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Isolation of Cellulolytic Bacteria from Peat Soils as Decomposer of Oil Palm Empty Fruit Bunch

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ABSTRACT

The objectives of the study were to find out potential strains of cellulolytic bacteria isolated from two tropical peat soils and to study the potency of the isolated bacteria to decompose oil palm empty fruit bunch (EFB). The study was carried out in two stages: (1) isolation of cellulolytic bacteria from peat soils and (2) testing the potency of isolated bacteria to decompose oil palm EFB. The cellulolytic bacteria were isolated from two peat soils, i.e. a natural peat soil (forest) and a cultivated peat soil (has been used as agriculture land). Isolation of cellulolytic bacteria was conducted by preparing a series dilution of culture solutions using a streak plate method in a carboxymethyl cellulose (CMC) selective medium. Isolates that were able to form clear zones surrounding their bacterial colony were further tested to study the potency of the isolates to decompose cellulose in oil palm EFB. The cellulolytic activity of the selected isolates were further determined via production of reducing sugars in an oil palm EFB liquid medium using Nelson-Somogyi method. The results showed that there are six isolates of cellulolytic bacteria that have been identified in two tropical peat soils used in the current study. Two isolates were identified in a natural peat soil (forest) and four isolates were identified in a cultivated peat soil. The isolates collected are identified as *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. Among the isolates, an isolate of GS II-1 produces the highest concentration of reducing sugars, namely 0.1012 unit mL^{-1}or 101 ppm, indicating that the isolate of GS II-1 is highly potential to decompose oil palm EFB. Therefore, the isolate of GS II-1 can be used as a decomposer in the bio-conversion processes of oil palm EFB.

Keywords: Isolation, bacteria, cellulolytic, oil palm empty fruit bunch (EFB), peat soil

ABSTRAK

Bahan penyusun tanah gambut di daerah tropis berasal dari kayu-kayuan yang komponen terbesarnya adalah selulosa. Penelitian bertujuan untuk mendapatkan strain unggul bakteri selulolitik asal tanah gambut dan mengetahui potensinya dalam mendegradasi TKS (Tandan Kosong Sawit). Penelitian dilakukan secara bertahap yaitu: isolasi dan uji potensi bakteri selulolitik asal tanah gambut. Bahan yang digunakan sebagai sumber isolat adalah tanah gambut alami (hutan) dan tanah gambut terganggu (yang telah digunakan untuk budidaya tanaman). Isolasi dilakukan menggunakan seri pengenceran dengan metode cawan gores pada media selektif Carboxymethyl cellulosa (CMC). Isolat yang membentuk zona bening disekitar koloni dilanjutkan dengan uji potensinya dalam mendegradasi selulosa pada TKS. Isolat– isolate terseleksi diuji aktivitas selulolitiknya melalui pembentukan gula reduksi pada media cair TKS menggunakan metode Nelson-Somogyi. Hasil isolasi diperoleh 6 isolat bakteri selulolitik, 2 isolat berasal dari tanah gambut alami dan 4 isolat dari tanah gambut terganggu. Isolat-isolat tersebut teridentifikasi sebagai *Bacillus* sp., *Pseudomanas* sp., dan *Staphylococcus* sp. Hasil uji gula reduksi diperoleh 1 isolat (GS II-1) yang berpotensi tinggi dalam mendegradasi TKS dengan konsentrasi gula reduksi yang dihasilkan 0,1012 unit per ml setara 101 ppm, sehingga dapat digunakan sebagai agen perombak dalam biokonversi TKS.

Kata kunci: Bakteri, gambut, isolasi, selulolitik, tandan kosong sawit,

INTRODUCTION

A great prospect of palm oil in the world vegetable oil trading has encouraged Indonesian government to extend oil palm plantation in Indonesia. Since 2006, Indonesia has become the largest palm oil producing country with the number of Crude Palm Oil (CPO) production up to 20.4 million ton year^{-1} (Ditjen Perkebunan 2007). The expansion of oil palm plantation in Indonesia has been dominated by both plantations owned by state or private companies and plantations owned by communities. The total area of oil palm plantation in Indonesia in 2017 is about 11.7 million ha with 35.4 million tons of CPO production (Ditjen Perkebunan 2016).

