Characterization of crude cellulase enzyme produced by Bacillus licheniformis P12 isolate

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Abstract. Microbial enzymes utilization in industrial application recently has become extensive. One enzyme that is widely used in industry is the cellulase which is able to hydrolyse the glycosidic β-1,4 bonds present in cellulose. In previous studies, isolation of a cellulose degrading bacteria P12 from Mount Merapi spring water was carried out which had the highest cellulolytic activity (2.326 ± 0.219 U/mg). This research aims to identify P12 isolates molecularly using the 16S-rRNA gene, and characterize the cellulase produced. A descriptive quantitative design was used in this research. The P12 isolate was revealed to be Bacillus licheniformis based on 16S rRNA analysis with 99% homology. The concentration of ammonium sulfate 70% saturation can precipitate cellulase enzymes with purification folds of 6 times with specific activities 0.0103 U/mg. Cellulase enzyme fractionated with ammonium sulfate at this research was optimum at pH 7 and temperature 50°C.

1. Introduction
Cellulases are the enzymes that hydrolyse β-1,4 linkages in cellulose, the most abundant renewable biological resource. In nature, cellulases are expressed by a wide spectrum of microorganisms such as fungi, bacteria and protozoans [1]. Several screening and isolation steps of cellulase-producing microbes from nature is an important way to get novel cellulases. Generally, cellulase-producing microbes are isolated from soil samples obtained from forest and nature reserves, hot water springs, compost, sewage, animal manure and bovine rumen [2]. Microbial cellulases have attracted much interest as they are potential enzymes for application in various industries [3].

Cellulase production using bacteria offers several advantages such as higher growth rates, more adaptable to extreme environments, produce enzyme more effectively and less inhibited by hydrolyzed material. Indonesia’s diverse natural sources are potential to explore in order to find the source of cellulolytic enzymes-producing microorganisms. Microorganisms screening from various sources, such as forest [4], deserts [5] and hot springs [6] has been conducted. One of a very active volcano in Indonesia, Mount Merapi, was selected in this research as it performed multiple eruptions in the last 10 years. This causes damaged impacts to ecosystems, biodiversity and decreased ecological functions. In the present study, the screening of some cellulose producing bacteria, isolation and identification, as well as the characterisation of crude cellulase produced are reported.
2. Materials and Method

2.1. Isolation of cellulase producing bacteria
Water sample were taken from spring water of Mount Merapi. After performing serial dilutions and two steps enrichment, 0.1 mL was taken from each dilution, spread method was carried out on CMC-agar plate and then incubated for 24 hours at 55°C. Then each single bacterial colony with different morphological characteristics was taken and a quadrant streaking technique was performed on another CMC agar plate and kept for incubation at 55°C for 24 hours. This procedure was conducted to obtain pure bacterial isolates.

2.2. Screening of cellulase producing bacteria
Each plate that contain bacteria isolate was flooded with 1% congo red for 15 minutes and then rinsed with 1 M NaCl for another 15 minutes. Cellulase activity assay was observed from the cellulase index obtained. Isolates with the highest cellulase index were selected for further investigation. The cellulolytic index or cellulase activity index is obtained using the following formula:

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\text{Cellulolytic index} = \frac{\text{Diameter of clear zone (mm)} - \text{Diameter of bacterial colony (mm)}}{\text{Diameter of bacterial colony (mm)}}
\]

2.3. Morphological and biochemical characteristics of isolate
Morphological and biochemical characteristics of colour, shape, elevation, surface, gram staining, motility, endospore production, catalase was carried out.

2.4. Molecular identification using 16S rRNA gene
Prior to sequencing, the ribosomal rRNA gene (also referred to as rDNA) was amplified using the polymerase chain reaction (PCR) technique in which two universal primers were used for amplification: forward primer 27f (5' - AGAGTTTGATCCTGGCTCAG - 3') and reverse primer 1492r (5' - TACG GYTACCTTGGTTACGACTT-3') were incorporated in the reaction mixture. Each sample was sequenced in the sense and antisense directions using the same universal primers previously used. Sequences were further analysed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was carried out using MEGA7 software.

