

Ecological Diversity of Microbial Consortium Feces of Beef Cattle and Lignite Coal

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ABSTRACT

Increasing energy demand is not alongside the availability of limited fossil fuels. Alternative and renewable energy sources are not only an option to overcome energy problems but also essential to minimize global warming. Another critical and promising renewable energy source is biomass-derived from livestock feces. Beef cattle feces contain a microorganism consortium that can be used as a starter with coal media to form biogas. Indonesia recently developed coal waste processing into renewable energy, such as biogas. This study aimed to overview the ecological diversity of microbial consortium of beef cattle feces, lignite coal waste, and a combination of livestock and lignite coal waste under mesophilic conditions. This research is an explorative method, the data obtained were analyzed descriptively. The process of formation was carried out anaerobically on a bottle containing the rumen fluid medium. The fermentation process lasted 42 days at 39°C of temperature. After that, the sample was electrophoresis, followed by next-generation sequencing (NGS) method. NGS data is processed with the MG-Rast website. This study demonstrates the ecological diversity of microbial consortium of beef cattle, lignite coal waste, and a combined consortium. The results showed ecological diversity in the form of taxonomy dominated by bacteria, eukaryotes, and archaea.

1. Introduction

Beef cattle feces is a waste that has not optimally used. If not managed properly, it will pollute the river environment is to increase Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and reduce dissolved oxygen (DO) and can cause greenhouse gases. Feces is a livestock waste that contains organic materials that can be converted into energy sources in biogas form (Olvera and Lopez-Lopez 2012; Pap and Maróti 2016). Facilitate biogas formation; beef cattle feces can be handled by culturing beef cattle feces consortium as biogas starter with lignite coal media. Microorganisms in feces come from the digestive tract of beef cattle such as *Enterobacter*. Bioaugmentation and biostimulation process can increase the amount of microorganism from feces beef cattle and coal more than 10¹⁰ CFU/ml so that can be used as biogas starter.

Biogas production can be carried out under different temperature process that affected biogas formation, such as competition of microbial community and process stability (Stolze Y *et al.* 2015). The biogas formation process is divided into four continuous and simultaneous phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. A community of complex anaerobic microbes performs all of those phase. The first three phases are carried out by bacteria, while the last phase is performed by archaea (Jünemanna *et al.* 2017). Biogas component is methane (CH₄), carbon dioxide (CO₂), nitrogen (N₂), hydrogen (H₂), oxygen (O₂), and hydrogen disulfide (H₂S) (Ohmiya K *et al.* 2005; Stolze Y *et al.* 2015).

Lignite coal is one type of coal that has calorific value 4,000-8,300 Btu/lb, 10-50% ash, 23-35% carbon, 0.4-1.0% sulfur, and 120±20 ppm chlorine (Brian *et al.* 2008). Most of the low range of Indonesia's coal consists of hydrogen-rich component (Davis RC *et al.* 2007), so that has a good potential as substrate to utilize directly for biogenic CH₄ generation from various starter such as beef cattle feces consortium,

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lignite coal waste, and combination of beef cattle feces consortium and lignite coal waste. Based on various starter used, the diversity of microbes form during incubation period for 42 days in 39°C can be observed. However, most of these microbes and their role in biogas production are not discover yet. A good understanding of the composition and activities of diverse microbial communities is very important to optimize the fermentation process. To find out the diversity of microorganisms in the sample, molecular methods are used. The molecular method used is Next Generation Sequencing (NGS) technology. NGS-based metagenomics explains the analysis of the genomic content of a whole microbial community. It provides insight into its structure and function, resulting in information about organisms that are not easily cultured (Jünemanna *et al.* 2017). NGS is the latest applied science technology tool. This tool has a great ability in produce DNA sequences throughout the genome of an organism. NGS technology can also quickly capture the variety and number of microorganisms from a genomic environment in the microbial community. Data generated through NGS is assisted by analysis using MG-Rast servers. The MG-Rast server analyzes each metagenome dataset involving a comparison of known sequence data. In this research, the biogas digester's microbial community with starter microbial beef cattle feces and microbial starter of coal were analyzed by applying the metagenomic approach. The results obtained were compared with taxonomic community profiles inferred from the biogas digester with a combined microbial starter from beef cattle and coal feces.