Oil palm produces oil as the main product and other byproducts including empty fruit bunch (EFB). Empty Fruit Bunch (EFB) is the main waste of palm oil industry, which is about 20% to 27% of the processed Fresh Fruit Bunch (FFB) (Sivalingan 1983; Darnoko 1992; Ditjen PPHP 2006; Hastuti 2009). Empty Fruit Bunch (EFB) is a lignocellulosic waste that contains 45.95% cellulose, 22.84% hemicellulose, and 16.45% lignin. Those three substances belong to glucose polymers and polyphenol polymers, which are difficult to decompose (Darmoko *et al*. 1993). Empty Fruit Bunch (EFB) contains predominantly cellulose, consisting of 40% to 60% cellulose (Sivalingan 1983; Darwis e*t al*. 1988; Gusmawartati 1999; Erwinsyah *et al*. 2015). The study of Chandel *et al*. (2007) suggested that in nature, cellulose and hemicellulose are often bound to lignin; as a result microbial groups that are able to decompose cellulose would be able to decompose lignin as well. For instance, Clostridium, Cellulomonas, Trichoderma, Penicillium, Fusarium, and Aspergilus are microbial groups that possess cellulolytic activity.

Cellulose is a natural polymer that consists of glucose units. Hydrolysis of cellulose produces glucose, which further can be converted into ethanol, organic acids, single-cell protein, or other compounds via bio-conversion processes. Darnoko (1992) suggested that utilization of oil palm solid waste via bio-conversion processes would give an added value to the waste. Bio-conversion is a decomposition process of organic matter into simple organic compounds via microbial activity or its products. Some microbes are able to produce cellulase enzyme in response to the presence of cellulose in their environment. Microbes that are able to hydrolize cellulose are called cellulolytic microorganisms, such as bacteria, fungi and actinomycetes.

The presence of microbes in soil is affected by physical, chemical, and biological conditions of the soil, therefore, the soil condition plays an important role in the selectivity of soil microbes. Bacteria are the predominant microbial group in soil. Cellulolytic bacteria are heterotroph bacteria including saprophytic bacteria that are able to hydrolyze cellulose into glucose monomer. The cellulolytic bacteria are small in size (0.5-1.0 µm) and prokaryotic with a single cell. Their small sizes give a benefit in the decomposition processes of organic matter, *i.e.* the bacteria have a larger surface area to contact with the substrate in comparison to fungi and actinomycetes. In addition, the cellulolytic bacteria have a high reproductive rate, consequently, they have a rapid population growth and enzyme production (Baharuddin *et al*. 2010). Isolation process of specific microbial species is started with the isolation of the species from other micro organisms living in the same environment, and further continued with growing the isolate in a pure microbial culture. An approach that is commonly used to isolate microbes is *enrichment culture technique* (Madigan *et al*. 2000). This method uses a specific medium and condition that are suitable for specific microbial species (Hurst *et al*. 1997).

Indonesia has a large area of tropical peat soils. The peat soils in tropical area are mainly formed from wood residue. This wood residue contains mainly cellulose, which is difficult to decompose. Due to the low pH of peat ecosystem, it is unlikely that microbes such as cellulolytic bacteria can live in. In general, bacteria grow optimally at neutral pH range (*i.e.* pH 6 to 7). Each microbial species has its own specific pH requirement to grow on. A cellulolytic bacterium such as *Cythopaga* grows optimally at pH 6.1 to 9.1, whereas *Sporocythopaga* grows optimally at pH 5.6 to 6.0 (Sutedjo e*t al.* 1991). We hypothesize that cellulolytic bacteria that are isolated from peat soils would be able to decompose oil palm EFB.

The study aimed at isolating potential strains of cellulolytic bacteria derived from tropical peat soils and studying the potency of the isolated bacteria to decompose oil palm EFB.

