Isolation and Identification of Biogas-Producing Methanogenic Bacteria from Cow Manure

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Abstract. Biogas is a methane-rich gas produced from microbial digestion of waste (agricultural, sewage, and landfill) which can be used for power production. The low rate of biogas production from an anaerobic digester becomes a problem in cow manure processing increasing. Biogas production is influenced by the biomass of methanogenic bacteria in the conversion of organic matter contained in a digester, so other methanogenic bacteria are needed to accelerate the rate of biogas production, namely methanogenic bacteria from the anaerobic digester of cow manure. Bacterial isolation was carried out by isolating samples of the anaerobic digester of cow manure in fluid thioglycolate media using the pour plate method in a vial tube. Those samples were incubated at 37 °C in an anaerobic chamber where after isolation, the bacteria were identified through several biochemical tests. Based on research conducted, a single colony of methanogenic bacteria is gram negative bacterium where the results of the isolates show that the bacteria of the *Methanobacterium* genus. The highest biogas production was obtained from the addition of 15%v/v of bacterial isolate. It can be seen from the volume of biogas produced with the volume 40 mL for 14 days of the fermentation process.

Keywords: Biogas production · Cow manure · Identification · Isolation · Methanogenic bacteria

1 Introduction

Cow manure is an organic waste produced from the rest of the metabolism of cattle that is no longer used [1]. Cow manure contains 14.05–19.00 hemicellulose (%), 15.31–29.00 cellulose (%), and 13.97–16.00 lignin (%) [2]. In one day, each adult cow can produce as much as 20–30 kg of solid waste and 100–150 L of liquid waste [3]. The total cattle production in Indonesia is 177.6 million kilograms per day [1].

This livestock waste will increase along with the increase of livestock business in Indonesia. Therefore, alternative organic waste processing especially in cow manure is needed to obtain more valuable debris, namely, processing cow manure into biogas through an anaerobic digester [5, 6].

The biochemical reaction of organic matter into biogas is divided into three stages, namely the hydrolysis stage, the acid formation stage (acidogenesis, acetogenesis), and

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the methane formation stage (methanogenesis) [7]. Each step is carried out by the cooperation of various obligate and facultative anaerobic microorganisms who helped produced methane in those stages. The biochemical reaction of organic matter into biogas is divided into three stages, namely the hydrolysis stage, the acid formation stage (acidogenesis, acetogenesis), and the methane formation stage (methanogenesis) [7], where each step is carried out by the cooperation of various obligate and facultative anaerobic microorganisms. The biogas produced in the anaerobic digester is closely related to these microorganisms to assist or catalyse the biogas formation process [8]. If one category of microorganisms inhibits work, the process of biogas formation in the anaerobic digester can also be hampered [9].

In practice, the biogas production process from the cow manure digester takes time because the microorganisms that convert cow manure into biogas are limited. In addition, the process is conducted only by microorganism that requires anaerobic digester substrate. Microorganisms' activity influences the rate of biogas production in the anaerobic digester [10]. The higher the action of microorganisms, the higher the rate of biogas production in the anaerobic digester.

Different proportions of methane gas were obtained from the addition of isolated bacteria grown on NA (Nutrient Agar) and RGCA (Rumen fluid-glucose-cellobiose agar) media, which were tested using a Gas Chromatography (GC) tool [11]. The proportion of methane gas on the fourteenth day on the RGCA media increased by 10.47%, while the NA medium decreased on the fourteenth day to 1.06%. The proportion of methane gas is influenced by the biomass of methanogenic bacteria contained in it.

In these studies, the isolation of methanogenic bacteria from cow manure will be carried out, which plays a role in the biogas production process in the anaerobic digester of cow manure and identify the phenotype of the isolated bacteria. Methanogenic bacteria were obtained by isolating microorganisms from the anaerobic digester of cow manure using the pour plate method in an obligate anaerobic environment. The addition of methanogenic bacteria isolated from cow manure is expected to increase the rate of biogas production.

