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Research Article

Isolation, Screening, and Molecular Identification of Cellulolytic Bacteria From Supit Urang Municipal Landfill, Malang City

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ABSTRACT

The municipal landfills contain substantial amounts of lignocellulosic waste that have not been adequately utilized. This waste can be processed using cellulolytic bacteria. Cellulolytic bacteria play an important role in the degradation of cellulose-based materials. This study aimed to isolate and identify cellulolytic bacteria based on 16S rDNA sequence obtained from the Supit Urang municipal landfill, Malang City. Bacteria were isolated from soil by cultivating on 1% carboxyl methyl cellulose (CMC) agar media. The cellulolytic activity was analyzed semi-quantitatively with flooded 1% Congo red and then washed with 1 M NaCl three times. Cellulase activity assay was measured using the 3.5-Dinitrosalicylic Acid (DNS) method. The most potential isolate in cellulose decomposition was identified. The most potential isolate in cellulose decomposition was identified. Twenty isolates of cellulolytic bacteria were obtained from the sample and sixteen isolates formed the clear zone. The cellulolytic index ranged between 0.26 and 1.52. Five isolates, SL1, SL2, SL10, SL16, and SL19 had the highest cellulolytic index. Isolate SL2 had the highest cellulase activity at 0.071 U/mL. Based on the 16S rDNA sequence, SL2 was identified as *Bacillus amyloliquefaciens* F98 with a similarity of 99.02%. This potential isolate has prospects for the biodegradation process from agricultural waste, which can be processed into valuable products.

Keywords: Bacillus amyloliquefaciens F98, Cellulolytic bacteria, Cellulase activity assay

Introduction

The increase in population has an impact on increasing the amount of waste. Indonesia produces 17,931,137.45 tons of waste/year [1]. The most common types of waste are food waste, wood, twigs, and trees, which contain lignocellulose. Lignocellulose can be used as a renewable resource for biofuel. Lignocellulosic biomass can be processed as a source of energy and other useful products because the presence of multi-carbon components and their derivatives can be converted into added-value materials for the synthesis of sugar, alcohol, lipids, etc. [2]. Lignocellulosic biomass consists of cellulose, hemicellulose, and lignin [3]. The main component of lignocellulose is cellulose. Cellulose can be converted into various products, such as bioethanol. To convert cellulose into products, cellulose needs to be separated from the hemicellulose and lignin components. The separation process of lignocellulosic components into products generally uses chemical methods that use many toxic chemicals, complicated procedures, and require relatively high costs [4]. This process can also be carried out biologically using microorganisms like bacteria that produce enzymes. Microorganism enzyme is a promising and sustainable approach because microorganisms can simultaneously break down lignocellulosic components without damaging the environment [5].

The potential of bacteria as agents used in biological methods to convert lignocellulose into useful products is cellulolytic bacteria. Cellulolytic bacteria can degrade cellulose because they have cellulase enzymes. The cellulase enzyme has

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three components. The components are endoglucanases (β-1,4-D-glucan-4-glucanohydrolase, EC 3.2.1.4, carboxymethyl cellulase, EC), exoglucanases (β-1,4-D-glucan-4-glucohydrolase, EC 3.2.1.91, cellobiohydrolase, CBH), and cellobiose (β-D-glucoside glucohydrolase, EC 3.2.1.21, β-1,4-D-glucosidase). These enzyme components have their respective functions and produce the main products in the form of glucose, cellobiose and cello-oligosaccharides [6]. The endoglucanase component breaks the β-glucosidic bond. Cellobiose breaks the non-reducing cello-oligosaccharide chain or crystalline region and produces cellobiose; then exoglucanase will hydrolyze cellobiose into glucose [7].

