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THE ROLE OF BACTERIAL AND ARCHAEA IN DETERMINING THE METABOLIC PATHWAY OF BIOGAS FERMENTATION AT LOW TEMPERATURES

Papel de las bacterias y las arqueas en la determinación de la vía metabólica de la fermentación del biogás a baja temperatura

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ABSTRACT

The challenge in achieving large-scale biogas production still lies in the biogas fermentation process at low temperatures. Our goal was to delve into the metabolic pathway behind the formation of biogas at these lower temperatures, focusing on the dominant bacterial and archaeal communities. Employing a batch system with activated sludge inoculum at 10°C, we fermented cow manure at 12°C for 150 days. Through genetic sequencing and taxonomic analysis using OTUs from the 16S rDNA gene, we investigated bacterial and archaeal species. Correlation analysis between their abundance was conducted using Pearson correlation and t-tests via IBM SPSS Statistics. Our findings revealed a biogas production of around 0.74 L/day, with CH_4 levels surpassing 0.45 L/g VS. Peak efficiency occurred between day 60 and 110, reaching its apex on day 90. *Clostridium cellulovorans* dominated, ranging from 13.9% to 27%, followed by *Terrisporobacter petrolarius*, around 16.2% to 23%. Specifically, the formation of biogas (CH_4) predominantly occurred through the H_2 pathway, led by significant hydrogenotrophic Archaea OTUs like *Methanocorpusculum sinense* (ranging from 4.95% to 37.10%) and *Methanobrevibacter millerae* (with relative abundances between 2.00% and 11.20%)

Keywords: Environmental condition, Hydrolytic bacteria, Methane formation pathway, Microbial metabolic function.

RESUMEN

El reto para lograr la producción de biogás a gran escala sigue residiendo en el proceso de fermentación de este a bajas temperaturas. Nuestro objetivo fue profundizar en la ruta metabólica que subyace a la formación de biogás a bajas temperaturas, centrándonos en las comunidades bacterianas y arqueas dominantes. Empleando un sistema discontinuo con inóculo de lodos activados a 10°C, fermentamos estiércol de vaca a 12°C durante 150 días. Mediante secuenciación genética y análisis taxonómico utilizando OTU del gen 16S rDNA, investigamos las especies bacterianas y arqueas. El análisis de correlación entre su abundancia se llevó a cabo mediante la correlación de Pearson y pruebas t a través de IBM SPSS Statistics. Nuestros resultados revelaron una producción de biogás de alrededor de 0,74 L/día, con niveles de CH_4 superiores a 0,45 L/g VS. El pico de eficiencia se produjo entre los días 60 y 110, alcanzando su ápice el día 90. Predominó *Clostridium cellulovorans*, con un rango del 13,9% al 27%, seguido de *Terrisporobacter petrolarius*, con un rango del 16,2% al 23%. En concreto, la formación de biogás (CH_4) se produjo predominantemente a través de la vía del H_2, liderada por importantes OTU de arqueas hidrogenotróficas como *Methanocorpusculum sinense* (entre el 4,95% y el 37,10%) y *Methanobrevibacter millerae* (con abundancias relativas entre el 2,00% y el 11,20%).

Palabras clave: Bacterias hidrolíticas, Condiciones ambientales, Función metabólica microbiana, Vía de formación del metano.

INTRODUCTION

The processing and utilization of organic waste to be used as biogas is a support for achieving a new and renewable energy mix. The Indonesian government is trying to find alternative energy sources to reduce dependence on petroleum energy. Utilization of domestic waste of 21.88 million tons/year is a challenge for the government to create biogas as renewable energy while reducing environmental pollution.

The Ministry of Environment and Forestry in 2021 noted that household domestic waste contributed significantly (85 %). Support also came from the Ministry of Energy and Mineral Resources, which succeeded in building 25.157 biodigester units in various regions. The selected area has low humidity (RH<50 %) and high temperature (28-35°C). The selection of these places is an ideal condition for the growth of fermenting microorganisms.

Domestic waste found in areas with a temperature of < 20°C sometimes must be transported to the biodigester, which requires time and money in the transportation process. A breakthrough is needed to establish waste treatment at low temperatures so that there are no waste distribution costs. A quarter of Indonesia has a low temperature (10-20°C) so that the biogas potential in that area needs to be optimized.

