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Isolation of Cellulolytic Bacteria from the Soil Surrounding a Final Landfill Site

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Abstract. Cellulolytic bacteria are capable of producing cellulase enzymes, enabling them to break down cellulose into simpler compounds using cellulose as a growth substrate. These bacteria are commonly found in various environments containing cellulose, such as landfill sites where abundant organic materials are present. This research employed the cup-plate isolation method with the aim of obtaining cellulolytic bacterial isolates from the soil surrounding a final landfill site. Successful isolation of cellulolytic bacteria was achieved from soil samples taken in proximity to the disposal site and cultured using selective CMC media. Two purified isolates were obtained, and their cellulase enzyme activities were examined. Isolate LA2 exhibited higher cellulase enzyme activity than isolate LA1, forming a clear zone index of 0.7778. Further quantitative analysis of isolate LA2 revealed its highest enzyme activity at the fifth minute, measuring 0.098 u/ml with a specific activity of 0.29 u/mg protein. Based on physiological characteristics, Gram staining, and spore staining, isolate bacteria LA2 were identified as Pseudomonas sp.

Key words: Bacteria, Enzyme, Cellulase, Cellulolytic.

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INTRODUCTION

Cellulose is one of the most abundant biopolymer compounds on Earth, with a chemical formula of C6H10O5 [1][2]. This compound exhibits slow degradation characteristics, necessitating external assistance to accelerate its degradation process. Microorganisms are organisms capable of aiding in cellulose degradation [3]. Microorganisms utilize cellulose as a substrate for growth [4]. These microorganisms, known as cellulolytic bacteria, can produce cellulose and employ it to break down cellulose into simpler glucose compounds [5][6]. Environments rich in cellulose, such as landfills, harbor significant potential for cellulolytic bacteria habitats [5]. Hence, it is plausible to discover cellulolytic bacteria in the soil surrounding landfill sites [7].

Several researchers have successfully isolated cellulolytic bacteria from sources such as rice husks [8], textile waste [9], sago wastewater [10], diaper waste [11], and organic waste [12]. The isolated bacteria demonstrated cellulase enzyme activity, showcasing their ability to utilize cellulose in waste, thereby expediting the cellulose decomposition process. This phenomenon can mitigate negative environmental impacts [13]. Furthermore, cellulase enzymes produced by bacteria possess advantages over those produced by other organisms, including animals and plants, as they are purer, more stable, and have a faster production time [14][15].

Exploration of enzymes produced by bacteria continues to align with the evolving needs of industrial products. This pursuit is concurrent with the depletion of natural resources due to excessive utilization [16]. Bacterial cellulase enzymes find applications in various industries, necessitating continuous development in bacterial cellulase enzyme research. The diversity of bacterial isolates may also be influenced by the distinct habitats, prompting this study to isolate cellulolytic bacteria from a landfill site in the Bogor region. The objective of this research is to isolate cellulolytic bacteria originating from a landfill site.

MATERIALS AND METHODS

Research Procedure

This research was conducted from January 12 to March 5, 2018, in the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural Institute.

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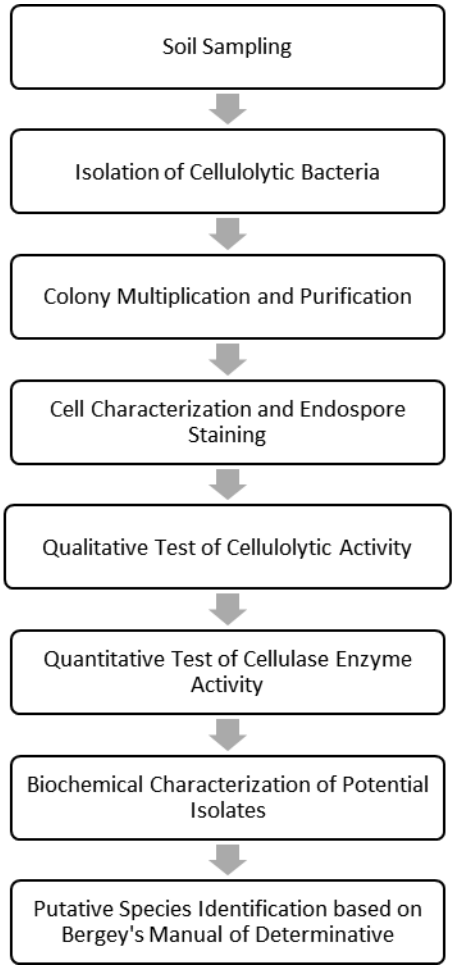
The research procedures are outlined in Figure 1.