2 Materials and Methods

2.1 Microorganism Isolation

The isolation stage of microorganisms begins by taking 1 mL of cow manure filtrate, mixing it with 99 mL of physiological solution, and then homogenizing it. The dilution series used for the solution were 10^{-4}, 10^{-6}, and 10^{-8}. Microorganisms are carried out in a serum bottle made of glass equipped with a rubber stopper and an aluminum cap. Conditions in the serum bottle are maintained to prevent the entry of oxygen into the growth medium of microorganisms. Isolation of microorganisms was carried out under environmental conditions without oxygen in the anaerobic chamber. This tool is in the form of a closed, clear glass box connected to a Nitrogen (N_2). In the device, there is also a valve that functions to open the lid of the nitrogen flow. Nitrogen will be flowed continuously during the isolation of microorganisms to create oxygen-free conditions. Anaerobic chamber sterilization using 70% alcohol and UV light.

Each dilution was taken as much as 1 mL and inoculated on fluid thioglycollate media added to agar in a serum bottle. The serum bottle containing the sample of microorganisms was fed with nitrogen gas for five minutes to remove the oxygen that had entered the bottle. Samples were inoculated using the pour plate method and incubated on a hot plate at 37 °C (48 h).

2.2 Characterization

The identification was carried out to determine the genus and the characteristics of the isolated methanogenic bacteria. Identification was conducted by observing the colony morphology, Gram staining, and several biochemical tests such as indole test, nitrate test, sugar fermentation, gelatine hydrolysis, casein hydrolysis, MR test, VP test, and nitrate test. The identification result then were compared with the Bergey's Manual of Determinative Bacteriology [12].

2.3 Biogas Production Test

Cow manure was used in a 2:1 ratio of water and cow manure, then it was mixed into 250 mL anaerobic digester with various additions of methanogenic bacteria isolates 0%v/v, 5%v/v, 10%v/v, and 15%v/v to determine the effect of adding bacterial isolates to the volume of biogas produced.

3 Result and Discussion

3.1 Methanogenic Bacteria Isolation

Methanogenic bacteria were isolated using a thioglycolate medium with agar added. Isolation was carried out by incubating the media and isolates in an anaerobic chamber at 37 °C. From the bacterial isolation process, one colony was obtained from the anaerobic digester of cow manure with a dilution of 10^{-16}. The isolates were then observed macroscopically and microscopically. Several parameters of macroscopic morphological observations included colony shape, surface, colony edge, colony color, and colony size.

Morphology of the bacterial isolates obtained spherical in shape, the surface of the colony is raised, with flat edges, white and small in diameter. Macroscopic and microscopic observations of the morphological characteristics of bacterial colonies need to be carried out to facilitate bacterial identification.

The principle of bacterial isolation is to separate or move certain bacteria from their environment, which is then grown on artificial media to obtain pure cultures. The sample needs to be diluted to reduce the number of isolated microbes to be observed later and the specific number of microorganisms known. In addition, sample dilution can facilitate colony calculations.

The gram staining results showed that the colonies of methanogenic bacteria isolated from the anaerobic digester of cow manure were Gram-negative bacteria. Gram-negative bacteria have a cell wall structure with a high lipid content, making it difficult to maintain

Table 1. Methanogenic bacteria colony biochemical test results from cow manure anaerobic digester

No.	Biochemical Test	Biochemical Test Result
1	Catalase Test	–
2	Indole Test	+
3	Starch Hydrolysis	+
4	Casein Hydrolysis	+
5	Gelatinase Hydrolysis	+
6	Carbohydrates Fermentation	+
7	MR Test	+
8	VP Test	–
9	Citrate Test	+

the crystal violet color. Alcohol solvents used in gram staining can easily damage the outer membrane of gram-negative bacteria, which have a relatively thin peptidoglycan layer to not retain the dye.

3.2 *Methanogenic* Bacteria Identification

Identification of bacteria using biochemical tests was carried out to determine the physiological properties and bacterial species of the bacterial isolates obtained. This physiological property is related to the metabolic processes that occur in the cells of a bacterium, where each bacterium has different biochemical activities. Differences in protein and fat metabolism, carbohydrate metabolism, enzyme production, ability to utilize compounds, and others are some of the factors that help identify bacteria according to their physiological properties. In this biochemical test, nine tests were used, namely indole test, nitrate test, catalase test, citrate test, sugar (glucose) fermentation, gelatin hydrolysis, casein hydrolysis, starch hydrolysis, and Methyl Red and Voges Proskauer (MR-VP) tests (Table 1).