The process of biogas formation is more efficient at high temperatures (Guo et al., 2019) but most of the earth's surface is cold (De Maayer et al., 2014). Low temperature is a major obstacle in the development of large-scale biogas. Thus, research related to the development of efficient biogas at low temperatures needs much attention.

Closing the research gap so far, we want to analyze the metabolic pathways for biogas formation at low temperatures (12°C). The sample we used was cow manure with activated sludge inoculum which was stored for a long time at 10 °C. The fermentation process is carried out through a batch system at a temperature of 12°C. We observed active microorganisms at low temperatures in the early stages. The next stage is determining the dominant population of bacteria and archaea as well as testing its correlation with environmental factors.

The purpose of this study was to analyze the metabolic pathways of biogas formation based on the dominant population of bacteria and archaea. This will give an idea of which species play the main role in the biogas metabolism process at low temperatures. This research provides preliminary guidance for the efficient development of biogas at low temperatures for the future.

MATERIALS AND METHODS

Raw material

The raw material used in this research is cow dung obtained from cattle farms in Jambangan village, Kawedanan sub-district, Magetan, East Java, Indonesia. The sample had a volatile solid content (VS) of 80.5 % and a total solid content (TS) of 21.20 %. The biogas fermentation process was carried out using an inoculum in the form of activated sludge which was stored at a low temperature (10 °C) for a long time. The inoculum had a TS of 6.43 % and a VS of 61.38 %.

Fermentation process

The fermentation system was divided into three parts, namely: cooling system, fermentation system, and storage system. The cooling system used a water chiller type FL-0250 (5-35°C), and Wuxi Guanya Refrigeration Technology Co. Ltd. (LNEYA). The fermentation reactor was made of glass with a volume of around 15 L. The fermented biogas was stored in a low-pressure wet gas storage cabinet with a volume of 8 L.

The fermentation process was carried out at a constant temperature of 12 °C with a batch system. The sample:inoculum ratio was 0.75:1 (VS/VS). In this study, the amount of inoculum was 300g (VS) and cow manure was 225g (VS). Then, this amount was added with water to a final volume of 10 L. Sampling was carried out every ten days for non-biological analysis such as: TS, VS, COD, NH_2-N, pH, Volatile Fatty Acid (VFA), and samples for biological analysis were stored at temperature -20°C. Biogas was stored in gas storage which is equipped with a volumetric meter scale.

Non-biological analysis

Biogas analysis was carried out every ten days. The composition of biogas (methane, CH_4 and CO_2) and ammonia nitrogen were analyzed using gas chromatography (GC2400; Perkin Elmer Inc.). TS determination was carried out using an air-drying oven equipped with a thermostat (DON-IF45; Bioevopeak Co., Ltd). Samples were put into 50-100 g cups and weighed, then dried in the oven at 100-105 °C for 1 hour. This was repeated until a stable dry weight was obtained. VS analysis using a box-shaped high-temperature resistance furnace (SRJX-8-13; Changzou Zhongjian Precision Instrument Co., Ltd). analysis was carried out at 550-600 °C.

VFA analysis was carried out through the Volatile Fatty Acids Analyzer (EZ7200; Hach Lange GmbH), and COD analysis was carried out through the Fast and Portable COD Analyzer (PeCOD L50; Mantech Inc.). pH was analyzed using a pH meter (S400-STD-Kit; Mettler Toledo). The instrument was operated according to its instruction manual.

Biological Analysis

DNA Extraction.

Sample DNA was extracted using the ZymoBIOMICS DNA Mini Kit. The procedure used refers to Zymo Research (Irvine, CA, US). DNA levels were tested using electrophoresis on 2 % agarose gel in 1x TAE buffer (20 mM acetic acid, 40

mM Tris base, 1 mM EDTA, pH 7.5). DNA was stained using ethidium bromide (EtBr) 0.1 %, and visualization using UV Transilluminator. DNA extracts were stored at -20 to -80 °C

Microbial community analysis with Next Generation Sequencing (NGS).

