Isolation and Characterization of Cellulolytic Bacteria Diversity in Peatland Ecosystem and Their Cellulolytic Activities

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Abstract. Peatlands are terrestrial wetland ecosystems formed from piles of organic matter that decompose into organic deposits. Peat soil has a high potential to produce cellulose which, can be reused by cellulolytic bacteria. This study aims to find out the potential strain of cellulolytic bacteria isolated from peatland ecosystems. The method used was experimental, sequentially, the stages are isolation and screening for cellulolytic bacteria, quantitative testing of cellulolytic activity, characterizing the morphology and physiology of bacteria, and the identification of bacteria based on Bergey's Manual of Determinative Bacteriology. The screening results obtained seven isolates of cellulolytic bacteria capable of hydrolysed cellulose on 1% Carboxy Methyl Cellulose (CMC) Agar Medium, namely SPS1, SPS2, SPS 3, SDG1, SDG 2, SPW1, and SPW4. Three of seven isolates obtained the highest cellulolytic index sequentially, namely SPS2 of 2.82, SPS3 of 2.65, and SDG1 of 2.47. The cellulolytic activity was indicated by the value of a halo zone around the colonies on 1 % CMC medium after being dripped with Congo red. The halo zone is an early indication to determine the ability of bacteria to decompose cellulose. Based on Bergey's Manual of Determinative Bacteriology showed that the three isolates had the same characteristics as the genus Bacillus, Lactobacillus and Corynebacterium.

1. Introduction
Peatlands are terrestrial wetland ecosystems with high water content, generally consisting of carbon, hydrogen, nitrogen, and phosphorus. Peatlands form by the accumulations of organic matter components that are naturally not completely decomposed and degraded under acidic and anaerobic conditions [1]. Peatlands have an abundant organic matter with compositions of 95% organic fraction and less than 5% inorganic fraction [2].

Many microorganisms can live and colonize every layer of peatlands, such as bacteria, fungi, and actinomycetes. Bacteria are microorganisms that can decompose organic matter in peatlands and use them as growth nutrients [3]. One of them is Cellulolytic bacteria. Cellulolytic bacteria can hydrolyze cellulose complexes into simpler molecules such as oligosaccharides and glucose. Naturally, cellulolytic
bacteria can hydrolyze cellulose in aerobic and anaerobic conditions. Under aerobic conditions, the hydrolysis process will produce air and carbon dioxide, while under anaerobic conditions, it will produce hydrogen molecules and methane compounds. Anaerobic cellulolytic bacteria only grow on sources of cellulose and its cellulolytic products [4]. Therefore, these bacteria cannot grow on oligosaccharides, monosaccharides, and polysaccharides derived from non-glucose. In general, the classification of cellulolytic bacteria is based on oxygen requirements grouped into two parts, namely aerobic and anaerobic bacteria. Aerobic cellulolytic bacteria include *Pseudomonas*, *Cellvibrio*, *Cellulomonas*, *Bacillus*, *Actinomyces*, *Streptomyces*, *Microbispora*, *Thermomonospora*, and *Acidothermus*. While anaerobic cellulolytic bacteria such as *Ruminococcus*, *Clostridium*, *Caldocellum*, *Bacteroides*, and *Acetivibrio* [5].

Cellulolytic bacteria can produce cellulase enzymes that can hydrolyze cellulose into glucose. Cellulase production occurs because of the response or direct contact of cells to cellulose in the environment. Cellulase is an enzyme complex that forms a synergistic system that gradually converts cellulose into an energy source in biomass [6], [7] reported, many species of microorganisms are capable of biosynthesis of cellulose. However, cellulolytic bacteria more utilize because they have many advantages, such as faster and more stable enzyme production, high biomass, and a rapid incubation period. Therefore, until now studies on the exploration and screening of cellulolytic bacteria from various sources are still being carried out, one of which is from peatland ecosystems.

2. **Methodology**

2.1 **Sampling**

The sample was obtained by the purposive sampling method. Sampling locations were determined randomly based on soil surface conditions. Samples were taken from three different stations, namely the ground surface, the soil at a depth of 10 and 20 from the ground surface. Samples were taken using a 2-inch pipe with a length of 40 cm and a pointed end. When sampling, a Pipe is inserted into the soil to a depth of 10 and 20 cm. Soil that enters the pipe cavity is then taken aseptically and put into sterile sample bottles. Furthermore, the measurement of physical factors at the sampling location was carried out based on the value of moisture, pH, and temperature.

