Bioconversion of lignin and methane production from Corn cobs (Zea mays) treated by lignin-degrading bacteria

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Abstract. Corn cobs are one of the potential feedstocks consisting of cellulose, hemicellulose and lignin, which provide potential lignocellulose biomass to be converted into renewable energy such as biogas through anaerobic digestion (AD). However, the recalcitrant structure of corn cobs lignocellulose makes it resistant to microbial access to the cell wall, and therefore the effective pre-treatment needs to be conducted. The biological pre-treatment using lignin-degrading bacteria is one of the promising bioconversion processes which will help to break down the lignocellulose structure. This study aims to analyse the ability of bacteria, Agrobacterium sp., Lysinibacillus sphaericus and Paenibacillus sp. in degrading lignin of corn cobs and therefore will enhance the methane released from AD. The ability of bacteria to degrade lignin was observed by analysis of total reducing sugar, total soluble phenols, lignin content, and weight loss, while the methane production was determined by the biochemical methane potential (BMP). The percentage of lignin content of untreated and pre-treated corn cobs with bacteria Agrobacterium sp., L. sphaericus and Paenibacillus sp. is 18.34%; 9.66%; 11.48% and 9.06%, respectively. The methane concentration (specific methane production) produced by using inoculum of Agrobacterium sp., L. sphaericus and Paenibacillus sp. with the addition of pre-treated corn cobs are 1.79%; 1.16% and 2.51%, respectively. These results were higher than the inoculum with the addition of untreated corn cobs.

1. Introduction
Lignocellulosic biomass is the most abundant renewable natural resource, which is the main component of plant cell walls. Lignocellulose biomass in general consists of cellulose (30-60%), hemicellulose (20-40%) and lignin (15-25%) [1] which can be used as feedstock for the production of bioenergy and other biobased products. The use of agricultural residues, especially lignocellulosic biomass, is crucial for environmentally friendly sustainable development [2]. Corn cobs are one of the renewable sources of biomass feedstock which consist high amount of lignocellulose (45% cellulose, 35% hemicellulose and 15% lignin) [3,4]. However, due to the recalcitrant structure of lignocellulose, especially lignin [5], the use of agricultural residues such as corn cobs is the major issue creating trouble in the efficient utilization, such as for the development of bioenergy products and value-added chemicals. Therefore, it is necessary to optimize the efficient process of pre-treatment, which can use physical, chemical or biological agents [6]. Compared to physical or chemical pre-treatment, the use of biological agents, however, could help to break down the lignocellulose structure environmentally friendly [7].
Lignin is an aromatic compound that mainly includes three groups, i.e., guaiacyl (G), syringyl (S) and hydroxyphenyl (H) \[8,9\]. The most common depolymerization of lignin through the enzymatic process is the use of extracellular oxidative enzymes produced by brown and white fungi \[10\]. The use of fungal, however, requires a long period of incubation and needs more effort to be genetically modified \[11,12\]. Previous research used the pine biomass from soil inoculum, which contains municipal solid waste (MSW) \[13\], and found that some bacteria were able to degrade lignin and produce methane. By using bacteria lignin degrader, lignocellulose of corn cob biomass, therefore, can be depolymerized and enhanced methane production through anaerobic digestion (AD).

The aim of this study was to determine the ability of lignin-degrading bacteria (\textit{Agrobacterium sp.}, \textit{Lysinibacillus sphaericus}, and \textit{Paenibacillus sp.}) to degrade lignin and enhance the methane production from corn cob. The assay of total reducing sugar and total soluble phenols were used to identify the effect of lignin breakdown toward the existence of both cellulose and hemicellulose in lignocellulosic biomass. In contrast, the biochemical methane potential (BMP) test was used to measure the rate of methane release from AD.

2. Material and methods

2.1. Microorganism and growth media
Bacterial lignin degrader strains (\textit{Agrobacterium sp.}, \textit{Lysinibacillus sphaericus}, and \textit{Paenibacillus sp.}) were originally collected from the Department of Chemistry, University of Warwick. The bacteria were then cultured and maintained using LB (Luria Bertani) agar media at 30 °C and stored at 4 °C in Bioindustry laboratory, Department of Agroindustry, Universitas Brawijaya, Indonesia.

2.2. Feedstock and pre-treatment
Corn cobs were collected from local corn farmers in Malang, Indonesia. Corn cobs were chopped into small sizes (about 1 cm) and air-dried for 48 h. Samples were placed into the jars and doubled sterilized (121 °C for 1 hour). The sterilized corn cobs samples were then inoculated with each bacteria strain, which was suspended in M9 minimal media and incubated for 7 days at 30 °C at incubator shaker (180 rpm). The processes diagram in preparing bacterial strains for pre-treatment is shown in Figure 1.

