Screening and Identifying of Cellulolytic Bacteria from Alas Purwo National Park

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Screening and Identifying of Cellulolytic Bacteria from Alas Purwo National Park

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Abstract. This research aimed to screen and identifies cellulolytic bacteria from Alas Purwo National Park. Soil samples were taken from five points in each location by indirect sampling method. Bacteria were isolated with CMC (Carboxymethylcellulose) agar. The result had successfully obtained 16 isolates which produced cellulase. The cellulase activity index assay of bacteria used CMC agar modified with adds 1 % YME (Yeast Malt Extract) and then was incubated during one day at room temperature. Bacterial colonies were soaked with Congo red 1 % and rinsed with NaOH 10 % to visualize the halo zone formed. Three isolates with highest cellulase activity index were identified by observing the colony morphology, cell morphology, and the biochemical characters. The results of this research showed that the three highest potential isolates producing cellulase were BPA-B, BSA-D, and BPA-A with cellulase activity indexes respectively 10.67, 9.86, and 8.16. BPA-B, BSA-D, and BPA-A each have the similarity with genera *Pimelobacter*, *Micrococcus*, and *Cellulomonas*.

Keywords: Alas Purwo National Park, cellulase activity index, cellulolytic bacteria

INTRODUCTION

Alas Purwo National Park is geographically located at the eastern tip of Java Island South coast between 8.44°44'S and 114.22°60'E with an area of 44 037.30 ha. Based on the type of ecosystem, the area of Alas Purwo National Park is grouped into bamboo forests, coastal forests, mangrove forests, plantations, natural forests, and feeding ground [1]. The soil type in Alas Purwo National Park consists of four groups namely, red lithosol mediteran land complex, gray regosol soil, grumusol gray soil, and dominated by alluvial soil hidromorf. Leaves that fall on the ground cause high cellulose content [2]. Cellulose content on soil is used as a carbon source.

Almost half of the land on earth contains carbon components of cellulose. Cellulose is one of the carbon components of polysaccharides [3]. All carbon in the form of polysaccharides is mineralized with enzymes from microorganisms. One of the enzymes essential for the carbon cycle process is the polysaccharide hydrolysis enzyme. The enzyme that hydrolyzes the polysaccharide in the form of cellulose is cellulase. Cellulase can be found in soil microorganisms, one of them was cellulolytic bacteria. Cellulolytic bacteria are bacteria that have the ability to convert cellulose into oligosaccharides using cellulase enzyme [4]. This enzyme is used as a production material in the industry. Cellulase from bacteria is an extracellular enzyme consisting of the endo-β-1,4-glucanase complex, exo-β-

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1,4-glucanase, and β-1,4-glucosidase [5–7]. It can have good prospects for fuel from lignocellulose by combining cellulase and lignocellulase systems. In addition to the prospect being used as a fuel, cellulase enzymes can increase the production of bioethanol or biofuel [9].

Screening and identifying cellulolytic bacteria need to be done because of the lack of indigenous cellulolytic bacteria data in Indonesia. This study aims to screen cellulolytic bacteria from Alas Purwo National Park that has the potential to produce cellulase. The result of this research is used to increase the isolate database of cellulase enzyme producer.

MATERIALS AND METHODS

Soil Sampling

The sampling process took place in Feb 2017. Soil sampling was conducted on four locations in Alas Purwo National Park. Selection was based on vegetation homogeneity and physicochemical parameter test. Air humidity was measured using sling psychrometer (Bacharach), ground temperature was measured using a thermometer (Yenaco), light intensity using lux meter (IEEE, LX1010B), pH, and soil moisture using soil tester (Takemura DM-13). Soil sampling was carried out randomly from center point by five samples in each location using handheld soil sampler (LaMotte 1055/EP). Soil sampler was inserted 10 cm deep from the ground surface of leaf litter or grass. Five soil sample of each location is composed and stored in sterile plastic. Then, the sample is stored in a closed container and taken to the laboratory for two days.

