The specific activity of the enzyme is the number of enzyme units per mg of protein, where the specific activity of the enzyme is calculated using the following formula:

$$\text{Specific Activity} = \frac{\text{Total Activity (U)}}{\text{Total Protein (mg)}}$$

$$\text{Total Activity (U)} : \text{Enzyme volume (ml) x Activity (U/ml)}$$

$$\text{Total Protein (mg)} : \text{Enzyme volume (ml) x protein content (mg/ml)}$$

2.5. **Mannanase enzyme characterization**

The characterization of the mannanase enzyme has done by the determination of the enzyme activity at the optimum temperature, the determination of the enzyme activity at the optimal pH, the determination of the temperature stability of the mannanase enzyme, the determination of the pH stability of the enzyme, the substrate specification test by the mannanase enzyme, and the effect of the mannanase enzyme on the addition of metal ions.

2.5.1. **Determination of optimum temperature and temperature stability**. Determination of the optimal temperature of the enzyme is by testing it at temperatures ranging from 30-70 °C in 0.05M citrate buffer, and 0.2 ml of glucomannan substrate for 30 minutes. The thermal stability of the enzyme was determined by incubating 0.2 ml of the enzyme in 0.2 ml of 0.05M citrate buffer pH 6 without substrate at several temperature variations between 30-70 °C for 30 minutes, then incubating again with 0.5% glucomannan substrate added. After that, the activity was tested and measured with a UV-Vis spectrophotometer at λ 50 nm.

2.5.2. **Determination of optimum pH and pH stability.** Optimum pH of enzyme activity was determined at pH 4, 5, 6, 7, 8, 9, 10 under standard test conditions of 0.05 M citrate buffer pH 6. The enzyme reaction on the substrate was incubated at 45 °C for 30 minutes by giving 0, 5% glucomannan substratedissolved in buffers. The effect of pH on enzyme stability was determined using the same buffer system.
in the pH range of 4.0-10. Then the enzyme activity was tested and the absorbance was measured using a spectrophotometer at \( \lambda = 540 \) nm.

2.6. Data analysis
The results of the research is aimed to obtain bacteria capable of producing the mannanase enzyme and will be described in a descriptive form, the parameters observed include the activity of the mannanase enzyme, optimum temperature, optimum pH, temperature stability, pH stability, substrate specifications, and the effect of ions will be presented quantitatively.

3. Results and discussion

3.1. Isolation of mannanase enzyme producing bacteria
After obtaining pure cultures from multiple screenings with the streak plate method, the bacterial isolates were tested using Congo red 1% again to confirm the success of the expected manolytic properties of the bacteria.

![Figure 1. M2 isolate colonies produced the largest clear zone.](image)

| Isolate Name | cd1 (mm) | cz1 (mm) | cd2 (mm) | cz2 (mm) | xdd (mm) | xdl (mm) | Clear Zone Size (mm) | Manolytic Index |
|--------------|----------|----------|----------|----------|----------|----------|----------------------|----------------|
| M 1          | 10       | 12       | 40       | 42       | 11       | 41       | 30                   | 2.7            |
| M 2          | 10       | 10       | 38       | 38       | 10       | 38       | 28                   | 2.8            |
| M 3          | 10       | 10       | 37       | 37       | 10       | 37       | 27                   | 2.7            |
| M 4          | 10       | 41       | 39       | 58       | 25.5     | 48.5     | 23                   | 0.9            |

*Colony diameter

*Clear zone diameter

*Average colony diameter

*Average clear zone diameter

Measurement of clear zone based on manolytic index (MI). The manolytic index (MI) was obtained by comparing the clear zones, with the diameter of the colonies, as measured using a ruler. Table 1 shows that the manolytic index of M2 is higher than M1, M3, and M4, with a clear zone size of 28 mm, and a manolytic index of 2.8 mm. The second-largest manolytic index was found in sample M1 with a clear zone size, and the manolytic index was 30 mm and 2.72 mm, respectively, while the lowest manolytic index was found in isolate M4 with a clear zone size, and the manolytic index was respectively, also 23 mm, and 0.90 mm. The result of the clear zone is directly proportional to the enzyme activity in the bacterial isolate.

3.2. Bacteria identification
Molecular identification to determine the genus and species in bacteria using the 16S rRNA gene marker. After obtaining the DNA sequence from the M2 isolate, it was then compared with the NCBI database using Blast search (Basic Local Alignment Search Tool). The of the M2 sample showed the highest similarity with *Acinetobacter baumannii* Strain EH, having a similarity level of 99.67%, with the percentage of the overall analysis value (query coverage) reaching 99%. Based on the similarity, the M2
sample was named *Acinetobacter baumannii* Muzakki. From these results, it is then constructed in compiling a phylogenetic tree with the results as shown in Figure 2.