Bacterial and archaea communities were detected using the 16S rDNA gene. NGS analyzes were performed using the Illumina MiSeq Platform (Illumina, San Diego, CA, US). Bacterial and archaeal communities were amplified with primary bacteria 341F/806R (Roggenbuck et al., 2014) and the archaeal primer Arch519F/Arch915R (He et al., 2016). The first stage of PCR aimed to amplify the coverage area of the V4 16S rDNA gene of archaea, V4-V5 of the bacterial 16S rDNA gene at ~1400 bp.

The PCR reactions were predenatured at 98°C (three minutes), denatured at 98°C for 10 seconds, annealing at 55°C at 35 seconds, and elongation 72°C at 40 seconds. Sequencing was performed at ~1400 bp using the Illumina MiSeq platform based on standard Axil Scientific procedures.

DNA Sequence Data Analysis.

DNA analysis included nitrogen base quality, DNA size, and missamplified taxa. High-quality sequences based on the Genomes Online Database (GOLD) use the UCHIME algorithm to detect chimeras, in order to obtain effective tags (Edgar et al., 2011)

Operational Taxonomic Units (OTU).

OTU grouping refers to UPARSE v7.0.1001. The DNA sequences had a nitrogen base similarity sequence of 97 % based on operational taxonomic units (OTU). DNA sequences that often appear in one OTU are called representative sequences. Archaea and bacterial 16S rDNA gene sequences were classified based on taxonomic assignment according to the Greengene database (DeSantis et al., 2006) using the RDP classifier algorithm V2.2.

Correlation analysis

The correlation between the abundance of bacteria and archaea in their respective communities was examined through Pearson correlation, using a two-sided t-test performed on IBM SPSS Statistics version 26 (SPSS Inc., Chicago, IL, USA). Note that ** Correlation is significant at the 0.01 (2-tailed) level; * Correlation is significant at the 0.05 (2-tailed) level.

RESULTS

Fermentation abiotic conditions

The relationship between time and abiotic conditions during the biogas fermentation process at a temperature of 12°C can be seen in (Table 1).

Table 1. Changes in abiotic conditions during biogas fermentation at 12 °C

Day	Total solid (%)	Volatile solid (%)	NH_2-N (mg/L)	pH	CH_3COOH (mg/L)	Propionic acid (mg/L)	Butyric Acid (mg/L)	COD (mg/L)
0	5.80 ± **0.10**	65.50 ± **1.20**	470 ± **120**	7.20 ± **0.03**	1400 ± **140**	260 ± **80**	420 ± **50**	5200 ± **170**
10	5.70 ± **0.15**	65.40 ± **1.40**	704 ± **100**	7.10 ± **0.04**	1750 ± **120**	380 ±**70**	390 ± **50**	5400 ± **200**
20	5.40 ± **0.15**	64.70 ± **1.12**	672 ± **120**	7.10 ± **0.02**	1950 ± **340**	405 ± **80**	370 ± **40**	4902 ± **210**
30	5.20 ± **0.30**	64.60 ± **1.23**	673 ± **124**	7.20 ± **0.06**	2003 ± **240**	420 ± **95**	320 ± **30**	5210 ± **190**
40	5.10 ± **0.20**	64.55 ± **1.25**	670 ± **120**	6.95 ± **0.07**	2300 ± **140**	464 ± **80**	310 ± **30**	4040 ± **270**
50	5.40 ± **0.15**	65.10 ± **1.25**	655 ± **220**	6.90 ± **0.04**	2600 ± **270**	475 ± **80**	260 ± **25**	4045 ± **170**
60	5.25 ± **0.25**	64.90 ± **1.22**	646 ± **120**	6.30 ± **0.09**	2650 ± **180**	560 ± **94**	210 ± **30**	4030 ± **230**
70	5.60 ± **0.10**	64.50 ± **1.20**	657 ± **170**	6.85 ± **0.10**	2800 ± **140**	570 ± **80**	160 ± **30**	4010 ± **220**
80	5.30 ± **0.15**	65.10 ± **1.28**	638 ±**200**	6.65 ± **0.11**	2850 ± **240**	630 ± **84**	125 ± **35**	4044 ± **240**
90	5.20 ± **0.20**	63.00 ± **1.20**	589 ±**170**	6.75 ± **0.12**	1700 ± **140**	690 ± **98**	94 ± **25**	4010 ± **270**
100	4.70 ± **0.20**	63.50 ± **1.01**	540 ±**160**	6.50 ± **0.08**	985 ± **160**	560 ± **78**	85 ± **25**	3020 ± 210
110	4.65 ± **0.24**	63.60 ± **1.20**	535 ±**200**	6.75 ± **0.04**	974 ± **100**	530 ± **78**	64 ± **15**	3300 ± 130
120	4.80 ± **0.22**	63.50 ± **1.23**	530 ±**170**	6.65 ± **0.05**	987 ± **100**	505 ± **80**	57 ± **25**	3150 ± 210
130	4.50 ± **0.20**	63.70 ± **1.20**	525±**200**	6.50 ± **0.06**	958 ± **190**	480 ± **78**	38 ± **10**	3120 ± 110
140	4.70 ± **0.24**	63.40 ± **1.25**	520 ±**110**	6.45 ± **0.07**	940± **185**	484 ± **78**	26 ± **25**	3024 ± 110
150	4.60 ± **0.24**	63.30 ± **1.10**	495 ±**110**	6.40± **0.08**	930 ± **230**	465 ± **98**	14 ± **15**	3010 ± 210