Isolation and Screening of Cellulolytic Bacteria

A total of 25 g of composite soil from each location were homogenized with 225 mL of aquadest in Erlenmeyer (Pyrex). Then, the soil is agitated using a shaker (GFL MbH D-30938, Burgwedel) at a rate of 90 rpm for 15 min. After that, the supernatant was taken and macro dilution until 10^{-7}. Furthermore, bacterial was isolated using pour plate technique with pure CMC media; Carboxymethylcellulose, 10 000 mg · L^{-1} (PT Brataco) and agar powder 20 000 g · L^{-1}. The culture was incubated for 24 h at 27 °C in incubator (Heraeus Instrument). Prior to the screening, the grow cultures were stocked in NA (Nutrient Agar (Oxoid)). Screening is done by soaking bacterial colonies and media with Congo red (XCWY) 1 % for 10 min. Then the immersion solution was replaced with 10 % NaOH (Oxoid) and allowed to stand for 15 min. After submersion, the solution is removed, and the halo zone will appear. The visible halo zone is measured and calculated by the formula [10]:

$$\text{Relative enzyme activity index (ICMC)} = \frac{Halo\ zone\ diameter}{Colony\ zone\ diameter}$$

Identification of Bacteria

Bacterial isolates grown on CMC media were inoculated on NA medium at room temperature. The bacteria in the NA medium were used as insulating stock. Then, the bacteria growing on the NA medium were inoculated with a stab at the CMC medium for modification with the composition (Carboxymethylcellulose 1 % and Yeast Malt Extract 1 %). Three screening bacteria with potentially high cellulase production were inoculated on NA media. Identification of bacteria was based on macroscopic characters of colony, microscopic characters of cells, and also biochemical characterization. Macroscopic characters based on characteristic shape, size, color, texture, edges, and elevation of colonies. Microscopic characters were observed based on Gram characteristics with Gram staining, endospores staining with green Malachite dyes, and cell shape. Biochemical characterization of bacteria used ID Kit-12 A/B, catalase test, starch test, motility test, oxidative test, and 37 °C temperature. Yeast malt extract, Gram kit staining, green Malachite, and ID Kit-12 A/B were purchased from Oxoid. The results of identification are matched using Bergey's Determinative of Bacteria 9th edition.

RESULTS AND DISCUSSIONS

Each location of Alas Purwo National Park has vegetation dominated by different plants. Savanna vegetation is dominated by *Cyperus rotundus*, Sadengan bamboo forest is dominated by *Bambusa jacobsii*, Pancur bamboo forest is dominated by *Bambusa arundinaceae*, and tropical rainforest is dominated by *Switenia macrophylla*. The physicochemical parameter data of location is shown in Table 1.

Vegetation dominance indicates the homogeneity of the soils [11]. The cellulose nutrients in the soil for cellulolytic bacteria from four locations are litter or dead plant. The soil moisture is important for bacterial growth. Different type of soil will make a different bacterial community [12]. Therefore, the physicochemical of soil and air measurement are needed.

Related to the physicochemical parameter from each location, the cellulolytic bacterial isolated was classified as mesophilic bacteria because of the temperatures from each location have rates range from 28.4 to 31.6 °C. The mesophilic bacteria temperature is below 50 °C [13].

TABLE 1. Vegetation dominant and physicochemical parameters of location in Alas Purwo National Park.

Locations (code)	Dominant Vegetation	Rate of Soils pH	Rate of Soils Humidity (RH %)	Rate of Soils Temperature (°C)	Rate of Airs Humidity (RH %)	Rate of Light Intensity (Lux)
Savanna (SAV)	*Cyperus rotundus*	6.3 ± 0.48	80	31.6 ± 1.94	70	3 000 ± 0
Sadengan bamboo forest (BSA)	*Bambusa jacobsii*	4.75 ± 0.46	80	29.2 ± 0.83	80	459 ± 104.84
Pancur bamboo forest (BPA)	*Bambusa arundinaceae*	5.45 ± 0.44	80	27.6 ± 1.34	91	868.4 ± 390.63
Tropical rain forest (HHT)	*Switenia macrophyla*	5,4 ± 0.45	80	28.4 ± 0.89	72	620 ± 76.48

The result of isolation of cellulolytic bacteria from the fourth soil samples of vegetation obtained sixteenth different isolates that potential to produce cellulase. The potency of cellulolytic bacteria is based on the result of the relative enzyme activity index (I_{cmc}). From the sixteenth isolates, it was selected to be three isolates with the highest I_{cmc} value, shown in Table 2. The control used CMC media without a bacterial culture to indicate there is no enzyme activity. The halo zone forming because of the activity of cellulase to hydrolysis cellulose become glucose [14].