![Figure 1. Process diagram in preparing bacterial strains.](image-url)
2.3. Total reducing sugar, total soluble phenols, pH and weight loss
The corn stover treated with 3 (three) bacteria species (Agrobacterium sp., Lysinibacillus sphaericus, dan Paenibacillus sp.) were analysed to determine the ability of bacteria to degrade lignocellulose. Total reducing sugars assay [14] was used to identify the amount of sugar released from pre-treated corn cobs. While the amount of soluble phenols were measured colourimetrically using the Folin-Ciocalteau method [15]. Estimation of lignin content was measured using the Chesson method (Datta, 1981), further assays such as pH and weight loss were also conducted by Nurika et al. [16] to support the evidence of lignocellulose degradation by the bacteria.

2.4. Anaerobic digestion (AD) set-up
Pre-treated corn cobs were used as substrate and added to 250ml bioreactor with the inoculum at a substrate: inoculum ratio of 6:1. The digestate was obtained from a full-scale mesophilic AD-treating cattle manure at Balai Besar Pelatihan Peternakan (BBPP) in Batu city, Indonesia and degassed for 48 h at 37°C. The collected digestate was sieved through a 1 mm screen to remove larger particles. All bioreactors were operated in batch culture for 28 days.

2.5. The Biochemical Methane Potential (BMP) test
The BMP test used in this study was performed based on Suhartini et al. [17]. All the sample was carried out in three replicates over 28 days. The samples were placed in 250 ml bottle with a working volume of 40 ml. All samples were heated by a water bath maintained at 37°C ± 0.5°C. The pressure was measured daily using a digital manometer. The inoculum only was used as the control blank and □-cellulose as positive control. Determination of biogas volume based on gas pressure measurements per day. Then biogas volumes were calculated using the following equation [18]:

\[
\text{Biogas Volume (ml)} = \frac{(P \times \text{Vol}) \times V_m}{(R \times T)}
\]

Where, P is the pressure in the bottle (kPa); Vol is the volume of the bottle (ml); V_m is the molar volume of an ideal gas (22.414 L mol⁻¹); R is the ideal gas constant (8.314 m³ Pa K⁻¹ mol⁻¹); and T is incubation temperature (°C).

The specific methane potential was calculated to interpret the BMP test result using formula as reported by Strömberg et al. [19] as follows:

\[
\text{SMP} = \frac{V_h - V_{bg}}{m_{IS} - m_{B}} \times \frac{m_{IS}}{m_{VS,SS}}
\]

Where, SMP is specific methane potential, obtained from normalization methane volume (m³ CH₄/kg VS); V_h is the cumulative methane volume from reactor with substrate and inoculum, V_{bg} is methane volume from reactor with inoculum (blank sample); m_{IS} is the mass of VS of inoculum added in the sample; m_{B} is the mass of VS of inoculum added in the blank sample; m_{VS,SS} is the mass of substrate added in the reactor.

3. Results and discussion
3.1. Total reducing sugar (TRS), total soluble phenols (TSP), pH and weight loss
In this study, the change of sugars and soluble phenols represent the degradation of lignocellulose on corn cobs degraded by the bacteria. The measurement of these parameters will also give an idea of how the breakdown of lignin will affect cellulose and hemicellulose. Compared to the untreated sample (control), the total reducing sugar of the pre-treated samples is significantly increased (Figure 2a).
highest sugar released obtained from sample inoculated by *Paenibacillus sp* followed by samples treated by *Agrobacterium sp* and *Lysinibacillus sphaericus* about 1.962 mg g⁻¹; 1.631 mg g⁻¹ and 1.367 mg g⁻¹, respectively. The results of TRS indicated *Paenibacillus sp* could degrade sugars from both cellulose and hemicellulose more than samples treated by other bacteria. According to López-Mondéjar et al. [20], the genus *Paenibacillus* was known to produce cellulolytic and hemicellulolytic enzymes, both for industrial and agricultural applications. *Paenibacillus sp* was able to grow on lignin media do not require additional nutrients [21].

The value change of total soluble phenols between untreated and pre-treated samples depicted insignificantly different on releasing the soluble phenols (Figure 2b). This is probably due to the type of phenols which were detected only the soluble compounds, while some other types of phenol could not be detected. Further results on the change of lignin concentration between untreated and pre-treated samples showed the untreated sample contains lignin (18.34%) higher than the pre-treated sample with *Paenibacillus sp, Agrobacterium sp* and *L. spaeircus*, which showed a decrease of 9.06%, 9.66% and 11.48% respectively (data not showed). The reduction of lignin content on samples treated by *Paenibacillus sp* decreased about 50.50% compared to the untreated sample. The decrease of lignin on the pre-treated sample indicated the presence of enzymes capable of depolymerizing lignin into its derivative. According to Weselowski et al. [22], the ability of *Paenibacillus sp* to degrade lignin was confirmed by growing the bacteria on minimal media equipped with methylene blue and lignin mimetics which aims to detect the activity of ligninolytic enzymes. As a result, there was an oxidation zone (clear halo) around the colony, indicating the metabolism of lignin. Compared to another study Rashid et al. [13], the percentage of lignin on pre-treated samples in this study is higher, which is probably due to the use of a different type of feedstocks.