![Phylogenetic tree](image)

**Figure 2.** Phylogenetic tree of bacteria sample M shows the relationship with bacteria found in Gene Bank.

### 3.3. Mannanase enzyme purification

The results of the purification of the enzyme can be concluded that the specific activity of the enzyme increases because the higher the level of purity, the increase in enzyme activity of the crude extract; precipitation of ammonium sulfate 70%; and dialysis respectively have a specific activity of 0.330 with a purity level of 1 while the resulting yield is 100%; 1,925 with a purity level of 5 while the resulting yield is 32%; 2,379 U/mg with a purity level of 7 while the resulting yield is 27%.

| Purification Step           | Enzyme Volume (mL) | Total Activity (Unit) | Total Protein (mg) | Specific Activity (U/mg) | Yield (%) | Purity Level |
|-----------------------------|---------------------|-----------------------|--------------------|--------------------------|-----------|--------------|
| Enzyme Crude Extract       | 500                 | 2462,121              | 7457,357           | 0.330                    | 100       | 1            |
| Precipitation Ammonium Sulphate 70% | 18                 | 788,258               | 409,395            | 1,925                    | 32,015    | 5,832        |
| Dialysis                   | 15                  | 681,692               | 287,595            | 2,370                    | 27,687    | 7,179        |

### 3.4. Characterization of mannanase

#### 3.4.1. Temperature and optimum pH and stabilities

The optimal temperature for the activity of the mannanase enzyme is 45 °C (Figure 3A), whereas in previous studies the mannanase enzyme isolated from *Aspergillus* had an optimum temperature of 75 °C [10], *Bacillus subtilis* WY34 had an optimum temperature of 65 °C [11, 12] *Alkaliphilic bacillus sp.* N16-5 has an optimum temperature of 70 °C [13] *Bacillus subtilis* Strain MAN-511 has an optimum temperature of 30 °C [14, 7]. The enzyme is stable at 35-50 °C, but its activity decreases at 55-70 °C after 30 minutes of incubation (Figure 3B), in several previous studies the enzyme produced by *Aspergillus mannanase* is stable at 65-70 °C[10, 11] *Lichtheimia ramose* has stability at 40-75 °C [15], and *Bacillus sp* N16-5 has stability at 60-75 °C[13].
The optimum pH for the activity of the mannanase enzyme was pH 7 (Figure 4A), whereas in previous studies conducted on Bacillus sp. SM-1.4 has an optimum pH of 7 [14], Streptomyces costaricanus 451-3 has an optimum pH of 6 [16], Eupenicillium javanicum has an optimum pH of 6.5 [17]. The enzyme showed stability in the pH range of 5-7 (Figure 4B), in several previous studies conducted by Bacillus subtilis which was stable at pH 4.5-7 [18], Aspergillus terreus was stable at pH 4-7 [19], Streptacidiphilus luteoalbus is stable at pH 4-8 [2], Aspergillus niger is stable at pH 3-7 [12].

3.4.2. Substrate specificity test on mannanase enzyme, and effect of metal ions on enzyme activity

The highest mannanase enzyme activity was found on the guar gum substrate with a relative activity 137.52% (Figure 5A), which was similar to the mannanase enzyme activity produced by Aspergillus niger on the same substrate [12], then the second-highest enzyme activity was found on the konjac substrate, with a relative activity of 96.812%, while the lowest relative activity was porang flour with a relative activity of 70.055%.

The experimental results showed that the addition of Mn2+ ions to MnCl2 resulted in the highest increase in activity among all treatments, namely 118.468% (Figure 5B), this is the same as that carried out by previous studies on Bacillus Subtilis WY34 [11], and Bacillus subtilis BE [18], that the stability of the enzyme against metal ions increased with the addition of Mn2+ ions which resulted in the formation of metalloenzymes, this was due to the occurrence of strong bonds. EDTA had no effect on enzyme activity, having similarities with the mannanase enzyme produced by the bacterium Bacillus subtilis strain MAN-511 [7], while K+ ions in KCl, and Na+ ions in NaCl, decreased the activity of the tested enzymes.
4. Conclusions

A new mannan-degrading microbe was found in *porang* rotting on moist soil. Molecular identification using 16S-rRNA sequence analysis showed that the isolate belongs to *Acinetobacter baumannii*. The mannanase enzyme produced has the characteristics of an optimum temperature of 45 °C, is stable at a temperature of 35 °C - 50 °C, the optimum pH is 7, and is able to be stable at a pH of 5-7. The best substrates for these bacteria are guar gum, and *konyaku*, while the presence of metal ions that can increase the rate of enzyme activity are Mn$^{2+}$ ions in MnCl$_2$.

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