MATERIALS AND METHODS

The research was conducted at Laboratory of Soil Science, Faculty of Agriculture; Laboratory of Microbiology and Research Laboratory of Enzyme, Fermentation and Bio Molecular, Faculty of Mathematics and Natural Sciences, University of Riau. The study was consisted of two stages, *i.e.* (1) isolation of cellulolytic bacteria from tropical peat soils and (2) an experiment to test the potency of isolated bacteria to decompose oil palm EFB.

Tropical peat soils used in the current study were derived from The Peatland Experimental Station, Faculty of Agriculture, University of Riau, which is located at Rimbo Panjang Village and Cagar Biosfer Giam Siak Kecil-Bukit Batu (CG GSK-BB).

Isolation and Purification of Cellulolytic Bacteria

Isolation of cellulolytic bacteria from tropical peat soils was performed by preparing a series dilution of enrichment culture solutions with a dilution factor up to 10^{-4} of pure culture solution. Dilution of pure culture solution was conducted by mixing 1 g of soil sample and sterilized physiologic saline solution (NaCl 0.85%) in a test tube, and homogenizing the soil suspension for 1 minute using a vortex shaker. Isolation of cellulolytic bacteria was performed using a streak plate method (Hadioetomo 1990) in a modified carboxymethyl cellulose (CMC) selective medium (Aaronson 1970). After that, one inoculating loop of isolated cellulolytic bacteria was grown on a plate containing CMC using a streak plate method, and incubated at room temperature for four days with upside down plate position.

The bacterial colonies grown on CMC are considered as cellulolytic bacteria. Afterwards, the bacterial colonies were purified in a similar CMC medium using a streak plate method, and incubated for four days at room temperature. The purification was conducted for 3 to 4 times until a single colony of cellulolytic bacteria was obtained, which was shown by a uniform morphology in color, size, and form of the colony. The pure isolate of cellulolytic bacteria was further collected in a nutrient agar (NA) medium and stored in a freezer. After that, the pure isolate of cellulolytic bacteria was ready to be used for the next experiment.

Potency Test of Cellulolytic Bacteria

To know the potency of the isolate of cellulolytic bacteria, each isolate collected was tested for their cellulolytic activity in a CMC solid medium and oil palm EFB liquid medium. To test the potency of cellulolytic bacteria in a CMC solid medium, one inoculating loop of a 24 h isolate suspension was grown and incubated for three days. After that, the isolate of cellulolytic bacteria was stained using 0.1% Congo Red to see a clear zone that was formed (Teather dan Wood, 1982). About 15 mL of 0.1% Congo Red was added to the test culture medium and the culture medium was let to stand for 1 h. After that, the culture medium was washed two times using 15 mL of 1 M NaCl and the culture medium was let to stand for 15 minutes. Diameter of the colony and diameter of the clear zone that were formed were measured using a caliper. Cellulase activity was tested based on cellulolytic index obtained. Cellulolytic index is a ratio between diameter of the clear zone and diameter of the colony. The higher the cellulolytic index, the higher the potency of the isolate to produce cellulase enzyme is. The cellulolytic index is calculated using the following formula (Kader dan Omar 1998):

Cellulolytic Index (CI) = diameter of clear zone (mm) – diameter of colony (mm)/diameter of colony (mm)

To test the potency of cellulolytic bacteria in oil palm EFB liquid medium, one inoculating loop of isolate was mixed in a pre-culture medium (5 mL of oil palm EFB liquid medium), and incubated at room temperature for 24 h. Afterwards, 1 mL of pre-culture medium was put in an erlenmeyer containing 10 mL of oil palm EFB liquid culture medium (10% EFB powder), and incubated at an orbital shaker at 160 rpm for 72 h. After that, the culture medium was separated from supernatant using a centrifuge at 5000 rpm for 20 minutes. Reducing sugars that were produced by the isolates of cellulolitic bacteria were measured using spectrophotometer using Nelson-Somogyi Method (Sudarmadji *et al*. 1984).

Identification of bacteria was performed using a conventional approach including simple morphological characterization and physiological characterization. Morphological characterization was carried out by identifying bacterial colony including color, margin, consistency and elevation of the colony, whereas physiological characterization was identified by gram staining method using a microscope. Gram-positive bacteria have a purple color, whereas negative gram bacteria have a pink color (Hadioetomo 1993).