2.5. Production of crude cellulase enzyme
Enzyme production was carried out by inoculating 1 colony of bacteria isolate into 2 mL of liquid CMC media then incubated at 50°C at shaker waterbath (100 rpm for 26 hours). Dilution of 1 to 10 was conducted prior to incubation with the same condition previously stated. Inoculation was done in 1L flask with maximum 10% media volume of total volume of the flask. As the final step, the biomass and supernatant was then separated by centrifugation at 4°C, 5000 rpm for 15 minutes.

2.6. Ammonium sulfate precipitation and dialysis
The crude extract of cellulose was fractionated using ammonium sulfate (NH₄)₂SO₄ using concentration range of 60-80%. For each fractionation treatment, stirring at low temperature (4°C) was done for 15 minutes before centrifugation at 4°C, 7000 rpm for 15 minutes. The supernatant produced was then discarded. The dialysis was carried out by suspending the precipitation in phosphate buffer solution pH 8 in a cellophane bag then immersing it in phosphate buffer solution 0.05 M pH 8 for overnight at 4°C in stirring condition. The soaking buffer was replaced every 6 hours. Dialysis was stopped if all ammonium sulfate salts had been removed by testing it using BaCl₂ and HCl solution. Sulfate ions (SO₄²⁻) will be formed as BaSO₄ white deposits.

2.7 Determination of cellulase activity
Cellulase activity was measured by measuring the reducing sugars as the products of cellulose hydrolysis. 1 mL of crude enzyme was mixed with 1 mL of 1% CMC in 0.05 M citrate phosphate buffer solution then incubated at 50°C for 30 minutes. 2 mL of DNS reagent was added afterward and heated
in boiling water for 5 minutes and then cooled. Finally, the absorbance measurements were carried out at a wavelength of 540 nm [9].

One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 μmol glucose per minute under the assay conditions. The control of enzyme and appropriate substrate were included in all tests. Each experiment was repeated three times and the mean value was plotted for each experiment.

2.8. Determination of optimum pH

Determination of optimum pH was carried out by measuring the activity of cellulase in the pH range 3-9. The process was conducted by preparing 1 mL of 1% CMC dissolved in 0.05 M citrate phosphate buffer (pH 3-6), 0.05 M phosphate buffer (pH 6-8), and 0.05 M Tris HCl buffer (pH 9) then added with 1 mL of enzyme. Incubation was carried out at a temperature of 50°C for 15 minutes. Cellulase activity assay was then conducted as previously described. The optimum pH was determined based on which pH gave the highest cellulase activity [10].

2.9. Determination of optimum temperature

Determination of the optimum temperature was carried out by measuring the activity of cellulase enzymes at optimum pH conditions from the previous stage. The variation of temperatures ranged between 30-60°C. The optimum temperature was determined based on which temperature gave the highest cellulase activity [10].

2.10. Effect of ions on enzyme activity

The effect of ions to enzyme activity was carried out by measuring the activity of cellulase enzymes in several ions solution: KCl, CaCl₂, MnCl₂, MgCl₂, and NaCl, each with a concentration of 10 mM. The process was done by dissolving 1 mL of 1% CMC in the optimal pH buffer previously obtained. 10 mM ion solution was then added along with 1 mL of the enzyme. Cellulase activity assay was then conducted as previously described. Data on enzyme activity are plotted in graphs with ions as abscissa and cellulase activity as ordinate axes [11].

3. Results and Discussion

3.1. Isolation of cellulase producing bacteria

There were 4 isolates of cellulolytic bacteria and isolate P12 had the highest cellulolytic index (2.326 ± 0.219) among the other isolates. Isolate P12 which had white feature (as can be seen on milk color), had shape of point colonies, irregular elevation, rough surface, lobate margin, gram negative bacteria, was capable to form endospore, catalase-positive and had coccus shape.