Soil sampling was carried out by extracting soil at a depth of 10-20 cm, and the soil was then placed in aluminum foil. Isolation of cellulolytic bacteria was conducted using the spread plate method on CMC media. One gram of soil sample was diluted in 9 ml of 0.85% NaCl solution. The final dilution of 0.1 ml was then inoculated onto CMC medium and incubated for 24-48 hours [17][18][19].

Colonies that developed were purified using the quadrant streak method on NA media and incubated for 24-48 hours [20]. Each colony was labeled differently. Single colonies obtained from streak plates were streaked onto NA media as pure isolates and stored for subsequent testing.

Characterization was performed using gram staining. Gram staining aimed to determine the type of cell membrane possessed by the bacteria. The prerequisite for gram staining was that the bacteria should be approximately 24 hours old. If the bacteria were gram-positive, the process continued with endospore staining [21].

All bacterial isolates were inoculated into CMC medium for 48 hours at room temperature. After 48 hours of bacterial inoculation, Congo red was applied to observe the presence of clear zones and identify potential isolates. Subsequently, the index of clear zones was measured using the formula [22]:

$$Index\ of\ clear\ zone = \frac{Clear\ zone\ diameter\ (mm) - Colonies\ Diameter\ (mm)}{Colonies\ Diameter\ (mm)}$$

Quantitative analysis of cellulase enzyme was conducted using the Miller method (2009) [23]. Bacteria were inoculated into 20 ml liquid CMC media and incubated for approximately 6 hours (shaker 120 rpm). After incubation, measure the OD using a normal OD spectrophotometer (0.6-0.8). The remaining sample was centrifuged, and the supernatant was taken (crude enzyme).

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Prepare a 0.05 M pH 7 phosphate buffer, a 5 mg/ml glucose standard solution, DNS reagent, Bradford reagent, and a 1 mg/ml BSA standard solution with the prepared materials. From the BSA standard solution (Table 1). The preparation of the glucose standard solution using the Bernfeld method [24] (Table 2) was given variations in concentration.

Tabel 1. Variations in BSA Standard Concentrations

Concentration BSA (mg/ml)	Stock BSA (ml)	Aquades (ml)
0	0	1
0,1	0,1	0,9
0,2	0,2	0,8
0,3	0,3	0,7
0,4	0,4	0,6
0,5	0,5	0,5

Tabel 2. Variations in Glucose Standard Concentrations

Concentration Glucose Solution (mg/ml)	Stock Glucose Solution (ml)	Aquades (ml)
0	0	5
0,1	0,1	4,9
0,2	0,2	4,8
0,3	0,3	4,7
0,4	0,4	4,6
0,5	0,5	4,5

The absorbance of each concentration variation was measured using a spectrophotometer with a wavelength of 550 nm for glucose and 595 nm for BSA. Then, a linear regression equation was obtained and used for the measurement of glucose concentration and protein content. The absorbance measurements at these wavelengths were plotted on the Y-axis, with glucose concentration/protein content plotted on the X-axis. Quantitative testing of cellulase enzyme activity in crude enzyme involved several treatments: sample, blank, control, and glucose standard curve. Protein content measurement was performed with sample treatment and BSA standard curve [25].