There was a decrease for TS and VS on day ten, indicating that organic matter had started to be consumed by microorganisms. A drastic decrease for TS occurred on day 100, but for VS occurred on day 90. Changes in pH were not too significant. The decline is steady and the low is 6.30. The highest COD content reached 5400ppm on day ten. This condition was reached when the organic solids were hydrolyzed to become smaller and soluble in water. A drastic decrease in COD occurred on day 100.

The highest Ammonia Nitrogen (NH_2-N) value was recorded on day ten, which was 704 mg/L. This is achieved when the greatest degradation of protein into NH_2-N through protease enzymes. The highest decrease in NH_2-N occurred at day 90, and the decline occurred regularly until 150 days.

Propionic acid (mg/L) had the highest increase on day 90 of 690 ± 98 mg/L. There was a consistent increase from day ten to day 90. Propionic acid was formed after the degradation of NH_2-N, glucose, maltose, xylose, fructose and long chain fatty acids.

Butyric Acid is a short chain fatty acid. The formation process occurs after the degradation of long chain fatty acids. There was a consistent decline on day 150, and the highest decline was recorded on day 60.

Acetic acid (CH_3COOH) had a peak increase on day 80 of 2850 ± 240 mg/L. This shows the maximum degradation of propionic acid, butyric acid, and pentanoic acid to CH_3COOH. There was a regular increase from day ten to day 80, and a 40 % decrease on day 100.

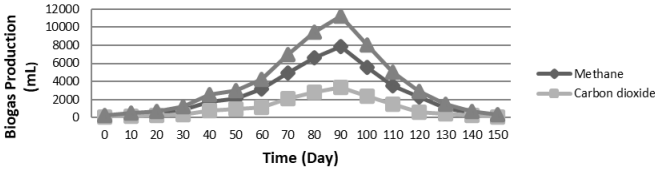

Figure 1. Biogas production per ten days at 12 °C

Observation of the amount of biogas was carried out for 150 days (Fig.1); analysis of the composition of CH_4 and CO_2 was carried out every ten days. The average amount of biogas produced from the fermentation process at low temperatures was 0.74 L/d with the amount of CH_4 above 0.45 L/gVS. Observations were also made in 5 intervals from days 61 to 110. Peak performance occurred on day 90.

Number and classification of bacteria

The bacterial OTUs in this fermentation were grouped based on the 16S rDNA gene sequence. 25 OTU representative bacterial sequences were found. The mean relative abundance (*ra*) was chosen to see changes in *ra* during fermentation.