Examples of cellulolytic bacteria are *Sporocytophaga* sp., *Cellulomonas* sp., *Bacillus subtilis*, and *Burkholderia* sp. [8-12]. Research by Masngut et al. [11] stated that B6.2 isolates isolated from a landfill showed the highest cellulase activity, 19.797 U/mL. The enzymes produced by cellulolytic bacteria can be used to degrade waste into fermentable sugar so that it can be used as raw material for bioethanol production. In this study, cellulolytic bacteria were isolated from the Supit Urang municipal landfill in Malang City. The waste in landfills contains a high cellulose component, so many cellulolytic bacteria are found as indigenous bacteria [11].

This study aimed to isolate, screen, and molecularly identify cellulolytic bacteria from the Supit Urang municipal landfill in Malang City. This research is important to obtain bacterial isolates as agents that can degrade cellulose so it can be processed into useful products.

Material and Methods

Sample collection

Soil samples under the solid waste were taken from the Supit Urang municipal landfill, Malang City, at three different points taken compositely with a soil depth of ±10 cm. The physicochemical parameters measured were soil and air temperature measurements at the sampling location and pH measurements at the Microbiology Laboratory, Brawijaya University. The measurements of C-organic, N-total, C/N ratio, and organic matter levels were carried out at the Chemistry Laboratory, Muhammadiyah University of Malang.

Isolation and screening of cellulolytic bacteria

Twenty-five grams of soil was taken and then suspended in an Erlenmeyer flask with 225 mL of 0.85% NaCl solution. Next, a dilution series up to 10^{-6} was carried out. As much as 0.1 mL of the inoculum from each dilution series was taken and then put into a petri dish containing CMC-agar medium for cellulolytic bacteria using the pour plate technique, then incubated at 30°C for 2x24 hours. The composition of the selective media in 1000 mL of media is CMC 10 grams; yeast extract 4 grams; KH_2PO_4 4 grams; Na_2HPO_4 4 grams; $MgSO_4.7H_2O$ 0.2 grams; $CaCl_2.2H_2O$ 0.001 gram; $FeSO_4.7H_2O$ 0.004 grams; and agar powder 15 grams [12]. After the bacterial colonies grew, the number of colonies was counted as total plate count (TPC). Bacterial colonies were characterized by measuring colony size and observing the overall shape of the colony, edges, elevation, texture, consistency, optical characteristics, and pigmentation. Next, the single colonies were purified using the streak plate technique and transferred to a CMC-agar slant and gram staining was carried out.

Purified bacterial isolates were tested semi-quantitatively to see their ability to produce cellulase enzymes. Bacterial isolates were flooded with 1% (w/v) Congo red solution for 15 minutes on CMC-agar media, which had been inoculated with bacterial culture on a blank disc and incubated for 2x24 hours at 30°C and washed with 1 M NaCl three times. Colonies that have cellulolytic ability form a clear zone after the staining process with Congo red [13]. The cellulolytic index can be determined by measuring the clear zone diameter and colony diameter. The cellulolytic index can be determined using the formula below.

$$A = \frac{B-C}{C}$$

A : Cellulolytic Index (CI)
B : Clear zone diameter (mm)
C : Colony diameter (mm)

Cellulase activity assay

Bacteria were inoculated on CMC-broth medium, then incubated on a rotary shaker at a speed of 120 rpm with a temperature of 30°C for 2x24 hours. The bacterial culture was centrifuged at 10.000 rpm for 10 minutes at 4°C. The resulting supernatant contains a crude enzyme. The activity of endo-β-1,4-glucanase was assessed by incubating a mixture containing 1% carbo-

xymethyl cellulose (CMC) in 1800 µL of 20 mM phosphate buffer (pH 7.0) with 200 µL of crude cellulase enzyme at 50°C. After 30 minutes of reaction, 2 mL of 3,5-dinitrosalicylic acid (DNS) solution was added and then heated to 100°C for 5 minutes to stop the reaction. The sample was cooled to room temperature, and its absorbance was measured at 540 nm. One unit of endo-β-1,4-glucanase activity is defined as the amount of enzyme that can hydrolyze CMC and release 1 g of glucose equivalent per minute of reaction at a temperature of 50 °C [14].

