Isolation and Identification of Indigenous Cellulolytic Bacteria from Sago Pith Waste at Palopo, South Sulawesi, Indonesia

Mamluatul Faizah^{1*}, Tri Ardyati², Suharjono²

¹Master Program of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

²Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Palopo, South Sulawesi, is one of the traditional industrial centers of sago processing. The accumulation of sago pith waste around industrial sites can pollute the environment. Some microorganisms can degrade the cellulose in sago pith waste. This study was aimed to evaluate the indigenous cellulolytic bacteria from sago pith waste as a biodegradation agent. Bacteria were isolated from sago pith waste and grown on a 1% Carboxyl methyl cellulose (CMC) agar medium. The cellulolytic activity was analyzed semiquantitatively using 1% Congo red and quantitatively using the 3,5-Dinitrosalicylic Acid (DNS) method at pH variations of 4, 5, and 6. The potential isolate was identified based on 16S rDNA sequence similarity. This study obtained 21 bacterial isolates where six isolates were A1D, A1E, A1I, A1K, A2A, and B1A had the highest cellulolytic index at 0.82 – 1.13. Among those six isolates, the A1E isolate had the highest cellulolytic activity, 0.54 U.mL⁻¹ at pH 6. The isolate A1E was identified as *Burkholderia cepacia* JCM 2799 with 99.73% similarity of 16S rDNA sequence.

Keywords: *Burkholderia cepacia*, cellulolytic bacteria, cellulase enzyme, sago waste.

INTRODUCTION

Sago (*Metroxylon sago* Rott.) is a native plant in Southeast Asia. Indonesia has the largest sago plantation in the world. South Sulawesi, especially Palopo City, is one of the traditional industrial centers of sago processing in Indonesia, which contributes significantly to the production of sago waste. In 2018, the area of sago plantations in South Sulawesi reached 4,383 ha with a productivity of 2,626 tons of sago [1]. Sago trees contain 20-30% starch and 70-80% pith [2]. Thus, the sago waste produced annually ranges from 1,838 - 2,100 tons.year⁻¹.

In general, the sago pith waste in the sago processing industry is not handled properly. It is only allowed to accumulate on the ground that causing acidity in the soil, polluting the environment and causing unpleasant odors [3]. Soil contamination caused by the sago pith waste requires serious attention, so it is important to degrade it by utilizing microorganisms. Sago pith waste contains 20% of cellulose [4]. Cellulose is a major component of plant cell walls, unbranched linear chains consisting of several thousand glucose units with β -1,4 glycosidic bonds. Cellulose is insoluble in water and has high mechanical strength, making it difficult to degrade [5].

Cellulolytic bacteria can degrade cellulose by producing extracellular cellulase enzymes. Cellulase is an enzyme that catalyzes the cellulolysis process (hydrolysis of cellulose) to be glucose, cellobiose, and cellooligosaccharides [6]. Cellulase enzymes produced by microorganisms play an important role in the biodegradation of cellulose and lignocellulose wastes to be more simple compounds. The hydrolysis of cellulose into glucose by cellulase enzymes acts synergistically including three enzymes, namely endo- β -1,4-glucanase, Cellobio-hydrolase, and β -glucosidase. Endo β -1,4-glucanase (EG; EC 3.2.1.4) attack amorphous regions and cleave the internal glycosidic bonds. Cellobiohydrolase (CBH; or Exo- β -1,4-glucanase, β -1,4-D-glucan-cellobiohydrolase, EC 3.2.1.91) attack the non-reducing cellooligosaccharide chain or crystalline regions and produce cellobiose, then the cellobiose will be hydrolyzed into glucose by β -glucosidase (BG; cellobiase, β -D-glucoside glucanohydrolase, EC 3.2.1.21) [7].

Several groups of bacteria such as *Cellulomonas*, *Pseudomonas*, *Thermoactinomyces*, and *Bacillus* [8], *Bacillus pumilis*, *B. licheniformis*, *B. cereus*, and *Pseudomonas aeruginosa* showing cellulolytic activity [9]. Meanwhile, *Serratia liquefaciens*, *Acinetobacter iwoffi*, *Bacillus Licheniformis*, and *Bacillus cereus* were reported as indigenous bacteria from sago waste [10]. Therefore, this study is important to obtain bacterial isolates that have potency as biodegradation agents to solve the problem of sago pith waste.

* Correspondence address:

Mamluatul Faizah

Email : mamluatulfaizah7@gmail.com

Address : Dept. Biology, University of Brawijaya, Veteran
Malang 65145

MATERIAL AND METHOD

Sago pith waste sampling

Sago pith waste was collected from the traditional industry of sago in Palopo, South Sulawesi at two locations, location A (2°53'34"S 120°10'18"E) and location B (3°2'20.20"S 120°12'33.77"E). The sample was taken from a pile of sago pith waste in the bottom, near the ground, and stored in a plastic bag in an isotherm box. The physicochemical parameter that measured including organic matter, C/N ratio, and pH of sago pith waste. The organic matter and C/N ratio were analyzed in Soil Laboratory, Faculty of Agriculture, Brawijaya University.