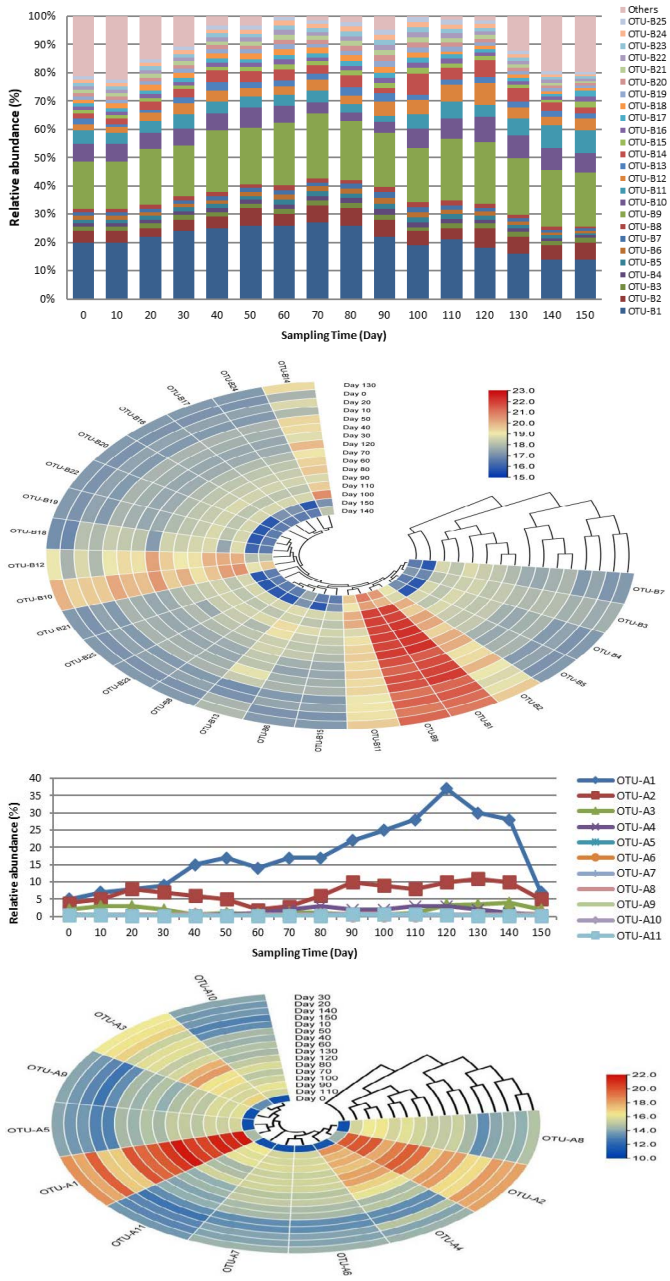

Figure 2. Visualization of microbial dynamics in biogas: depicting bacterial and archaea abundance (2a, 2c), colony counts (2b, 2d) accompanied by evolutionary relationships displayed in the central phylogenetic tree via MEGA11.

Accumulated relative abundance and number of colonies (fig. 2a and 2b) of 25 bacterial OTUs ranged between 79.7 % and 99.2 %, which were divided into: 19 Firmicutes, four Bacteroidetes, one Synergistetes, one Proteobacteria. The four phylum play a major role in hydrolytic fermentation. Representative order of 25 OTUs based on the Basic Local Alignment Search Tool (BLAST) using the NCBI Genbank database in identifying species similarity. The role of species

in metabolic processes was described based on the literature and the assistance of consultants (Table 2).

Bacterial OTU analysis in metabolic function was based on the order of 97 % nitrogen base similarity to the strain species from the closest comparison to the species group. First, we enlarge the Clostridium species. The most dominant *ra* was OTU-B1 which ranges from 13.9 % - 27 % (maximum 23 Log.CFU/g), based on the 16S sequence had 98 % similarity with *Clostridium cellulovorans* (Robert, et al., 1984). These bacteria break down glucose, cellulose, pectin, maltose into H_2, acetic acid, butyric acid, and CO_2.

During the fermentation process, the *ra* OTU-B1 was relatively constant in the range of 23 %, and the peak *ra* occurred on day 70. On day 90 there was a decrease. It was indicated by a decrease in dissolved sugar from glucose, cellulose, pectin, and maltose.