3.2. Molecular Identification using 16S rRNA gene

The relationship between P12 isolate and Bacillus licheniformis are shown in Figure 1. P12 isolates were closely related to Bacillus licheniformis strain 103D-012 (shown by highest bootstrap value of 64%). The bootstrap value is an alternative method for determining the trust level of the phylogeny tree. The higher the bootstrap value, the higher the level of confidence in the topology (branch) of the phylogeny tree. Bacillus licheniformis can be found naturally in soil, bird feathers and plant material. Bacillus licheniformis is generally known to cause food poisoning, food decay, and contamination of milk. These bacteria tend to form spores so that they can be used in the production of enzymes and antibiotics. The optimal growth temperature is 50°C but can also survive at higher temperatures. These bacteria are usually used in the biotechnology industry to produce many enzymes such as proteases, α-amylase, β-mannanase, keratinase, endoglucanase, glucoamylase, pectinase, and antibiotics [12].
3.3. Ammonium sulfate precipitation and dialysis

Ammonium sulfate precipitation is a first step, the most commonly used, of protein purification. This step is critical for concentration, fractionation and purification of protein. Several saturation percentages were used in order to obtain optimum saturation that achieve highest enzyme activity.

Table 1. Results of ammonium sulfate saturation

| Saturation of Ammonium Sulfate (%) | Enzyme Activity (U/mL) | Protein Content (mg/mL) | Specific Activity (U/mg) |
|----------------------------------|------------------------|-------------------------|-------------------------|
| 60                               | 0.003                  | 0.429                   | 0.0062                  |
| 70                               | 0.005                  | 0.430                   | 0.0107                  |
| 80                               | 0.002                  | 0.381                   | 0.0065                  |

Table 1 shows the activity of cellulase enzymes at several levels of saturation of ammonium sulfate. Cellulase enzyme activity increases along with the addition of ammonium sulfate. The decrease in enzyme activity at 80% concentration is because protein is assumed to be sufficiently precipitated so that at a concentration of 80% only a portion of the remaining enzymes that can be precipitated and the higher concentration of ammonium sulfate can reduce selectivity in the deposition process [13].

It was also reported that 70% ammonium sulfate can increase cellulase specific activity produced by *Bacillus amyoliquefaciens* DL-3 up to 533.40 U/mg [14]. For further step of dialysis, addition of ammonium sulfate to 70%, was then selected. Dialysis is done to separate small and unwanted components from a solution such as salts and ions by using passive and selective diffusion by semipermeable membranes to maintain the stability of the enzyme during storage [15]. Details for the cellulase purification are presented in Table 2.

Table 2 Purification of Cellulase Enzyme.

| Purification Step              | Volume (mL) | Enzyme Activity (U/mL) | Protein Content (mg/mL) | Specific Activity (U/mg) | Yield (%) | Purification Fold |
|--------------------------------|-------------|------------------------|-------------------------|--------------------------|-----------|-------------------|
| Crude Enzyme                  | 970         | 0.010                  | 0.340                   | 0.0303                   | 100       | -                 |
| Ammonium Sulfate 70% Precipitation | 800       | 0.005                  | 0.430                   | 0.0107                   | 45        | 0.354             |
| Dialysis                      | 50          | 0.004                  | 0.381                   | 0.0103                   | 38        | 0.340             |
Table 2 shows that the protein content increased during purification. The final purification fold of the enzyme fraction was 0.340 after ammonium sulfate precipitation and dialysis process. The decrease in purification fold after the dialysis stage is due to displacement of small molecules from the membrane to the exit of the membrane containing the buffer solution so that a buffer solution will move into the membrane and dilute the solution inside the membrane.