2. Materials and Methods

This study was explorative research, which consists of research conducted on the growth of biogas bacteria from a consortium of beef cattle feces, lignite coal waste and a mixture of a consortium of beef cattle feces, and lignite coal waste carried out in the laboratory scale. This study using molecular

analysis with Next Generation Sequencing method as a tool to see the diversity of microorganisms. The results of data analysis from the NGS were analyzed using the MG-Rast data server. In this study, the data discussed is taxonomic data based on domain, phylum, class, and genus. The data will be analyzed descriptively. The material used for this research were beef cattle feces taken from teaching farm Padjadjaran University, lignite coal from Berau, East Kalimantan, Indonesia, and rumen fluid from Cikuda slaughterhouse, Jatinangor.

The research process is by *in vitro* culturing beef cattle consortium and anaerobic adaptation to serum bottles containing rumen media (Ogimoto and Imai 1981) for two weeks. After two weeks, a starter is ready used for biogas fermentation. Fermentation was carried out on three serum bottles (as digester) with three replications. The first serum bottle is filled with liquid media and a consortium of beef cattle feces, the second bottle is filled with liquid media, and lignite coal waste and the third serum bottle is filled with a combination of beef cattle feces consortium and new lignite coal waste. The use of starter consortium of beef cattle feces is used by 4% of 100 ml of liquid media. The use of coal as much as 10% of 100 ml of liquid media. Samples were incubated at 39°C for 42 days. Then DNA extraction was then carried out by electrophoresis and continued with the next generation sequencing (NGS) method. The NGS data obtained were processed with the MG-Rast website (De Francisci *et al.* 2015). After obtaining the results of data through MG-Rast, the data is analyzed descriptively.

3. Results

This study analyzed the diversity of biogas forming microorganisms from the consortium of beef cattle feces, lignite coal waste, and a mixture of beef cattle feces consortium and lignite coal waste.

Table 1 showed that based on domains, microorganisms detected were dominated by bacteria as follows 99.59% in the microbial consortium of beef

Table 1. The NGS result based on domain

Taxonomy	Source		
	Beef cattle feces microbial consortium	Lignite coal waste	Beef cattle feces microbial consortium and lignite coal
Domain	Bacteria (98.89%)	Bacteria (99.68%)	Bacteria (98.57%)
	Eukaryota (1.05%)	Archaea (0.18%)	Archaea (1.34%)
	Archaea (0.05%)	Eukaryota (0.14%)	Eukaryota (0.09%)
	Unclassified sequences (0.01%)		Unclassified sequences (0.00%)
	Viruses (0.00%)		

cattle feces, 99.68% in lignite coal waste, and 98.57% in mixed feces of beef cattle and lignite coal waste.

Table 2 showed the analysis result on the phylum of samples. Based on phylum, the three samples were dominated by *Firmicutes*, as much as 72.58% in beef cattle feces consortium, 89.94% in lignite coal waste, and 97.65% in the mixture of beef cattle feces and lignite coal waste consortium.

Table 3 Showed the analysis result in class. In the sample of beef cattle feces consortium were dominated by *Bacilli* as much as 67.69% while in lignite coal waste and the mixture of beef cattle feces

consortium and lignite coal waste was dominated by *Clostridia* as much as 47.55% and 70.96%.

4. Discussion

The growth process of microorganisms influenced by environmental conditions, substrate, temperature, and pH. The presence of microorganisms formed greatly influenced the biogas formation. Taxonomy on the three samples was obtained by using the MG-Rast server (Table 1). Table 1 showed that based on domains, microorganisms detected were dominated by bacteria

Table 2. The NGS result based on phylum

Taxonomy	Source		
	Beef cattle feces microbial consortium	Lignite coal waste	Beef cattle feces microbial consortium and lignite coal
Phylum	<i>Firmicutes</i> (72.58%)	<i>Firmicutes</i> (89.49%)	<i>Firmicutes</i> (97.65%)
	<i>Synergistetes</i> (22.57%)	<i>Synergistetes</i> (5.58%)	<i>Euryarchaeota</i> (1.35%)
	<i>Arthropoda</i> (1.10%)	<i>Proteobacteria</i> (1.82%)	Unclassified (derived from Bacteria) (0.16%)
	<i>Proteobacteria</i> (1.03%)	<i>Planctomycetes</i> (1.69%)	<i>Planctomycetes</i> (0.15%)
	<i>Cyanobacteria</i> (0.096%)	<i>Verrucomicrobia</i> (0.53%)	<i>Tenericutes</i> (0.12%)
	Unclassified (derived from bacteria) (0.78%)	Unclassified (derived from bacteria) (0.23%)	<i>Proteobacteria</i> (0.12%)
	<i>Actinobacteria</i> (0.34%)	<i>Euryarchaeota</i> (0.18%)	<i>Elusimicrobia</i> (0.09%)
	<i>Bacteroidetes</i> (0.24%)	<i>Bacteroidetes</i> (0.18%)	<i>Actinobacteria</i> (0.08%)
	<i>Elusimicrobia</i> (0.13%)	<i>Actinobacteria</i> (0.15%)	<i>Bacteroidetes</i> (0.06%)
	<i>Lentisphaerae</i> (0.13%)	<i>Arthropoda</i> (0.11%)	<i>Arthropoda</i> (0.06%)
	<i>Euryarchaeota</i> (0.06%)	<i>Lentisphaerae</i> (0.06%)	<i>Verrucomicrobia</i> (0.04%)
	<i>Chloroflexi</i> (0.04%)	<i>Streptophyta</i> (0.03%)	<i>Acidobacteria</i> (0.04%)
	<i>Streptophyta</i> (0.04%)	<i>Elusimicrobia</i> (0.00%)	<i>Streptophyta</i> (0.03%)
	Unclassified (derived from unclassified sequences)	<i>Spirochaetes</i> (0.00%)	<i>Synergistetes</i> (0.03%)