The second dominant bacterial OTU was OTU-B9, which had *ra* range of 16.2 % - 23 % (maximum 21 Log. CFU/g). The similarity of 16S was 99 % *Terrisporobacter petrolarius* (Deng et al., 2015). These bacteria decompose monosaccharides into CO_2, and acetic acid. The pattern of increase in *ra* was similar to that of OTU-B1. This indicates that there was competition for the same substrate. The *ra* OTU-B9 increased to a peak (23 %) on day 70. There was a decrease on 80-100 days, and on day 110, it increased again. This indicates that OTU-B9 consumes the soluble sugar produced by OTU-B1.

OTU-B10 had the third dominant *ra*, which was 3.10 - 9.05 % (maximum 17 Log.CFU/g), had a similarity of 98 % *Turicibacter sanguinis* (Bosshard et al., 2002), bacteria that degrade maltose into lactic acid. The increase in *ra* occurred regularly at the beginning of the fermentation until the peak (9.05 %) on day 60 and 70. Then, there was a decrease and an increase in days 110 to 120. OTU-B1 and OTU-B9 competition for maltose substrate. Complete details regarding closest species/similarity, *ra*, substrate/product and genus /phylum can be seen in (Table 2).

Number and classification of archaea

in this study, the reported Archaea have *ra* values > 0.1. OTU-A1 was the most dominant Archaea with similarity (98 %) identical to *Methanocorpusculum sinence* (Zellner et al., 1989). This Euryarchaeota dominates with a *ra* of 4.95–37.10 % (maximum 22 Log.CFU/g) . The function of metabolism in the fermentation process is to convert CO_2 and H_2 into CH_4. The increase in ra Indicate fermentative activity of specific bacterial groups (or taxa)

OTU-A2 was identical to *Methanobrevibacter millerae* (99 %) with ra 2.00 % -11.20 % (maximum 17 Log.CFU/g). The pattern of increasing *ra*was almost the same, but the peak position of OTU-A2 was at 130 days. OTU-A4 had a 98 % similarity with *Methanobrevibacter ruminantium* (Smith and Hungate, 1958) which had a ra of 0.50 - 3.20 %. OTU-A5 had 99 % similarity to *Methanocorpusculum sinense* (Zellner et al., 1989) with *ra* 0.24 - 2.90 %. OTU-A6 showed (96 %) *Methanobacterium lacus* (Borrel et al., 2012). OTU-A7 similarity (94 %) was *Methanobacterium beijingense* (Ma et al., 2005) and OTU-A8 similarity (98 %) was identical to *Methanobrevibacter smithii.*(Balch et al., 1979).

OTU-A3 indicated by *Methanosarcina soligelidi* (Wagner et al., 2013) with 97 % rDNA similarity and having *ra* ranging from 0.70 - 3.60 % was Archaea that decompose CO_2, H_2, and acetic acid into CH_4. The highest increase in *ra* occurred on day 140. Referring to (Fig.1), the highest production of CH_4 was on day 90. This indicates that the role of OTU-A3 was below OTU-A1 and OTU-A2. The role of OTU-A3 was the same as that of OTU-A10 based on substrate consumption. OTU-A10 was a type of *Methanosarcina mazei* (Blotevogel and Fischer, 1989) with *ra* ranging from 0.47 - 3.20 %.

OTU-A9 was identical (90 %) to *Methanosaeta concilii* (Patel, 1984) which breaks down acetic acid to CH_4. This archaea competed with OTU-A3 and OTU-A10 in acetic acid consumption. OTU-A11 was the least dominant Archaea, OTU-A11 was identical to *Methanosphaera cuniculi* (Biavati et al., 1988).