Isolation and Screening of Cellulolytic Bacteria

Twenty-five grams of sago pith waste was suspended in 225 mL of 0.85% NaCl solution, and we made serial dilution until 10^{-7} . The aliquot of sample suspension 0.1 mL was spread on a Petri dish containing 1% CMC-agar medium and incubated at 30°C for 72 hours. The CMC-agar medium (g.L^{-1}) consist of CMC 10, Yeast Extract 4, KH_2PO_4 4, Na_2HPO_4 4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.001, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.004, and agar 15 at pH 6 [11].

The bacterial colonies that were grown on the CMC medium were observed and purified. The pure isolates were screened semi-quantitatively for cellulolytic producing enzymes using Congo red. The isolates then cultivate in 20 mL of 1% CMC-broth medium as much as 20 μL with equal cell density was 10^7 cells. mL^{-1} was taken and inoculated on blank disc paper and placed onto 1% CMC-agar medium and incubated at 30 °C for 72 hours. After incubation was completed, then flooded with 1% Congo Red solution for 15 minutes and it washed with 1M NaCl solution [12]. The cellulolytic index (CI) was determined with the formula 1.

$$CI = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diameter}} \dots \dots \dots (1)$$

Growth Curve and Crude Enzyme Production of Selected Bacteria

Broth culture of selected bacterial isolates (10%) were inoculated into 100 mL of 1% CMC-broth medium and incubated in a rotary shaker at 30°C, 120 rpm for 72 h. The bacterial culture as much as 5 mL were taken every 4 h for 7 days and optical density (OD) was measured using a spectrophotometer at 540 nm wavelength. The production of crude cellulase of each bacterium was determined at the exponential growth phase. The crude enzyme was obtained by

centrifugation of culture medium at 4°C, 10.000 rpm for 10 minutes. The supernatant was defined as a crude cellulase enzyme [13].

Cellulolytic Activity Assay

Cellulolytic activity of each selected bacteria was assayed quantitatively by incubated substrate of 1% CMC in the 1800 μL 20 mM buffer citrate (pH 4, 5, and 6) with 200 μL crude enzyme extract at 30°C for 30 minutes. The reaction was stopped by added 2 mL of DNS then boiled in a water bath at 100°C for 5 minutes. The sample was cooled at room temperature and its absorbance was measured at 540 nm. One unit of cellulase enzyme activity is defined as the amount of enzyme that releases 1 μmol glucose per mL per minute. The values of the cellulolytic activity were determined based on the glucose standard curve [13].

Identification of Bacteria Based on 16S rDNA

Bacterial chromosomal DNA was extracted using a Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZYMO RESEARCH, USA). The 16S rDNA sequence was amplified by Polymerase Chain Reaction (PCR) using universal primer 27f (5'-AGAGTTTGATCCTGGCTCAG3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') with PCR program: pre-denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s over 35 cycles and a final extension at 72°C for 5 min. The amplicon of 16S rDNA was confirmed by 1.5% agarose gel electrophoresis and visualized using a UV transilluminator. An amplicon of 16S rDNA was purified and sequenced in First BASE, Malaysia. The 16S rDNA sequences of bacteria were aligned with reference strains from the GenBank database using the MEGA V.6 program, and the phylogeny tree was constructed and inferred according to the Neighbor-joining algorithm and Tamura-Nei model [14].

Data Analysis

Semiquantitative data of cellulolytic activity was variance analyzed based on One-Way ANOVA. Meanwhile, quantitative data of cellulolytic activity were analyzed based on Two-Way ANOVA followed by Tukey test using SPSS 16 program.

RESULT AND DISCUSSION

Potential Isolates of Cellulolytic Bacteria

The study of cellulolytic bacteria from the sago pith waste was obtained 21 isolates where 16 isolates from location A and 5 isolates from location B. The density of cellulolytic bacteria at

location A 25×10^5 CFU.g⁻¹ was higher than location B 19.3×10^5 CFU.g⁻¹. The low bacterial density at location B is due to the lower organic carbon, nitrogen, C/N ratio, and organic matter in sago pith waste samples (Table 1). Organic matter plays an important role in microorganism density and pH [15]. The low pH in sago pith waste is due to the fermentation activity of microorganisms to form organic acids (acetic acid, pyruvic acid, and lactic acid) [16,17]. Carbon and nitrogen are macromolecules that have structural and functional roles in bacteria's cell components [18]. Based on similar research, the physicochemical parameter of sago pith waste contained 33.01% C-organic, 1.66% N-total, 20% C/N ratio [19], and pH 6 [17].