Growth curve of selected cellulolytic bacteria

Selected bacterial isolates were inoculated into 50 mL of CMC-broth media and incubated on a rotary shaker at 120 rpm for 24 hours. After 24 hours, 10 mL was taken and transferred to 90 mL of CMC-broth medium. Next, 5 mL of bacterial culture was taken every 4 hours and the absorbance was measured at 540 nm using a spectrophotometer. This stage is carried out until the bacteria reach the initial stationary phase [14].

Identification of cellulolytic bacteria using 16S rDNA

DNA extraction of the selected bacterial isolate was done using the Quick-DNA™ Fungal/ Bacterial Miniprep Kit (Zymo Research, USA). The 16S rDNA sequence was amplified using Polymerase Chain Reaction (PCR) with universal primers 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGTTACCTTGTTACGACTT-3'). The PCR program used was predenaturation at 94°C for 5 minutes (1 cycle), denaturation at 94°C for 30 seconds (35 cycles), annealing at 55°C for 30 seconds (35 cycles), extension at 72°C for 1.5 minutes (35 cycles), and final extension at 72°C for 5 minutes (1 cycle). The presence of the 16S rDNA amplicon was checked by 1.5% agarose gel electrophoresis and visualized using a UV transilluminator. The 16S rDNA amplicon was purified and sequenced at First BASE, Malaysia. The sequence was analyzed using the web-based Basic Local Alignment Search Tool (BLAST) program to find regions of similarity between sequences. Next, phylogenetic tree construction was created using MEGA X and inferred according to the Neighbor Joining algorithm and Tamura-Nei model.

Data analysis

The results of environmental physicochemical parameters were presented with the average results for each sampling point. The results of the cellulolytic index and cellulase activity assay were analyzed using the Kruskal-Wallis test and followed by the Mann-Whitney test using the SPSS 25 program.

Results and Discussion

Isolation and screening of cellulolytic bacteria

The physicochemicals of the soil sample are shown in Table 1. The average soil temperature is 25.1°C. This is slightly different from research by Chukwuma et al. [15], which states that the temperature in municipal solid waste landfills is in the range of 28–36 ◦C. Temperature influences reactions in the environment and can strengthen odors at landfill sites [16]. The pH factor indicates the acidity or alkalinity of an environment. The soil pH measurement results of 6.8 are in line with research by Chukwuma et al. [15], which states that the average pH in municipal solid waste landfills is 6.37. The measured pH is close to the neutral pH number. The C/N ratio shows a value of 12.35. According to the [17], this value is in the medium

Table 1. Physicochemical parameters

No.	Parameter	Value
1.	Soil temperature (°C)	25.1 ± 0.1
2.	pH	6.8 ± 0.08
3.	C-organic (%)	5.14 ± 0.44
4.	N-total (%)	0.42 ± 0.07
5.	C/N ratio	12.35 ± 0.89
6.	Organic matter (%)	8.86 ± 0.76

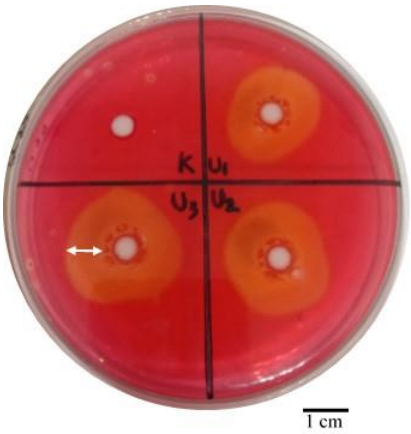


Figure 1. The clear zone of SL2 isolate formed in CMC-agar after stained with Congo red 1% and flooded with NaCl 1M. K : control. U : repetition. Arrows indicate clear zone formed.

category. The C/N ratio value shows the ratio of carbon and nitrogen in the sample. A low C/N ratio value indicates high nitrogen availability, while a high C/N ratio indicates high carbon availability.