3.4. Determination of optimum pH
Determination of optimum pH was carried out by measuring the activity of cellulase enzymes produced by dialysis in the pH range 3-9 (using different buffers) at intervals of 1 unit. Based on Figure 2, the results of the optimum activity of enzyme tests on pH indicate that pH 7 is the optimum pH of the cellulase enzyme produced by isolates P12. The H+ ion in the pH solution has an effect on the catalytic part of the enzyme which causes changes in the structure of the enzyme conformation. Changes in pH affect enzyme activity by changing the structure or residual charge of amino acids that function in binding to the substrate. A decrease in activity at some pH is likely due to changes in the charge of functional groups of amino acid residues in the enzyme. Some previous studies also showed neutral pH range as optimum pH [16,17].

![Figure 2](image.png)

**Figure 2.** Cellulase activities at several pH

3.5. Determination of optimum temperature
Determination of the optimum temperature was carried out by measuring the activity of cellulase enzymes from optimum pH dialysis results from the previous stage. The temperature variation used were 30-90°C with an interval of 10°C. The optimum temperature was determined based on the temperature that had the highest cellulase activity, as shown in Figure 3.
Figure 3 depicts the cellulase enzyme activity produced by P12 bacterial isolates increase to a temperature of 50°C and then decrease to a temperature of 90°C. This shows that the temperature of 50°C is assumed to be the optimum temperature for cellulase enzymes produced by bacterial isolates P12. Increasing the temperature will cause cellulase enzyme activity to increase because kinetic energy increases so that the interaction between the substrate and enzyme increases and produces more and more products. However, higher temperatures also cause breakdown of the secondary enzyme bonds due to kinetic energy that is too large from the enzyme molecule [18]. The cellulase enzyme produced by the optimum P12 isolate at 50°C has similarities with several isolates including Bacillus licheniformis B7 at 9.5 U/mL at 50°C, Bacillus subtilis YJ1 at an optimum temperature of 50-60°C [19], Bacillus sp. optimum at 50°C with enzyme activity of 0.37 U/mL [20].

3.6. Effect of ions on enzyme activity
The addition of ions was carried out to determine the effect of several ions on the activity of cellulase enzymes produced by bacterial isolates P12. The ions used are KCl, CaCl₂, MnCl₂, MgCl₂, and NaCl with a concentration of 10 mM.

Based on Figure 4, it shows that higher enzyme activity is achieved after being added with ions. The highest enzyme activity is obtained after addition with CaCl₂ which is 0.099 U/mL. While the addition of MgCl₂ and NaCl ions causes a decrease in cellulase enzyme activity to reach 0.083 U/mL. According to the study of Gaur and Sony [11] the cellulase enzyme produced by Bacillus vallismortis RG-07 was active in the addition of Ca²⁺, Mg²⁺, and Na⁺ ions with enzyme activities 184.4, 175.1 and 141.4% but
inhibited by Fe$^{2+}$, K$^+$ ions, Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Hg$^{2+}$ and Ni$^{2+}$. Previous study reported that cellulase enzyme activity produced by *Bacillus subtilis* with the addition of Mn$^{2+}$ ions produced the highest enzyme activity reaching 0.81 U/mL when compared with the addition of K$^+$, Na$^+$, and NH$_4^+$ ions resulting in low enzyme activity [21]. Cellulase enzyme activity produced by *Bacillus licheniformis* AU01 was increased with the addition of Ca$^{2+}$ (154%), Mg$^{2+}$ (108.50%), and Mn$^{2+}$ (108.33%). Thus, it can be concluded that Ca$^{2+}$ ions can increase the activity of cellulase enzymes in breaking down substrate into products [22].

4. Conclusions
P12 isolates from Mount Merapi springs are closely related to *Bacillus licheniformis* strain 103D-012 based on 16S rRNA test results. The concentration of ammonium sulfate 70% saturation can precipitate cellulase enzymes with purification folds of 6 times with specific activities 0.0103 U/mg. Cellulase enzyme fractionated with ammonium sulfate at this research was optimum at pH 7 and temperature 50°C. It is necessary to further identify the molecular weight of the purified cellulase enzyme by SDS-PAGE and Zymogram.

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