Table 1. Physicochemical parameter of sago pith waste

Parameter	Location A	Location B
C-organic (%)	38.9 ± 1.22	31.5 ± 3.25
N-total (%)	0.2 ± 0.06	1.2 ± 0.69
C/N ratio	179.7 ± 37.07	37 ± 28.69
Organic matter (%)	67.3 ± 2.10	54.5 ± 5.62
pH	5.9 ± 0.66	4.7 ± 0.35

Based on the cellulolytic index, there were six potential isolates consist of A1E, A1K, A1D, A2A, A1I, and B1A with cellulolytic index were 1.13, 0.97, 0.93, 0.88, 0.82, and 0.82, respectively (Fig. 1). The clear zone indicates the ability of bacteria to produce cellulase enzymes to hydrolyze cellulose substrates [20]. The similar research from sago waste reported that *Serratia liquefaciens*, *Acinetobacter iwoffii*, *Bacillus licheniformis*, and *Bacillus cereus* had cellulolytic index were 1.019, 2.009, 1.031, and 1.195, respectively [10]. Based on this study, the cellulolytic index of the A1E isolate was similar to the cellulolytic index of *Serratia liquefaciens*, *Bacillus licheniformis*, and *Bacillus cereus*.

Figure 1. Cellulolytic index of bacteria

Growth Curve of Selected Cellulolytic Bacteria

The growth curve of six cellulolytic bacterial isolates (Fig. 2), whole isolates were not showed an adaptation phase. The cell density of those isolates was increased significantly at the

logarithmic/exponential growth phase from initial incubation until 24 h. In the exponential growth phase, bacteria perform constantly at a maximum growth rate of cell division [13]. The bacterial cells had an optimal production of cellulase at the exponential growth phase [21]. It is due to cellulase as primary metabolites that have an important role in decomposing cellulose to be glucose as the carbon source for bacterial growth [22]. The stationary growth phase of cellulolytic bacteria occurred from 24 h until 64 h for A1D, A1I, A2A, and B1A, then followed by the death phase at 72 h, whereas A1E and A1K still in the stationary growth phase at 72 h. In the stationary growth phase, there is a balance between the number of live cells and dead cells [23].

Figure 2. Growth curve of cellulolytic bacteria

Each bacterial isolate with a density of 10^7 cells.mL⁻¹ at exponential growth phase (24 h incubation) had the highest potency for the production of cellulase enzymes. A similar study showed the exponential growth phase of cellulolytic bacteria occurs at 4 – 24 h incubation [13]. Another research was reported that the production of cellulase enzymes at 24 h incubation [24]. The exponential growth phase was considered at the incubation time of 36 h. Bacterial cultures have a significant cell mass at the exponential phase that can be expected the cellulase enzymes production more quickly [25].

Cellulolytic Activity of Bacterial Isolates

The A1E isolate at optimum pH 6 had the highest cellulolytic activity for 0.54 U.mL⁻¹ (Fig. 3). The isolate A1K, A1D, and A1I at optimum pH 6 had a cellulolytic activity for 0.35 U.mL⁻¹, 0.27 U.mL⁻¹, and 0.22 U.mL⁻¹, respectively. While A2A and B1A isolates at optimum pH 5 had cellulase activity for 0.31 U.mL⁻¹ and 0.33 U.mL⁻¹, respectively. A similar study was reported that *Cerenna* sp. and *Pseudomonas aeruginosa* at optimum pH 6 had cellulase activity 0.928 U.mL⁻¹ and 1.554 U.mL⁻¹, respectively [26]. *Bacillus subtilis*, *B. brevis*, *Paenibacillus* sp., and

Cellulomonas sp. are active at optimum pH 5.5 – 7 [24]. The Cellulase activity of *Citrobacter* sp. from pretreated bagasse was optimum at pH 6 [27].

The pH is a factor that has a significant effect on the stability of enzymatic reaction [28]. The enzymes that are produced from different bacteria allow them to have different optimum pH for their activity. When the pH changes, the enzyme's active site, and the substrate can alter because of the ionization. It influences the rate of the binding substrate to the active site of the enzyme [29]. Based on this research, at pH 6 and pH 5, the active site of the cellulase can be ionized optimally. Therefore, its activity can be optimal, as well.

Figure 3. Cellulolytic activity of bacteria at pH variation

Species of Potential Cellulolytic Bacteria

Potential cellulolytic bacteria A1E isolate from sago pith waste was identified phylogenetically based on 16S rDNA sequence similarity. Figure 4 showed that the A1E isolate was identified as *Burkholderia cepacia* JCM 2799 with 99.73% similarity of 16S rDNA sequence (Fig. 4). Previous studies also reported that *Burkholderia cepacia* from banana peel waste was potential as a cellulose decomposer with cellulolytic index 1.36 [30].

Figure 4. Phylogeny tree that showed relationship of A1E isolate and reference bacterial strains based on 16S rDNA sequence according Neighbor-joining algorithm

Burkholderia is a genus of lignocellulolytic bacteria [31, 32], and it is known to have cellulolytic activity [33]. *Burkholderia cepacia* from the soil in Xiling mountain has a cellulolytic activity of 2.76 U.mL⁻¹ at optimum temperature 60°C and pH 5 [34]. The different enzyme activity of bacteria can be influenced by the different conditions in the medium such as temperature, pH, carbon source, nitrogen source, enzymes, and substrate concentration [29].

CONCLUSION

The A1E indigenous isolate of sago pith waste from Palopo, South Sulawesi, had the highest potency as cellulolytic bacteria. The cellulase activity 0.54 U.mL⁻¹ was optimum at pH 6. The A1E isolate was identified as *Burkholderia cepacia* JCM 2799 with 99.73% similarity.

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