Cellulolytic bacteria isolated from the soil of the Supit Urang municipal landfill obtained 20 isolates with a cell density of 6.6×10^3 CFU/g. Research by [18] showed that the number of cellulolytic bacterial cells isolated from the Tsomgo Lake, Panthang, Mao, and Shillong Peak areas ranged between 1.91×10^5 to 5.2×10^5 CFU/g at temperatures of 20°C. Study of [19] obtained $4.7 \pm 3.5 \times 10^6$ CFU/g cellulolytic bacterial cells isolated from decomposed *Coffea arabica* pulp. The number of cellulolytic cells obtained in this study was lower than in other studies.

All of the bacterial isolates were screened for cellulolytic activity on CMC-agar medium. Among them, 16 isolates formed a clear zone. The diameter of the clear zone of SL2 was 28.22 mm (Figure 1). The formation of a clear zone shows that cellulolytic bacterial isolates can hydrolyze cellulose as the carbon source in the medium [20]. On agar media, Congo red and cellulose interact with each other. Congo red attaches to 1,4-β glycoside bonds within cellulose, causing a red color. The halo zone around the bacterial colony signifies cellulose breakdown into monosaccharides. When bacteria secrete cellulase, the cellulose is degraded into disaccharides, glucose, and organic acids. It means cellulose releases 1,4-β glycoside bonds. Congo red cannot bind to glucose and, leading to a colorless area [21] [22].

The index of the clear zone formed was calculated and the data was shown in Figure 2. The cellulolytic index ranged between 0.26 and 1.52, indicating variations in cellulase enzyme production by the isolates. Five isolates had the highest cellulolytic index, i.e. SL1, SL2, SL10, SL16, and SL19, which had significant differences compared to other isolates. These isolates were chosen for the next screening stage for cellulase enzyme activity assay.

Cellulase activity assay

The enzyme activity test was carried out using the DNS method. Based on the results, isolates SL2 showed the highest enzyme activity, the value is 0.071 U/mL, and had significant differences compared to other isolates (Figure 3). Study of Yang et al. [9] showed that BY-2 had the highest enzyme activity at 2.41 U/mL in media with pH 4. Another study of Li et al. [23], stated that isolate M2 had the highest enzyme activity at 20.20 ± 0.74 U/mL in media with pH 5. The cellulase enzyme is a complex enzyme that gradually cuts cellulose chains into glucose [24]. Bacteria release extracellular cellulase enzymes, which are proven by the cellulase activity of the supernatant used. [25]. If the value of the cellulase enzyme activity is high, it means that bacteria have a good ability to hydrolyze cellulose.