Identification of cellulolytic bacteria was carried out for each isolate referring to Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994).

RESULTS AND DISCUSSION

Isolation and Selection of Cellulolytic Bacteria

Isolation and selection of cellulolytic bacteria from two tropical peat soils taken from two locations (natural peat soil and cultivated peat soil) resulted in 6 isolates of cellulolytic bacteria that have been purified using a streak plate method in a CMC selective agar medium (Table 1).

Table 1 showed a different number of isolates of cellulolytic bacteria derived from the two locations of soil sampling, *i.e*. two isolates were identified in natural peat soil, whereas four isolates

Table 1. Cellulolytic bacterial isolates identified in two tropical peat soils.

Soil Sample	Code of Isolate	Number of Isolate
Natural peat soil (forest)	GS I-1; GS I-2	2
Cultivated peat soil	GS II-1; GS II-2; GS II-3; GS II-4	4

were identified in cultivated peat soil. The different condition of environment between the two soils may lead to a different number of cellulolytic bacteria isolated from the soils. The less number of isolates of cellulolytic bacteria identified in natural peat soil compared to that in cultivated peat soil may be due to the extreme condition of natural peat soil environment. In general, natural peat soils in Indonesia have a low pH range (pH < 4) besides the low amount of available macro-nutrients and micro-nutrients in the soils, therefore, it is unlikely that the microbes can grow optimally. Soil pH affects microbial activity and predominant microbe living in the soil. Sutedjo e*t al.* (1991) suggested that an optimum pH required for bacterial growth is at neutral pH range (pH 6 to 7); when the soil acidity increases, the number of bacteria tends to decrease in contrast to an increase of the number of fungi. The study of Chen *et al*. (2004) observed that cellulase activity of *Sinorhizobium fredii* (measured as CMCase production) was optimum at pH 7.0, and the study of Ariffin *et al. (*2006) indicated that activity of cellulolytic bacteria *Bacillus pumilus* EB3.l. (measured as CMCase production) was optimum at pH 6.0. The study of Verma *et al.* (2012) showed that cellulase activity of *Bacillus subtilis* was optimum at pH 6.5 to 7.5. In addition to soil pH, above ground vegetation may also contribute to determine the number of cellulolytic bacteria in soil. Diversity of above ground vegetation would affect

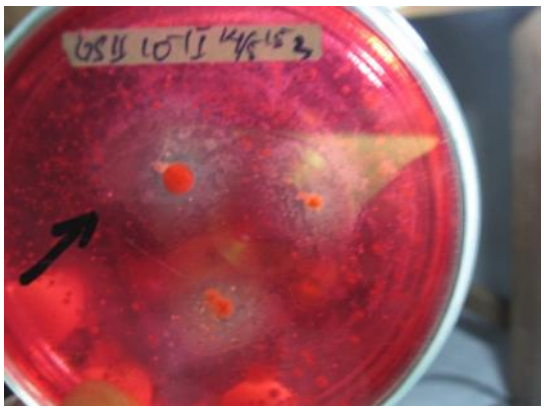

Figure 1. Clear zones of one tested isolate in a CMC agar medium.

the growth and number of soil microbes via organic matter availability derived from root metabolic activity. Newman (1995) suggested that the contents of soil organic matter and soil minerals are the factors influencing population of soil microbes, in addition to soil water content, soil temperature and soil acidity (pH).

Potency Test of Cellulolytic Bacteria

The potency of cellulolytic bacteria to produce cellulase enzyme can be identified from the formation of clear zones surrounding the bacterial colony grown in a CMC selective medium (Figure 1). Table 2 showed that the highest ratio of clear zone was observed for isolate GS II-1, *i.e*. 2.36. Table 2 also showed that isolate GS II-1 is the only one isolate that has cellulolytic index > 2. Rachmiati (1995) suggested that the formation of clear zones indicates the potency of bacteria qualitatively to dissolve insoluble substrate. Similarly, Nurmalinda *et al*. (2013) also suggested that the potency of microbes to convert substrate can be identified from the size of clear zones that are formed in a growth medium. A large clear zone that is formed indicates that the

Table 2. Cellulolytic activity of cellulolytic bacterial isolates in a CMC solid medium determined based on the formation of clear zones.