![Figure 2](image-url)

**Figure 2.** The effect of different types of bacteria as biological pre-treatment for the change of lignocellulose breakdown parameter: (a) total reducing sugar (mg g⁻¹); (b) total soluble phenols (mg g⁻¹); (c) pH; and (d) weight loss (%).
The degradation of lignocellulose can be influenced by the pH of the substrate as well as the change of pH during incubation. Especially when the biological pre-treatment of biomass is performed by fungi, hence the change of pH during the incubation is mostly decreased, indicating the releasing of organic acid compounds, which play a role in the breakdown of lignocellulose. However, in this study, the pattern of pH obtained showed different modes between samples (Figure 2c). After pre-treatment for 7 days, corn cobs treated with lignin-degrading bacteria had a lower pH than the pH before pre-treatment (pH 7). According to Yuansah et al. [23], a gradual decrease in pH occurs along with the length of the fermentation process. The decrease in pH is caused by the activity of microbial, which produce enzymes to break down the substrate into sugar, which is then converted to organic acid. Corn cobs sample treated by *Paenibacillus* sp had the lowest pH among other three bacteria. According to Jones et al.[24], *Paenibacillus* sp is one of the bacteria that produce cellulase that can tolerate in a wide range of pH, where this bacteria can be active in the pH range of 5.5–7.5.

The change of weight loss between untreated and pre-treated samples showed in Figure 2d. The increased weight loss percentage on pre-treated samples indicated the changes in the structure of lignin or lignocellulose which caused the decomposition of cellulose, hemicellulose, and volatile components. The highest percentage of weight loss was obtained from samples treated by *Paenibacillus* sp (22.68%) or almost 4 folds compared to the untreated sample. The increase in weight loss can be due to the loss of lignocellulosic material, which is degraded by the bacteria. Zhou et al. [25] supported that the higher of weight loss percentage obtained thus, the higher of lignin content which degraded.

### 3.2 BMP test

The specific methane production was obtained from the calculation of BMP using formula (2) developed by Strömberg et al. [19]. The average methane obtained from day first to 28 days can be seen in Figure 3. The highest methane production was obtained from corn cobs sample treated by *Paenibacillus* sp (0.102 m³/kg VS), followed by *Agrobacterium* sp (0.059 m³/kg VS) and *Lysinibacillus sphaericus* (0.051 m³/kg VS). All the samples treated by bacteria produced SMP higher than untreated samples, indicating the bacteria were able to break down the lignin structure and, therefore, the methane concentration increased. There were two samples treated by *Paenibacillus* sp and *Agrobacterium* sp that produced more methane compared to (-cellulose as a positive control, which contained a high amount of sugar.

![Figure 3. Specific Methane Potential from untreated and pre-treated corn cobs by lignin-degrading bacteria.](image-url)
The average results of SMP showed that the highest value of SMP obtained from corn cobs treated by *Paenibacillus sp* followed with a sample treated by *Agrobacterium sp*. These two samples indicated that the bacteria could break down lignin and, therefore, more organic substrate provided, such as sugars derived from cellulose and hemicellulose, which was used as nutrition by the consortia bacteria in the AD system to produce methane. The positive control (*α*-cellulose) however released methane less than samples pre-treated by the bacteria as well as the inoculum (Figure 4). The low average SMP of inoculum in this study probably due to several factors such as the handling of inoculum, the storage condition before its applied to AD and the lack of trace element.

![Figure 4. Average SMP from untreated and pre-treated samples.](image)

According to Saha et al. [26], the activity of microbes in all stages of AD depends on substrate, size of particle or material, surface area and the type of AD system. The reduction of material size will increase the chance of microbes degrading the substrate. The use of material with a bigger size, however, will probably inhibit the activity of microbes in the degradation process and, therefore will reduce methane production. Lestarie [27] also stated that the more of organic or nutrient provided during the AD process, the more organic compound would be converted to produce more methane.

4. Conclusions
The lignin degrading bacteria *Agrobacterium sp.*, *Lysinibacillus sphaericus* and *Paenibacillus sp.*, shows activity in lignocellulose degradation of corn cobs indicated by the change of total reducing sugars, lignin, and weight loss. Further results also showed that the methane produced by the pre-treated samples released more methane than the untreated sample. The lowest concentration of lignin (9.06%) and the highest value of SMP (0.046 m³/kg VS) were obtained from corn cobs inoculated by *Paenibacillus sp*. Further studies are required to optimize the lignin breakdown and enhance methane production using a single microbe (lignin-degrading bacteria).

Acknowledgement
The authors would like to acknowledge the support research fund from Faculty of Agricultural Technology (FAT) through Hibah Penelitian PNBP BP2M-2021.

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