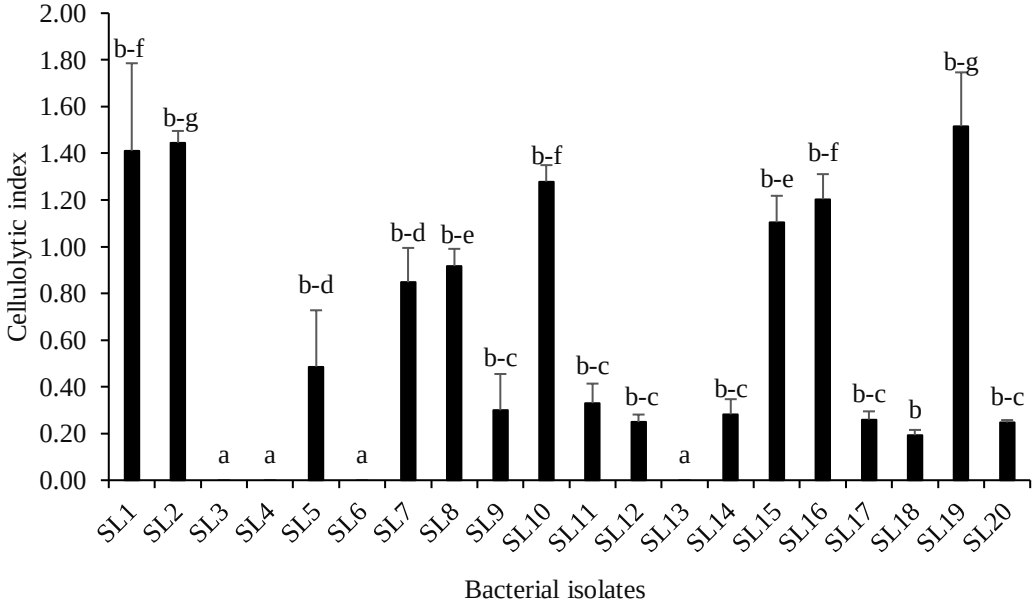


Figure 2. Cellulolytic index of bacterial isolates from Supit Urang municipal landfill

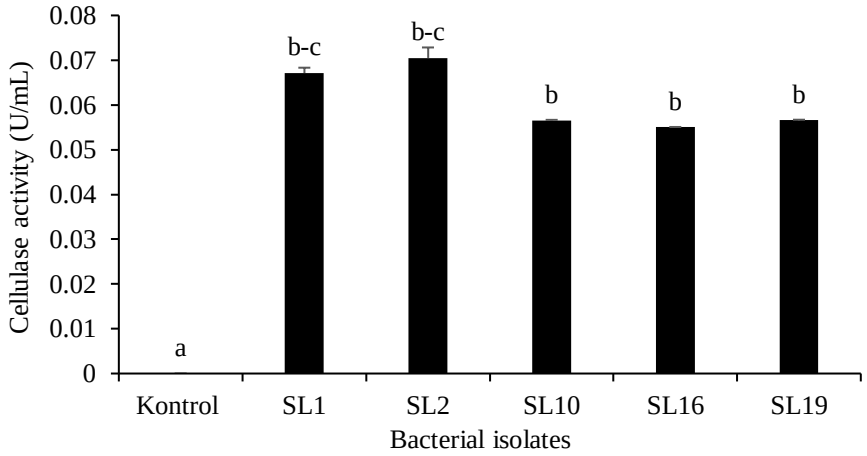


Figure 3. Cellulase activity assay of bacterial isolates from Supit Urang municipal landfill