2.2 **Isolation and screening cellulolytic bacteria**

Peat soil was weighed about 1 gram and put into a sterile test tube containing 10 ml of distilled water. Furthermore, the solution containing peat soil was diluted in series according to [8] method to a dilution of 10-6. Approximately 0.1 ml of the suspension was inoculated on the surface of the sterile Carboxy Methyl Cellulose (CMC) agar medium using the spread plate method. Then, CMC agar medium containing bacterial suspension was incubated in a bacterial incubator for 48 hours at 37°C.

2.3 **Cellulolytic Qualitative Activity**

Qualitative analysis of cellulolytic activity was done using CMC agar medium. Bacterial isolates were grown on medium containing 1% CMC and incubated at 37°C for 48 hours. After incubated, Petri plates were flooded with 0.1% Congo red reagent and left for 20 min. Then, Petri plates were washed with 1 M NaCl. A clearing zone called the halo zone is visible in Congo red for a positive test. The culture of bacterial isolates on CMC agar medium aims to determine the activity of cellulolytic bacteria. Bacterial isolates that produced a halo zone were declared capable of degrading cellulose and producing cellulase. Furthermore, the cellulolytic activity index (IS) was calculated based on the halo zone diameter and the colony diameter [9]. The degradation power of cellulose is classified based on the value of the cellulolytic index, namely low (less than 1), moderate (1-2), and high (more than 2) [10].

2.4 **Morphological characteristics of bacteria**

The morphological characteristics of bacteria include the shape, margin, texture, elevation and color of bacterial colonies.
2.5 Physiological characteristics of bacteria
The physiological characteristics of bacteria were carried out in two stages, namely gram staining and biochemical analysis. Gram staining was done to distinguish the gram properties of bacteria. Each bacterial were stained by the gram stain method following a standard staining protocol [11]. Bacterial biochemical analysis was carried out using several tests, such as a) motility test using Sulfide Indole Motility (SIM) medium, b) sugar fermentation ability test, and hydrogen sulfide production using Triple Sugar Iron Agar (TSIA) medium, c) catalase test using 3% H$_2$O$_2$ solution, d) selectivity of enterobacterial bacteria test using Mac-Conkey Agar medium, and e) the ability to degrade citrate using Simon's Citrate Agar (SCA) medium.

2.6 Identification of cellulolytic bacteria
Each bacterial isolate was identified based on morphological and physiological characteristics manually using Bergey's Manual of Determinative Bacteriology [12].

3. Results and Discussion
3.1 Isolation and screening of potential cellulolytic bacteria
Based on the results of bacterial isolation from peat soil, ten isolates were obtained that were able to hydrolyze cellulose. The ten isolates lived in different physical environmental conditions, sequentially the pH value ranged from 5-5.8, soil temperature ranged from 28-30°C, and soil moisture ranged from 55-65%. These results show that there is no significant difference for each layer of peat soil. However, cellulose production is highly dependent on various factors such as the amount of biomass, pH value, temperature, moisture, substrate, oxygen content, and incubation period. According to [13], several groups of cellulose-degrading bacteria were able to grow in the optimum pH between 5-6, the optimum temperature between 27-36°C, and the optimum moisture between 50-75%. Moisture, temperature, and pH are necessary factors that affect the activity of cellulose-degrading bacteria in the soil. [14] reported that most cellulolytic microbes live in the peat soil layer at a depth of 0-30 cm under aerobic conditions. Several species of cellulolytic bacteria can also survive in extreme conditions and produce extracellular cellulose. Some of these bacteria that reside in the subsoil under anaerobic growth conditions.

Based on macroscopic observations, several bacterial isolates had almost the same morphological characteristics, such as shape, margin, elevation, texture and color of bacterial colonies (Table 1). Bacteria are microorganisms that have various morphological characteristics. However, to determine the specific type of bacteria, it is necessary to analyze the biochemical properties of bacteria.