The measurement of cellulase enzyme activity in Units per ml (U/ml), meaning the amount of enzyme producing glucose per minute, was calculated based on the following formula (glucose concentration obtained from the x value through the equation of the glucose standard curve y = ax+b):

$$Cellulase\ Activity\ \mathrm{U/ml} = \frac{[glucose\ consentration]x\ fp}{BM\ glucose\ x\ Enzyme\ Volume\ x\ Time}$$

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Note BM : Molecule Weight
fp : Dilution Factor

The measurement of specific cellulase enzyme activity in U/mg of protein is calculated using the formula:

$$Specific\ cellulase\ activity\ \text{U/mg protein} = \frac{Cellulase\ activity\ (U/ml)}{Protein\ content\ (mg/ml)}$$

The measurement of protein content utilizes the values from the equation y=ax-b obtained from the graph with the formula:

$$Protein\ concentration\ mg/ml = \frac{Corrected\ OD\ sample\ -\ b}{a}$$

Biochemical characterization is performed through several physiological tests on potential isolates. Potential isolates are tested using media prepared based on the characteristics of the isolate cells. The isolates are cultured on each medium and incubated for 24 hours [26].

The determination of the species name of potential bacteria is carried out using Bergey's Manual of Determinative Bacteriology. The naming is based on the results of the biochemical tests conducted.

RESULTS AND DISCUSSION

Isolation of Cellulolytic Bacteria

Isolation of cellulolytic bacteria is performed using soil samples. Bacterial isolation is successfully carried out by serial dilution up to 1x10-5 and 1x10-6. Pure isolates were not obtained in the isolation results, so purification was necessary using the quadrant streaking method. Isolates were taken only from the 1x10-6 dilution because at the 1x10-5 dilution, colonies grew very densely.

Colony Multiplication and Purification

Purification results using the quadrant streaking method yielded pure colonies. However, in this study, the streaking method on slant agar was used, and it can be used as an isolate for cellulase enzyme activity testing. Two isolates were successfully purified and stored on slant agar media, as seen in Figure 2.

Figure 2. Isolation Results (Isolates LA1 and LA2)

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Cell Characterization and Endospore Observation

Gram staining was performed to determine the type of cell membrane the bacteria possess. Gram staining results for isolates LA1 and LA2 showed a red color and a monobasil shape, indicating that isolates LA1 and LA2 are gram-negative bacteria (Figure 3). Gram-negative bacteria have a thin peptidoglycan wall layer, so crystal violet is discarded during the decolorization process. When safranin is applied, gram-negative bacteria turn red. Testing was not continued in the endospore staining stage because the isolates are gram-negative, and endospores are only possessed by gram-positive bacteria [27].

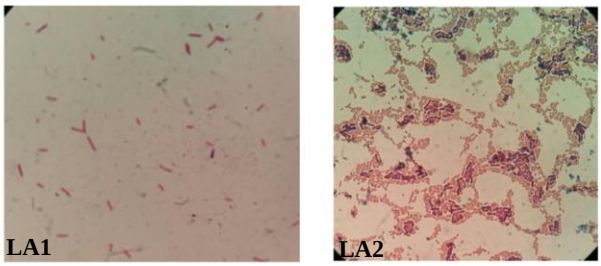

Figure 3. Results of Gram Staining Isolat LA1 and LA2

Qualitative Test of Cellulolytic Activity

Qualitative testing was done by counting the clear zone index that formed. Isolates used for qualitative testing were LA1 and LA2. Both isolates showed clear zones, indicating that LA1 and LA2 isolates have cellulase enzyme activity that can hydrolyze CMC into simple sugars (Figure 4). The clear zones indicate the absence of a reaction between *congo red* and simple sugars because *congo red* only reacts with CMC [23]. The clear zone index for LA1 isolate is 0.5384, while for LA2 isolate, it is larger at 0.7778, so LA2 isolate was selected as the potential isolate for further testing.