Table 3. The NGS result based on class

Taxonomy	Source		
	Beef cattle feces microbial consortium	Lignite coal waste	Beef cattle feces microbial consortium and lignite coal
Class	<i>Bacilli</i> (67.69%)	<i>Clostridia</i> (47.55%)	<i>Clostridia</i> (70.96%)
	<i>Synergistia</i> (22.7%)	<i>Bacilli</i> (42.18%)	<i>Bacilli</i> (25.84%)
	<i>Clostridia</i> (3.18%)	<i>Synergistia</i> (5.68%)	<i>Negativicutes</i> (1.87%)
	<i>Negativicutes</i> (1.54%)	<i>Deltaproteobacteria</i> (1.82%)	Unclassified (derived from Bacteria) (0.20%)
	<i>Insecta</i> (1.11%)	<i>Planctomycetacia</i> (1.72%)	<i>Planctomycetacia</i> (0.18%)
	Unclassified (derived from <i>Cyanobacteria</i>) (0.96%)	Unclassified (derived from bacteria) (0.24%)	<i>Mollicutes</i> (0.15%)
	<i>Deltaproteobacteria</i> (0.87%)	<i>Actinobacteria</i> (0.16%)	<i>Elusimicrobia</i> (0.11%)
	Unclassified (derived from bacteria) (0.79%)	<i>Bacteroidia</i> (0.15%)	<i>Actinobacteria</i> (0.10%)
	<i>Actinobacteria</i> (0.34%)	<i>Negativicutes</i> (0.11%)	<i>Gammaproteobacteria</i> (0.10%)
	<i>Bacteroidia</i> (0.16%)	<i>Insecta</i> (0.11%)	<i>Insecta</i> (0.08%)
	<i>Elusimicrobia</i> (0.13%)	<i>Verrucomicrobiae</i> (0.07%)	<i>Bacterioidia</i> (0.07%)
	Unclassified (derived from <i>Lentisphaerae</i>) (0.10%)	Unclassified (derived from <i>Lentisphaerae</i>) (0.06%)	<i>Methanobacteria</i> (0.07%)
	<i>Flavobacteria</i> (0.07%)	<i>Spartobacteria</i> (0.03%)	<i>Verrucomicrobiae</i> (0.05%)
		<i>Liliopsida</i> (0.03%)	<i>Solibacteres</i> (0.04%)

as follows 99.59% in the microbial consortium of beef cattle feces, 99.68% in lignite coal waste, and 98.57% in mixed feces of beef cattle and lignite coal waste. The presence of bacteria in the process of biogas formation was essential, beginning with the hydrolysis process as the first step in the degradation of complex organic substrates into a simple substrate. During hydrolysis, complex biopolymers such as cellulose, starch, proteins, and lipids were degraded to oligo and monomers by hydrolytic bacteria from the *Bacteroidetes* phylum and *Firmicutes* (Drake *et al.* 2002).

Table 2 showed the analysis result on the phylum of samples. Based on phylum, the three samples were dominated by *Firmicutes*, as much as 72.58% in beef cattle feces consortium, 89.94% in lignite coal waste, and 97.65% in the mixture of beef cattle feces and lignite coal waste consortium. Phylum *Firmicutes* had an essential role in the hydrolysis of biomass and in the fermentation process. *Firmicutes* were very important in cellulose degradation by the microbial community of biogas (Jaenicke *et al.* 2011). *Firmicutes* included all gram-positive bacteria. *Firmicutes* consisted of 26 families and 223 genera (Gibbons and Murray 1978). Forms of phylum *Firmicutes* cells were round, or straight, curved and helical or filaments with or without flagella and with heat-resistant or endospores (Bergey and Boone 2009). It could live under aerobic, facultative, or anaerobic conditions. Some members of the *Firmicutes* were thermophilic and or halophil bacteria. Most of *Firmicutes* were anoxygenic chemo-organotrophs. Most grew at neutral pH, while some were acidophiles or alkalyphilic (Bergey and Boone 2009).