| No | Isolate Code | Bacterial Colony Morphology | Shape | Margin | Elevation | Texture | Color |
|----|--------------|-------------------------------|-------|--------|-----------|---------|-------|
| 1  | SPS1         | Irregular                     | Curled| Flat   | Flat      | Matte, brittle | Milky |
| 2  | SPS2         | Circular                      | Entire| Flat   | Flat      | Slimy, moist   | Milky |
| 3  | SPS3         | Irregular                     | Undulate| Flat | Flat      | Slimy, moist   | Yellowish |
| 4  | SPS4         | Round                         | Entire| Convex| Flat      | Shiny, viscous | Milky |
| 5  | SDG1         | Irregular                     | Lobate| Flat   | Flat      | Matte, brittle | Milky |
| 6  | SDG2         | Irregular                     | Undulate| Flat | Flat      | Slimy, moist   | Yellowish |
| 7  | SPW1         | Punctiform                    | Entire| Flat   | Flat      | Slimy, moist   | Milky |
| 8  | SPW2         | Irregular                     | Entire| Flat   | Flat      | Slimy, moist   | Milky |
| 9  | SPW3         | Irregular                     | Entire| Flat   | Flat      | Slimy, moist   | Milky |
| 10 | SPW4         | Irregular                     | Lobate| Flat   | Flat      | Matte, brittle | Milky |

Based on Table 1, there are differences in the number of isolates obtained from the three sampling locations. The highest number of isolates was obtained from the surface of the peat soil than, the smallest number was from the peat soil with a depth of 20 cm from the soil surface. The density of
microorganisms in the soil peat caused many factors such as an abundance of nutrients, soil type, temperature, pH, moisture, and availability of O$_2$.

Table 2. Physiological characteristics of cellulolytic bacteria isolated from soil peat

| No | Isolate Code | Gram Stain | Cell Shape       | Biochemistry Tests |
|----|--------------|------------|------------------|--------------------|
|    |              |            | Rod-shaped       | Kat | Sulf | Ind | Mot | MCA | Cit | Glu | Suc | Lac | H$_2$S |
| 1  | SPS1         | +          | Rod-shaped       | +   | -    | -   | -   | -   | +   | +   | +   | -    |
| 2  | SPS2         | +          | Rod-shaped       | +   | -    | -   | -   | -   | +   | +   | +   | -    |
| 3  | SPS3         | +          | Rod-shaped       | +   | -    | -   | -   | -   | +   | +   | +   | -    |
| 4  | SDG1         | +          | Rod-shaped       | +   | -    | -   | -   | -   | +   | +   | +   | -    |
| 5  | SDG2         | +          | Rod-shaped       | -   | -    | -   | -   | -   | +   | +   | +   | -    |
| 6  | SPW1         | +          | Rod-shaped       | +   | -    | -   | -   | -   | +   | -   | -   | -    |
| 7  | SPW4         | +          | Rod-shaped       | +   | -    | -   | -   | -   | +   | +   | +   | -    |

Note: Kat: catalase, Sulf: sulfide, Ind: indole, Mot: Motile, MCA: Mac- Conkey, Cit: citrate, Glu: glucose, Suc: sucrose, Lac: lactose
(+) Positive test result
(-) Negative test result

The activity of cellulolytic bacteria in degrading organic matter was controlled by environmental conditions and the density of microorganisms. In the soil, cellulolytic bacteria will use degraded organic materials for growth. However, when it rains and wets the peat, most of the organic matter will be carried away by the water to the bottom layer of the peat. The high soil water content makes the peat soil deprived of oxygen needed by bacteria. As a result, aerobic and facultative anaerobic bacteria are difficult to survive. Another effect is hydrolysis by cellulolytic bacteria also inhibited [15].

3.2 Cellulolytic bacterial activity

The cellulolytic activity was indicated by the value of a clear zone around the colonies on CMC agar medium after being dripped with Congo red (Figure 1). The higher activity of the cellulolytic index produced by each isolate, the greater ability of the isolate to degrade cellulose (Table 3). Based on Figure 1, the results of the cellulolytic activity test showed that not all isolates obtained were able to hydrolyse the cellulose contained in CMC agar medium. Only seven isolates could hydrolyse cellulose and form a halo zone after dripped Congo red solution on the surface of the media. The ability of bacteria to hydrolyse cellulose declared in the cellulolytic activity index.