The three isolates selected were inoculated in NA media with incubation for one day. Visible form of macroscopic and microscopic characteristics of three bacteria is indicated in Table 3. Macroscopic characters observed are shape, size, color, texture, edges, and elevation. Macroscopic characters of bacterial colony are dominated by irregular forms. Obtained cellulolytic bacteria have two macroscopic colonies of large size, round, white bones, irregular edges and small, round, clear, irregular edges [15].

The microscopic characters of bacteria include Gram staining, endospore staining, and cell shape. The results of Gram staining are predominantly found Gram-positive with irregular rod-coccus form. The endospore stain has obtained in all isolate were not contain endospore (negative endospore).

Furthermore, the three isolates were performed to the physiological testing. Physiological test with ID 12A/B Kit shown in Table 4. Based on the test, three isolates can grow at 37 °C, catalase positive, motile, and oxidative hydrolysis. The BSA-D strain hydrolyzes ONPG, uses citrate, dilutes gelatin and inhibits malonate. The BPA-A strain performs fermentation of glucose, mannitol, sorbitol, rhamnose, sucrose, and arginine dihydrolase. The BPA-B strain can decarboxylate lysine, use citrate, dilute gelatin, and produce arginine dihydrolase. The results of macroscopic, microscopic, and physiological identification were matched on Bergey's Manual of Determinative Bacteriology 9th Edition.

Observed cellulolytic bacteria comprise several diverse physiological groups; first, fermentative anaerobes typically Gram-positive (Clostridium, Ruminococcus, and Caldicellulosiruptor) but containing a few Gram-negative

species (*Butryvibrio* and *Acetivibrio*); second, aerobic Gram-positive bacteria (*Cellulomonas* and *Thermobifida*); third, aerobic gliding bacteria (*Cytophaga* and *Sporocytophaga*) [9].

TABLE 2. Results of cellulase enzyme activity.

Locations	Strains	Relative Enzyme Activity Index (I_{cmc})
SAV	SAV-A	6.25 ± 2.16
	SAV-B	5.33 ± 1.44
	SAV-C	4.79 ± 1.57
	SAV-D	4.86 ± 1.32
BSA	BSA-A	6.66 ± 4.61
	BSA-B	6.58 ± 3.16
	BSA-C	7.88 ± 4.47
	BSA-D	9.86 ± 1.70
BPA	BPA-A	8.16 ± 1.13
	BPA-B	10.60 ± 1.57
	BPA-C	6.27 ± 2.56
	BPA-D	6.67 ± 2.51
HHT	HHT-A	2.00 ± 0.46
	HHT-B	2.08 ± 0.57
	HHT-C	1.86 ± 0.12
	HHT-D	2.25 ± 0.75
Control		N/A

TABLE 3. The results of macroscopic and microscopic characteristics of bacteria.

Strains	Colony Morphology						Cell Morphology		
	Shape	Size	Color	Texture	Margin	Elevation	Gram	Endospore	Shape
BSA-D	Irregular	Medium	White	Smooth with granule	Flat	Flat	Positive	Negative	Coccus
BPA-A	Irregular	Medium	White	Smooth	Lobate	Flat	Positive	Negative	Irregular rod-coccus
BPA-B	Irregular	Small	White	Smooth	Lobate	Raised	Positive	Negative	Irregular rod-coccus

The results of colony morphology, cell morphology, and biochemical characterization were determined with bacteria determination book. Percentage similarity gets from divide of same amount characteristic between the results data and the total of identification characteristic from determination books. Table 5 shows the identification results obtained genus name [16].