Table 2. Classification and metabolic roles of 25 bacterial and 11 archaea OTUs

Code	Closest species (similarity)	Access	Relative abundance (%)	Substrat/Product (References)	References for Substrat/product	Genus/ Phylum
OTU-B1	*Clostridium cellulovorans (98%)*	KF528156.1	13.9 – 27	Glucose, cellulose, pectin, maltose / H_2, acetic acid, butyric acid, CO_2	(Yang et al., 2022)	*Clostridium*/ Firmicutes
OTU-B2	*Clostridium Saudi (99%)*	NR_144696.1	2.4 - 7.1	Cellulose, glucose, hemicellulose / acetid acid, butyric acid	(Yang et al., 2022)	*Clostridium*/ Firmicutes
OTU-B3	*Clostridium butyricum (98%)*	OM698377.1	1.3 - 1.9	Glucose, starch, sucrose / butyric acid, acetic acid, H_2, CO_2	(Yang et al., 2022)	*Clostridium*/ Firmicutes
OTU-B4	*Clostridium beijerinckii (97%)*	NR_113388.1	0.8 - 2.0	Glucose, strach, sucrose/butyric acid, acetic acid, H_2, CO_2	(Yang et al., 2022)	*Clostridium*/ Firmicutes

Code	Closest species (similarity)	Access	Relative abundance (%)	Substrat/Product (References)	References for Substrat/product	Genus/ Phylum
OTU-B5	*Clostridium chartabidum (98%)*	X71850.1	0.7 - 2.2	Cellulose, fructose, sucrose, sucrose / butyric acid, acetic acid, H_2, CO_2	(Yang et al., 2022)	*Clostridium*/ Firmicutes
OTU-B6	*Clostridium lavalense (99%)*	LT223652.1	0.68- 2.05	Fructose, glucose, lactose / acetic acid, lactic acid, fumaric acid	(Yang et al., 2022)	*Clostridium*/ Firmicutes
OTU-B7	*Clostridium moniliforme (98%)*	KY079341.1	0.6 - 1.98	Peptone, arginine/ H_2, acetic acid, lactic acid, butyric acid	(Yang et al., 2022)	*Clostridium*/ Firmicutes
OTU-B8	*Clostridium celerecrescens (99%)*	AB601064.1	0.58 - 2.10	Cellulose, fructose, glucose/ acetic acid, butyric acid, H_2, CO_2	(Yang et al., 2022)	*Clostridium*/ Firmicutes
OTU-B9	*Terrisporobacter petrolearius (99%)*	ON778559.1	16.4 - 23.00	Fructose, glucose, maltose, xylase /CO_2, acetic acid	(Yang et al., 2019)	*Terrisporobacter*/ Firmicutes
OTU-B10	*Turicibacter sanguinis (98%)*	HQ646364.1	3.10 - 9.05	Maltose/ lactic acid	(Wu et al., 2022)	*Turicibacter*/ Firmicutes
OTU-B11	*Romboutsia timonensis (99%)*	NR_144740.1	2.00 - 8.05	Glucose, sucrose, fructose / acetic acid, lactic acid, H_2, CO_2	(Yang et al., 2019)	*Romboutsia*/ Firmicutes
OTU-B12	*Streptococcus gallolyticus (100%)*	MT012069.1	1.85 - 8.00	Cellobiose, protein, fructose, glucose / Lactic acid	(Yang et al., 2019)	*Streptococcus*/ Firmicutes
OTU-B13	*Ruminococcus gauvreauii (97%)*	LC269264.1	1.00 - 3.10	Galactose, glucose, fructose / acetic acid	(Wessels, 2022)	*Ruminococcus*/ Firmicutes
OTU-B14	*Bacteroides graminisolvens (99%)*	ON561145.1	1.95 - 7.50	Xylose, pectinose, glucose / acetic acid, propionic acid	(Wang et al., 2021)	*Bacteroides*/ Bacteroidetes
OTU-B15	*Cellulosilyticum lentocellum (97%)*	MW553583.1	1.20 - 2.05	Xylose, cellulose,, maltose / acetic acid, formic acid, H_2	(Zhu et al., 2016)	*Cellulosilyticum*/ Firmicutes
OTU-B16	*Tangfeifania diversioriginum (95%)*	NR_134211.1	1.10 - 2.10	Ribose, xylose, strach, fructose / VFAs	(Yang et al., 2019)	*Tangfeifania*/ Bacteroidetes
OTU-B17	*Sunxiuqinia faeciviva (97%)*	NR_108114.1	0.80 - 1.80	Tyrosine / VFAs	(Yang et al., 2019)	*Sunxiuqinia*/ Bacteroidetes
OTU-B18	*Atopostipes suicloacalis (97%)*	NR_028835.1	0.70 - 1.90	Maltose, lactose, glucose / formic acid, acetic acid, lactic acid	(Yang et al., 2019)	*Atopostipes*/ Firmicutes
OTU-B19	*Lactobacillus reuteri (100%)*	JX272059.1	0.65 - 2.01	Lactose, fructose, xylose, ribose / lactic acid	(Yang et al., 2019)	*Lactobacillus*/ Firmicutes
OTU-B20	*Pseudomonas caeni (99%)*	NR_116388.1	1.20 - 2.20	Fat / VFAs	(Yang et al., 2019)	*Pseudomonas* / Proteobacteria
OTU-B21	*Cloacibacillus porcorum (92%)*	LT223650.1	0.40 - 1.90	Amino Acid / acetic acid, formic acid, propionic acid	(Yang et al., 2019)	*Cloacibacillus*/ Synergistetes
OTU-B22	*Lactobacillus amylovorus (100%)*	AF133264.1	0.67 - 1.95	Glucose, galactose, fructose / lactic acid	(Yang et al., 2019)	*Lactobacillus*/ Firmicutes