Code of Isolate	Diameter of Clear Zone (mm)	Diameter of Colony (mm)	Cellulolytic Index
GS I-1	2.0	2.4	1.20
GS I-2	8.9	-	1.00
GS II-1	2.2	5.2	2.36
GS II-2	3.1	3.2	1.01
GS II-3	8.8	9.0	1.02
GS II-4	6.8	-	1.00

Table 3. Average concentrations of reducing sugars (unit mL^{-1}) produced by the cellulolytic bacterial isolates grown in oil palm EFB culture medium incubated for 72 h.

Code of Isolate	Concentration of Reducing Sugars (unit mL^{-1})
GS I-1	0.0818
GS I-2	0.0178
GS II-1	0.1012
GS II-2	0.0327
GS II-3	0.0168
GS II-4	0.0208

microbe has a great potency to convert substrate in the growth medium. In addition, Ochoa-Solano dan Olmos-Soto (2006) indicated that isolate that can form diameter of clear zone two times of the diameter of colony is a potential enzyme producer. The presence of cellulolytic activity of bacteria can be identified from the formation of clear zones surrounding the bacterial colonies grown in a CMC selective medium.

Potency Test of Cellulolytic Bacteria in EFB Liquid Medium

Cellulolytic activity of isolates in oil palm EFB liquid medium was determined based on the formation of reducing sugars (Table 3).

Table 3 showed that the concentrations of reducing sugars produced by each isolate of cellulolytic bacteria varied. Isolate of cellulolytic bacterium GS II-1 produced the highest concentration of reducing sugar, *i.e.*0.1012 unit mL^{-1}. The high concentration of reducing sugar produced by the isolate GS II-1 indicates the high activity of cellulase enzyme produced by the isolate. Cellulase enzyme is an agent of decomposition that has a specific characteristic to hydrolyze β-1.4-glicosidic bond from a cellulose chain and its derivatives. Deng and Tabai (1994) suggested that cellulase ezyme complexes in general consist of three major enzyme units, *i.e*. (1) endo-β-1.4-glucanase (Cx) that plays a role especially on the amorphous part of cellulose chains; (2) exo-β-1,4-glucanase (C1) or cellobiohydrolase that plays a role in the breakdown processes of crystalline part of cellulose chains; and (3) β-glucosidase, which is an enzyme unit that is important in the production of glucose derived from cellobiose breakdown. The results indicated that isolate GS II-1 produces a complete cellulase enzyme and there is a synergism between cellulase enzymes that are produced by the isolate, which involves natural cellulose as substrates, consisting of crystalline and amorphous parts. The study of Enari (1983) showed that the total activity of cellulase enzyme can be determined by measuring an activity of several enzymes that contain cellulose and produce glucose as the final product. In addition, the results showed that the isolate GS II-1 is a potential bio-fertilizer, *i.e.* an agent of decomposition of organic material of oil palm EFB, so that the use of isolate can shorten the composting period of oil palm EFB.

Identification of Cellulolytic Bacteria

Identification of morphological characteristics on the bacterial isolates includes color, shape, margin, and elevation (rise of colony surface) of the colony. The results of gram staining and morphological characteristics of the colony indicated that the isolates of cellulolytic bacteria identified in the current study have different characteristics (Table 4).

Table 4 showed that there are three genus of cellulolytic bacteria isolated from two tropical peat soils used in the current study, referring to Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994).

Genus Bacillus, which are expected to be present as isolates GS II-1 and GS II-4, have

Table 4. Morphological characteristics and the results of gram staining of cellulolytic bacteria isolated from two tropical peat soils.