BPA-B isolate was identified as *Pimelobacter* genus with similarity 85 %. *Pimelobacter* has characteristics cell Gram-positive, non-endosporic, and irregular-rod shape. *Pimelobacter* habitat is in soil with litter on the surface [16]. *Pimelobacter* has known can produce trehalose synthase. Trehalose synthase is an enzyme which converts maltose into trehalose. Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide that is widespread. Conversion of maltose into trehalose reached 80 % [17]. Another side of *Pimelobacter* research to produce cellulase enzyme has not known yet before. BPA-B isolate can produce cellulase enzyme by the relative enzyme activity (I_{cmc}) is 10.67 ± 1.57.

Identification result of BSA-D was genus *Micrococcus* with 80 % similarity. The characteristics colony were similar with *Micrococcus* is an irregular shape, white color, smooth texture, and flat while the same cell characteristics are Gram-positive and coccus shape. *Micrococcus* that produces cellulase has identified as *Micrococcus* sp. [18]. The bacteria can produce cellulase, xylanase, and carboxymethylcellulase but not showing the microscopic, macroscopic, and biochemical identifications of bacteria [19]. BSA-D has relative enzyme activities (I_{cmc}) is 9.86 ± 1.70. Based on Bergey's Manual of Determinative Bacteriology 9th Edition, there are some biochemical tests same between two species, which are *M. sedentarius* and *M. lylae* [16].

The similarity of BPA-A isolate was closed with genus *Cellulomonas* [16]. The same cell characters of BPA-A with *Cellulomonas* were Gram-positive, irregular-rod shape, and non-endosporic. *Cellulomonas* also produces exoglucanase/xylanase, endoglucanase, cellulase, and β-glucosidase [20–22]. It can produce extracellular, intracellular, and cell-bound enzyme types. BPA-A just measured the cellulase activity index by halo zone of CMC medium and calculated the relative enzyme activities (I_{cmc}). The relative enzyme activity of BPA-A is 8.16 ± 1.13. *Cellulomonas* sp. can use to electricity generations by the microbial fuel cell and photo-fermentation with consortium [23, 24].

TABLE 4. The results of biochemical characterization.

Physiological Tests	Strains		
	BSA-D	BPA-A	BPA-B
Lysin [a]	-	-	+
Ornithine	-	-	-
H_2S [b]	-	-	-
Glucose [c]	-	+	-
Mannitol [c]	-	+	-
Xylose [c]	-	-	-
ONPG [d]	+	-	-
Indole from tryptophan [b]	-	+	-
Urease [d]	-	-	-
VP [i]	-	-	-
Citric [e]	+	-	+
TDA[b]	-	+	-
Gelatin [f]	+	+	+
Malonate [g]	+	-	-
Inositol [c]	-	-	-
Sorbitol [c]	-	+	-
Rhamnose [c]	-	+	-
Sucrose [c]	-	+	-
Lactose [c]	-	-	-
Arabinose [c]	-	-	-
Adonitol [c]	-	-	-
Raffinose [c]	-	-	-
Salicin [c]	-	-	-
Arginine [h]	+	+	+
Amylum [d]	-	-	-
Catalyses	+	+	+
Motility	+	+	+
Temperature 37 °C	+	+	+
Oxidative	+	+	+

[a] decarboxylation; [b] production; [f]fermentation; [d] hydrolysis; [c] used of; [f]dilution; [g] inhibition; [h]dihydrolase; [i]acetoin production.

TABLE 5. Results of three strains identification.

Strains	Locations	Similarity (%)	Strain of Closest Match	Identification results
BSA-D	Sadengan Bamboo Forest	80	*Micrococcus*	*Micrococcus*
BPA-A	Pancur Bamboo Forest	80	*Cellulomonas*	*Cellulomonas*
BPA-B		85	*Pimelobacter*	*Pimelobacter*

CONCLUSIONS

It is concluded from this research that three of the highest potential productions of cellulase production were BPA-B, BSA-D, and BPA-A which has a cellulolytic index of 10.67, 9.86, and 8.16 respectively. Identification results BPA-B, BSA-D, and BPA-A each have in common with genus *Pimelobacter*, *Micrococcus*, and *Cellulomonas*. The identification can not conclude the species name because it needs a specific test as like as molecular sequencing and comparison with gene bank data.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8