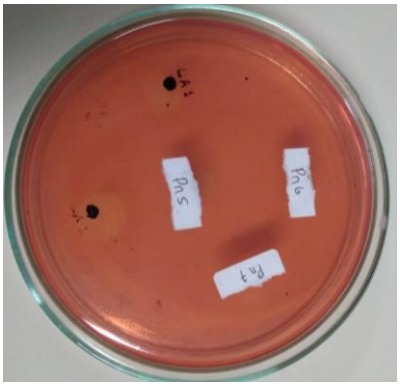

Figure 4. Clear Zones of Isolates LA1 and LA2

Cellulase enzyme activity in LA2 isolate for each U/ml of crude enzyme extract and specific activity for each U/mg of protein can be seen in Table 3. Unit and specific activity values were measured at different incubation times. The cellulolytic activity in the isolate is quite potent as it has high activity with a low enzyme concentration. After measurement, the bacterial cell absorbance of 0.915 indicates that the bacterial cells are in the log or exponential phase. The exponential phase correlates with high enzyme activity

results, meaning that when active cells divide, enzyme production also increases [28].

Based on the incubation time, both unit and specific activity decrease as the incubation time increases. This is due to the instability of enzyme activity [28]. However, enzyme activity still exists even though it is unstable (Table 3).

Incubation Time (Minutes)	Unit Activity (U/ml)	Specific Activity (U/mg)
5	0,098	0,29
10	0,020	0,06
20	0,018	0,05
30	0,010	0,03
40	0,008	0,02

Biochemical Characterization of Potential Isolates

Biochemical characterization is done by conducting physiological tests on bacteria according to cell characterization. Biochemical tests are performed to determine the type or species of a bacterium, as bacteria can be grouped based on the characteristics of their physiological activities [29]. In this experiment, physiological tests performed included glucose fermentation, oxidase, motility, H2S production, MR-VP, nitrate reduction, and colony pigmentation. These seven tests were conducted because the results of cell characterization indicated gram-negative monobasil bacteria. The results of these seven tests can be seen in Table 4.

Table 4. Biochemical Test Results

No	Test	Results
1	Oksidase	+
2	Glucose fermentation activity	-
3	MR-VP	-
4	Pigmen coloni	-
5	Motility	+
6	Produksi H_2S	-
7	Reduksi Nitrat	-

Table 6 shows that the LA2 isolate is unable to ferment glucose, as indicated by the negative test result. In the MR-VP test, after the addition of α-napthol and KOH, the color remained yellow, indicating no formation of acetyl methyl carbinol. This is consistent with the negative glucose fermentation test, as acetyl methyl carbinol can be formed from glucose fermentation. The colonies formed were not yellow, and the motility test indicated that the LA2 isolate is motile as its growth spread. The H2S test resulted in negative because there was no color change in the medium, meaning the isolate cannot ferment any type of

carbohydrate. The next test, nitrate reduction, showed a negative result, indicating that the bacterium cannot reduce nitrate to nitrite. From these chemical test results, the cellulolytic bacteria can be identified using the *Bergey's rods flow chart*.

Putative Species Base on Bergey's Manual of Determinative Bacteriology

According to the *Bergey's rods flow chart*, the identification of the gram-negative bacterium isolate LA2 is *Pseudomonas sp.* The specific species cannot be determined because several tests have not been conducted.

Conclusion

The isolation of cellulolytic bacteria was successfully performed from soil samples. Two isolates were successfully purified. Isolate LA2 exhibited cellulase enzyme activity greater than that of isolate LA1, forming a clear zone index of 0.7778. The LA2 isolate, which demonstrated higher activity, underwent quantitative testing, revealing the highest enzyme activity at the fifth minute, i.e., 0.098 u/ml, with a specific activity of 0.29 u/mg protein. Based on physiological characteristics, Gram staining, and spore staining, the bacterial isolate LA2 was identified as belonging to the Pseudomonas sp.

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