Code of Isolate	Morphological Characteristics					Gram Staining	
	Color	Size	Shape	Elevation	Margin	Gram	Shape
GS I-1	Yellowish cream	small	round	convex	entire	positif	round shaped like grapes
GS I-2	cream	small	round	convex	entire	negatif	rod-shaped
GS II-1	cream	small	round	convex	undulating	positif	rod-shaped
GS II-2	cream	small	round	convex	entire	negatif	rod-shaped like rice
GS II-3	cream	small	round	convex	entire	negatif	Small rod-shaped
GS II-4	cream	small	round	convex	undulating	positif	rod-shaped

morphological characteristics, namely cream color, round shape, undulating margin, and convex elevation. In addition, the cells of the isolates are in rod-shaped with physiological characteritistic as Gram-positive bacteria. Holt *et al.* (1994) indicated that *Bacillus* is Gram-positive bacteria that are in rod-shaped and inlcuded as aerobic bacteria or facultative anaerobic bacteria.The characteristics of *Bacillus* bacterial colony have a cream color and a round shape. The cells are in rod-shaped and straight, and often structured in pairs or chains with rounded-tips or square-tips.

Genus Pseudomonas, which are expected to be present as isolates GS I-2, GS II-2 and GS II-3, have macroscopic characteristics of the colony, namely cream color, entire margin, round shape and convex elevation. The microscopic characteristics of the isolates are in rod-shaped with physiological characteritistic as Gram-negative bacteria. The characteristics of bacterial colony identified in the current study have similar characteristics as genus *Pseudomonas*. Isolates of cellulolytic bacteria identified in banana fruit bunch waste used in the study of Hapsoh *et al.* (2014) showed similar characteristics as isolates of genus *Pseudomonas* identified in the current study, namely the isolates have white, milk-white, or cream color and the cells are in rod-shaped with physiological characteritistic as Gram-negative bacteria. Holt *et al.*(1994) indicated that bacteria genus *Pseudomonas* sp. have morphological characteristics, namely slightly yellowish color, the cells are in rod-shaped and straight with physiological characteritistic as negative gram bacteria. Many species of *Pseudomonas* are able to decompose polyhydroxybutirate and tend to be facultatively aerobic.

Genus Staphylococcus, which is present as the isolate GS I-1 identified in the current study, showed

Figure 2. Morphological characteristics of genus *Staphylococcus* colony.

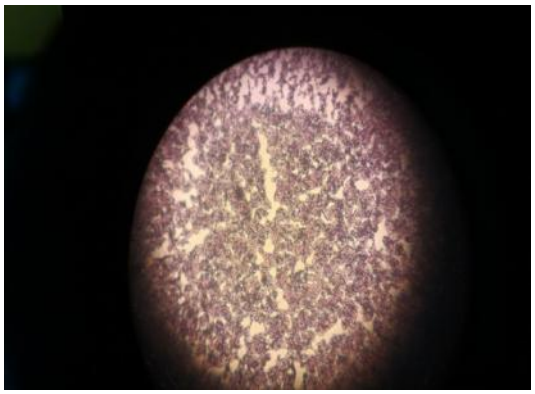

Figure 3. Gram-positive bacteria tested from isolate genus *Staphylococcus*.

similar characteristics as genus *Staphylococcus*, namely the cells are in round shape like grapes, with physiological characteritistic as Gram-positive bacteria, and the colony are in yellowish cream color, round shape, entire margin and convex elevation (Figure 2). Holt *et al* (1994) indicated that *Staphylococcus sp.*colony have morphological characteristics, namely milk-white color, somewhat creamy color, or sometimes orangeish yellow color, round shape with a lobate margin. The results of gram staining showed that the cells have physiological characteritistic as Gram-positive bacteria, which are present as coccus, diplococcus and staphylococcus (Figure 3).

CONCLUSIONS

The results showed that there are 6 isolates of cellulolytic bacteria that have been identified in two tropical peat soils used in the current study, namely 2 isolates are identified in a natural peat soil and 4 isolates are identified in a cultivated peat soil. The cellulolytic bacteria identified in the current study consist of 3 genus, namely Bacillus, Pseudomonas and Staphylococcus. Analysis of reducing sugars concentrations indicated that the isolate GS II-1 is highly potential to decompose oil palm EFB, therefore, the isolate can be used as a decomposer in the bio-conversion processes of oil palm EFB. The concentration of reducing sugars produced by the isolate is about 0.1012 unit mL^{-1} or 101.2 ppm.

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