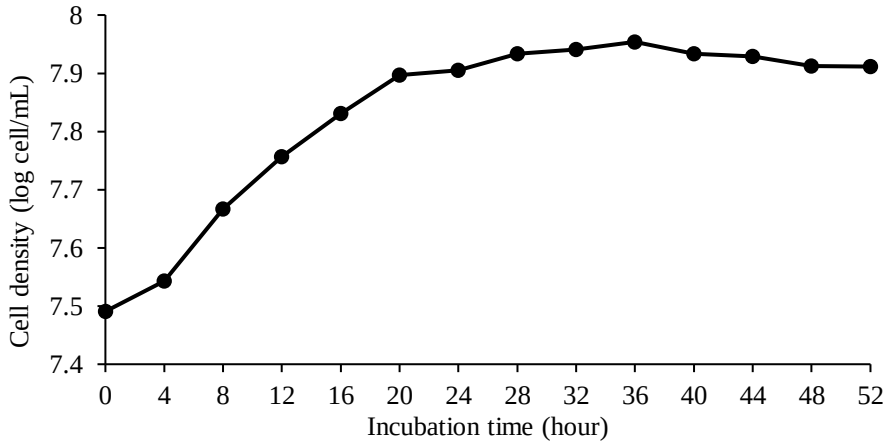
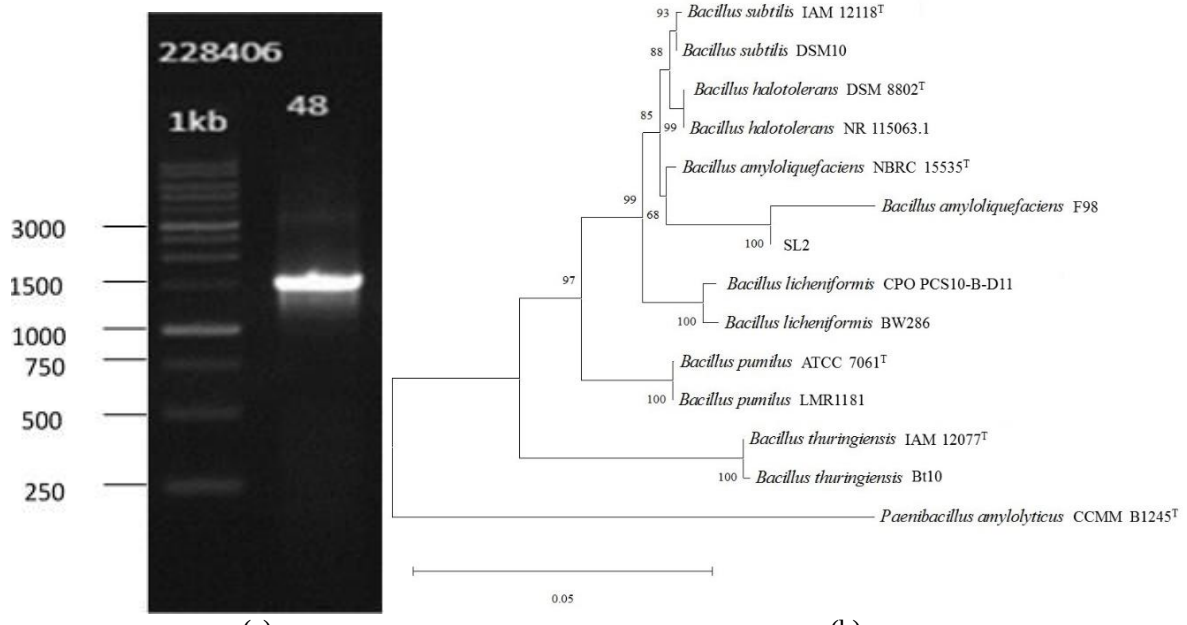


Figure 4. Growth curve of SL2 isolate



(a) (b)

Figure 5. Identification of cellulolytic bacteria based on 16S rDNA (a) visualization of PCR products by electrophoresis on agarose gel (b) molecular phylogenetic analysis of SL2 isolate using Neighbor Joining algorithm.

Growth curve of selected cellulolytic bacteria

The growth curve of the SL2 isolate shows that the lag phase is not visible because the adaptation phase of the SL2 bacterial isolate is relatively fast. The log phase starts at 0 hours to 20 hours. In the 20 to 52 hours, the graph tends to stabilize, which characterizes the stationary phase. The death phase on the graph cannot be observed, but it can be confirmed that the death phase occurs after 52 hours.

Identification of cellulolytic bacteria using 16S rDNA

The potential cellulolytic bacteria SL2 were identified based on the 16S rDNA gene. Figure 5 shows that the SL2 bacteria was identified as *Bacillus amyloliquefaciens* F98 with a similarity value of 99.02%. According to research by [26], *Bacillus amyloliquefaciens* has cellulolytic ability as indicated by the formation of a clear zone of 2.4 cm. Another study [27], found that *Bacillus amyloliquefaciens* M7 exhibited peak cellulase activity at 64.9 U/mL and demonstrated notable biopolishing efficacy on cotton fabric while minimizing weight loss to 4.3%. This cellulolytic bacteria can be used in biotechnology applications and various industrial processes.

Conclusion

The bacterial isolate that had the highest potential for cellulolytic bacteria was SL2, with cellulase activity at 0.071 U/mL. Based on molecular identification using 16S rDNA gene, SL2 was identified as *Bacillus amyloliquifaciens* F98. This bacteria can be used as agents that can degrade cellulose so it can be processed into useful products.

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