Table 3. Cellulolytic Activity Index

| No | Isolate Code | Cellulolytic activity index | Category |
|----|--------------|-----------------------------|----------|
| 1  | SPS1         | 2.25                        | High     |
| 2  | SPS2         | 2.82                        | High     |
| 3  | SPS3         | 2.65                        | High     |
| 4  | SDG1         | 2.47                        | High     |
| 5  | SDG2         | 2.43                        | High     |
| 6  | SPW1         | 0.73                        | Low      |
| 7  | SPW4         | 0.30                        | Low      |
[16] reported, cellulolytic colonies that produce extracellular cellulase will form a halo zone with a red background as a non-degradable area. The addition of about 0.1% Congo red solution in the cellulose degradation test to detect the halo zone, that can be degraded by cellulolytic bacteria. Congo red 0.1% solution is a dye that will diffuse into the agar medium and was absorbed by long chains of polysaccharides. Long-chain polysaccharides have D-Glucan bonds resulting from the cellulolytic activity. The process of administering 0.1% Congo red solution was carried out when the test bacterial colonies had grown with an incubation period of 48 hours at 37°C and neutral pH. The ability of bacteria to grow on CMC agar medium showed that these bacteria were able to utilize cellulose as a source of nutrition, especially as a carbon source. The halo zone is an early indication to determine the ability of bacteria to decompose cellulose. Qualitatively, the wider the halo zone formed, the greater the potential for cellulolytic bacteria. Potential cellulolytic bacteria isolates were obtained based on the area and brightness of the halo zone produced by each isolate [17].

Figure 1. The result of hydrolysis of cellulolytic bacteria from peat soil. clear zone (a) growth medium (b) bacterial colonies and (c) halo zone

Cellulose degradation in the environment occurs because cellulolytic bacteria have cellulase. The cellulase consists of three components such as, endo-1,4-β-D-glucanase (endo-cellulase), exo-1,4-β-D-glucanase (celllobiohydrolase), and 1,4-β-D-glucosidase (celllobiose), which were produced by various types of microorganisms [19]. Enzyme endo-1,4-β-D-glucanase functions each long glucose chain to be random. Complete degradation of cellulose that occurs with the help of microorganisms will release carbon dioxide (CO₂) and water under aerobic conditions. The hydrolysis process under anaerobic conditions will release CO₂, methane, and water. Therefore, cellulolytic bacteria can degrade cellulose because it produces 1,4-β-D-glucosidase in cellulose.

3.3 Identification of cellulolytic bacteria

Based on isolation and screening bacteria, seven isolates of cellulolytic bacteria were able to hydrolyze cellulose from peat soil. Furthermore, the seven isolates were identified based on the morphological (Table 1) and physiological characteristics of the bacteria (Table 2). Analysis of Bergey's Manual of Determinative Bacteriology showed that isolate with codes SPS2 had similar characteristics with the Bacillus genera [12]. The results of the analysis included rod-shaped cells, in pairs or in chains, Gram-positive and have spore, catalase-positive, non-motile, aerobic, or facultative anaerobes. Based on the biochemical analysis, five isolates showed negative results in the citrate, Mac-Conkey, indole, and sulfide tests. In the sugar fermentation test, the five isolates were able to hydrolyze glucose, sucrose, and lactose, and they also were unable to produce H₂S. [6] reported that bacteria with the genus of Bacillus were also found in soils with high organic content, one of which was peat soil.

Analysis of morphological and physiological characteristics was also identified on isolates with codes SPS3 and SDG1. Based on Bergey's Manual of Determinative Bacteriology, SDG3 isolates have similar characteristics with the Lactobacillus genera [12]. The characteristics possessed were rod-shaped cells, gram-positive and did not have spores, non-motile, catalase-negative, and unable to produce H₂S. The
biochemical test showed that the SDG3 isolate was able to hydrolyze glucose, lactose, and sucrose. Therefore, the results of the indole, sulfide, and Mac Conkey tests were negative.

Based on Bergey's Manual of Determinative Bacteriology, isolate SDG1 has similar characteristics with Corynebacterium genera [12]. The SDG1 isolate had physiological, such as rod-shaped cells, Gram-positive, catalase-positive, unable to produce H2S, catalase-positive, facultative aerobic or anaerobic, and non-motile. The results of the fermentation test showed that SDG1 isolate was able to hydrolyze glucose and did not produce gas, but some tests showed a negative result, such as indole, sulfide, Mac-Conkey, and Simon's citrate tests. [20] reported that the genus of Corynebacterium has keys characters, namely Gram-positive, cell rod-shape, and produces catalase enzymes. The genus Corynebacterium also could degrade cellulose contained in CMC agar medium.

4. Conclusion
Based on the results and discussion, it can be concluded that there are seven potential cellulolytic bacterial isolates from peatlands capable of degrading cellulose, namely SPS1, SPS2, SPS3, SPS4, SDG1, SDG2, SPW1, and SPW4. Three of the seven isolates obtained the highest cellulolytic index sequentially, namely SPS2 of 2.82, SPS3 of 2.65, and SDG1 of 2.47. The results showed that the three isolates had the same characteristics as Bacillus, Lactobacillus, and Corynebacterium.

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