The results of the class were slightly different (Table 3). In the sample of beef cattle feces consortium were dominated by *Bacilli* as much as 67.69% while in lignite coal waste and the mixture of beef cattle feces consortium and lignite coal waste were dominated by *Clostridia* as much as 47.55% and 70.96%, respectively. *Bacilli* and *Clostridia* classes were important bacteria that contribute to the decomposition of organic substrates. The results showed the importance of microbial communities metabolizing hydrogen in biogas formation (Wirth *et al.* 2012). The *Bacilli* class was generally gram-positive, and some grow under facultative anaerobic conditions (Bergey and Boone 2009). The *Clostridia* class contained gram-positive bacteria and negative gram stains. The class consisted of order *Clostridiales*, *Halanaerobiales*, and *Thermoanaerobacterales* (Bergey and Boone 2009).

In mixed sources of beef cattle feces consortium and lignite coal waste can be seen as a class of 0.07% *Methanobacteria*. *Methanobacteria* includes one anaerobic non-halophilic and thermophilic archaeobacteria. The special characteristic of methanogen groups is autotrophic or heterotrophic, the energy produced from oxidation of hydrogen, forms acetate with methane formation. Cells form balls, rods, and spirals, and are gram negative or positive.

In the MG-Rast analysis based on the genus, it showed that microbial consortium of beef cattle feces dominated by 64.34% *Bacillus*, followed by *Pyramidobacter* 25.28%, unclassified (derived from Bacteria) 2.48%, *Syntrophomonas* 1.35%, *Desulfovibrio* 0.97%, *Clostridium* 0.86% (Figure 1). Data based on the genus indicated that bacteria formed during 42 days of fermentation fluctuated, the dominance of bacteria presented in the hydrolysis stage. The bacteria contained in the hydrolysis stage were *Bacillus* and *Clostridium* (Wirth *et al.* 2012). This was in accordance with (Bryant and Burkey 1976) that microbes that had a role in the hydrolysis process were *Clostridium aceticum*, *Bacteriodes ruminicola*, *Bifidobacterium* sp., *E. coli*, *Enterobacter* sp., *Desulfurivibrio* sp., *Pseudomonas* sp., *Flavobacterium alkaligenes*, and *Aerobacter*. Observations based on the genus found *Lactobacillus* and *syntrophomonas* bacteria, which showed the acidogenesis process. Similarly, (Bryant and Burkey 1976) suggested that microbes that had a role in the acetogenesis were *Syntrophobacter* sp. and *Pelotomaculum* sp.

Based on MG-Rast result, the genus found in coal waste dominated by *Clostridium* 51.28%, followed with *Bacillus* 35.29%, *Pyramidobacter* 6.13%, *Desulfovibrio* 2.04%, *Planctomyces* 1.38%, and *Syntrophomonas* 0.56% (Figure 1). This was consistent with (Susilawati 2015), who stated that bacterial species from the *Clostridium* genus were geopolymer hydrolyzers in biogenic Coal Bed Methane (CBM) reservoirs. This showed that after applying the treatment and growing bacteria in waste, it still showed biogenic coal. The study results on beef cattle feces and lignite coal consortium were almost the same because the hydrolysis stage bacteria still dominated it.

Genus found in mixture of beef cattle feces consortium and lignite coal, namely *Clostridium* (72.37%), *Bacillus* (31.67%), *Lysinibacillus* (0.89%), *Thermosinus* (0.63%), *Caldicellulosiruptor* (0.39%), and *Lactobacillus* (0.29%). *Clostridium* genus in the