Methanogenic bacteria are bacteria that produce methane gas from hydrogen gas and CO_2 or acetic acid. Identification of bacteria is carried out in various biochemical tests to determine the species, where this biochemical test is an advanced stage of activity after knowing the cell shape and Gram staining of the isolated bacteria. From the results of biochemical tests, it can see what microorganisms possess reactions in their growth metabolism. Observation of metabolic activity is known from the ability of isolated bacteria to use and decompose complex molecules and simple molecules whose results will be used for bacterial identification.

Positive results on the indole test conducted by bacterial isolates indicated that these bacteria could hydrolyse the tryptophan present in methane and other gases formation. In addition, a positive result of the MR test indicates the presence and activity of methanogenic bacteria in the acid phase during biogas production. The positive test shown in the citrate test suggests the presence of citrate, which breaks down citrate into oxaloacetate and acetate, which is further converted into pyruvate and carbon dioxide, which produces ammonia with sodium citrate during biogas production.

A negative result on the catalase test indicates an obligate anaerobic bacterium, a characteristic of methanogenic bacteria. Furthermore, the casein hydrolysis and gelatin hydrolysis of bacterial isolates showed positive results in their biochemical tests. This indicates that the isolates can use the proteins in the trial [13, 14].

3.3 Biogas Production Test

Anaerobic digester of cow manure with the addition of 5% v/v, 10% v/v, and 15% v/v of methanogenic bacteria isolates started producing biogas on the third day with an additional volume of 1 mL, in contrast to the anaerobic digester of cow manure without the addition of bacterial isolates, which only started biogas production on the fourth day. The variation with the addition of 15% of methanogenic bacteria isolates produced the highest biogas volume of 12 mL on the ninth day, in contrast to the anaerobic digester with the addition of 10% isolates which had the highest biogas volume of 5 mL on the eleventh day. Meanwhile, in the digester with a 5% methanogenic bacteria accumulation variation, isolates produced a biogas volume of 4 mL on the eleventh day.

In the anaerobic digester of cow manure without adding methanogenic bacteria isolates, the total volume of biogas produced during 14 days of fermentation was 20.5 mL. Then the anaerobic digester with the addition of 5% methanogenic bacteria isolates produced a biogas volume of 28.5 mL. The accumulation of 10% methanogenic bacteria isolates produced a biogas volume of 31.5 mL, and the addition of 15% methanogenic bacteria isolates made a biogas volume of 40 mL.

The results of the biogas production test showed that the addition of methanogenic bacteria isolates affected the biogas production produced. The results of the biogas production test showed that the acquisition of 15% isolates could accelerate the formation of biogas by nine days, compared to an anaerobic digester without the addition of isolates. This was caused by adding the correct number of isolates to increase bacterial activity and gas production.

In the hydrolysis reaction, bacteria decompose organic matter and existing complex compounds into simpler compounds and change polymer compounds (glucose, fat, and protein) into monomer compounds (fatty acids, monosaccharides, amino acids) that no biogas is formed.

The results of the biogas production test with the addition of methanogenic bacteria isolates showed a peak in the accumulation of isolates with a variation of 15%. The rise shows an increase in biogas volume of 12 mL on the ninth day. The addition of a starter (EM-4) can accelerate the formation of biogas in a shorter time. The presence of other bacteria from the variation of the addition of 15% isolates in the anaerobic digester functions as a biocatalyst that can help the process of biogas formation. The addition of bacterial isolates with a variation of 15%v/v resulted in the volume of biogas with the highest quantity of 40 mL compared to other isolates.

In addition, environmental temperature plays an essential role in the growth of microorganisms in the anaerobic digester, which is related to the ability of bacteria to live in processing organic matter into biogas. The optimum temperature for the growth of anaerobic methanogenic bacteria is 25 °C–40 °C [15]. Therefore, the addition of methanogenic bacteria isolates from the results of bacterial isolation in an anaerobic digester of cow manure with a working temperature of 37 °C can indicate that bacterial isolates can accelerate bacterial activity, as seen from the increase in the volume of biogas produced.

Biogas production is one way that can be used to treat waste into something more useful. One of many problems in the biogas production is the length rate of the biogas production, that so we need something to boost the rate of production.

Isolation of microorganisms was performed from the anaerobic digester of cow manure. Characterization of the bacterial isolates was carried out and had characteristics such as the genus of Methanobacterium. The addition of methanogenic bacteria colonies into the anaerobic digester affected the volume of biogas, especially 15%v/v of bacterial isolates of the methanobacterium genus, which produced 40 mL of biogas volume.

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