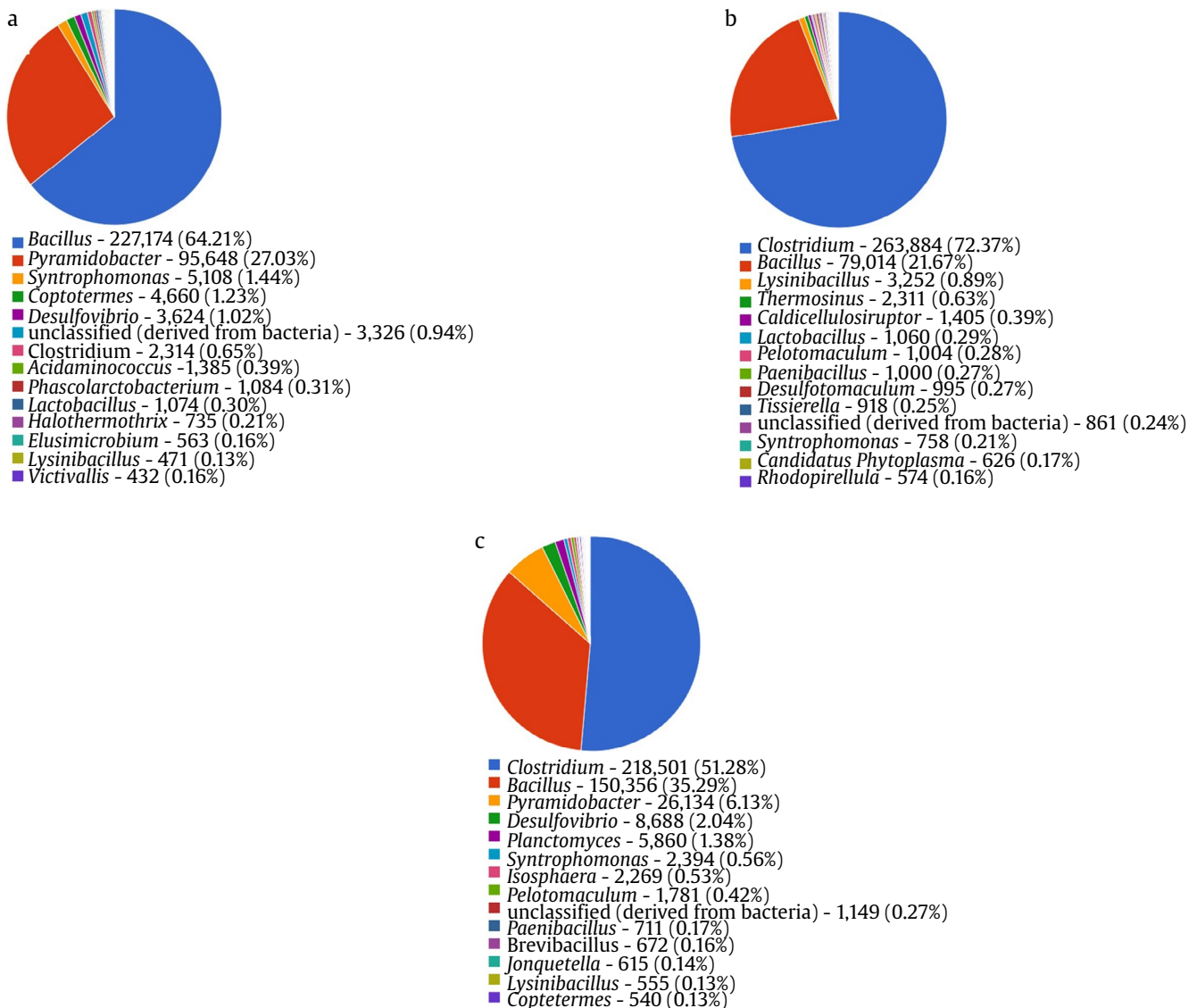


Figure 1. The NGS result based on genus. (a) The MG-Rast result on beef cattle feces consortium, (b) The MG-Rast result on lignite coal waste, (c) The MG-Rast result on mixture of beef cattle feces consortium and lignite coal waste. It can be seen that there was data fluctuation particularly in starter from coal waste microbes.

early stages of growth was generally gram positive, although in some species gram negative. When motile, it was commonly Peritrichous, the majority of species were oval or round endospores, which inflated cells. It generally produced a mixture of organic acids and alcohol from carbohydrates or peptones. It could metabolize carbohydrates, alcohols, amino acids, and organic compounds (Bergey and Boone 2009).

Genus dominated in lignite coal waste, and a mixture of beef cattle feces and lignite coal were *Clostridium* and *Bacillus*, while in beef cattle feces consortium was *Bacillus*. Genus *Clostridium* and

Bacillus were identified most dominant under mesophilic conditions.

5. Conclusion

The results of the metagenomic NGS data processed through MG-Rast can show ecological diversity of mesophilic temperature from the microbial consortium of beef cattle feces, microbes from coal and mixed starters which are complex starters, difficult to culture but can symbiosis to produce biogas according to the role of each microbe

in every phase. The exogenous anaerobic microbes used in this study and the original microbes are basically the same, at least at the phylum level.

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