Code	Closest species (similarity)	Access	Relative abundance (%)	Substrat/Product (References)	References for Substrat/product	Genus/ Phylum
OTU-B23	*Anaerosporobacter mobilis (98%)*	NR_042953.1	0.89 - 2.05	Galactose, fructose, cellulose / acetic acid, H_2, formic acid	(Yang et al., 2019)	*Anaerosporobacter/* Firmicutes
OTU-B24	*Saccharicrinis marinus (93%)*	NR_137404.1	0.92 - 2.01	Lactose, maltose, cellobiose / VFAs	(Yang et al., 2019)	*Saccharicrinis/* Bacteroidetes
OTU-B25	*Syntrophomonas zehnderi*	NR_044008.1	1.20 - 2.20	Amino Acid / acetic acid, formic acid, propionic acid	(Yang et al., 2019)	*Syntrophomonas /* Proteobacteria
Code	Closest species (similarity)	Access	Relative abundance (%)	Substrat/Product	References for Substrat/product	Genus/Phylum
OTU-A1	*Methanocorpusculum sinence* (98%)	NR_117148.1	4.95 - 37.10	CO_2, H_2 / CH_4	(Zhao et al., 1989)	*Methanocorpusculum/* Euryarchaeota
OTU-A2	*Methanobrevibacter millerae* (99%)	KP123404.1	2.00 - 11.20	CO_2, H_2 / CH_4	(Nkamga et al., 2017)	*Methanobrevibacter/* Euryarchaeota
OTU-A3	*Methanosarcina soligelidi* (97%)	AB973359.1	0.70 - 3.60	CO_2, H_2, Acetic acid/ CH_4	(Jha & Schmidt, 2017)	*Methanosarcina/* Euryarchaeota
OTU-A4	*Methanobrevibacter ruminantium* (98%)	KP123398.1	0.50 - 3.20	CO_2, H_2 / CH_4	(Li et al., 2021)	*Methanobrevibacter/* Euryarchaeota
OTU-A5	*M. Sinense* (99%)	NR_104804.1	0.24 - 2.90	CO_2, H_2 / CH_4	(Yang et al., 2019)	*Methanocorpusculum/* Euryarchaeota
OTU-A6	*Methanobacterium lacus* (96%)	NR_117917.1	0.15 - 1.98	CO_2, H_2 / CH_4	(Yang et al., 2019)	*Methanobacterium /* Euryarchaeota
OTU-A7	*Methanobacterium beijingense* (94%)	KP109878.1	0.28 - 1.96	CO_2, H_2 / CH_4	(Yang et al., 2019)	*Methanobacterium/* Euryarchaeota
OTU-A8	*Methanobrevibacter smithii* (93%)	LR590664.1	0.38 - 2.05	CO_2, H_2 / CH_4	(Yang et al., 2019)	*Methanobrevibacter/* Euryarchaeota
OTU-A9	*Methanosaeta concilii* (90%)	KM408635.1	0.78 - 2.34	Acetic acid / CH_4	(Yang et al., 2019)	*Methanosaeta/* Euryarchaeota
OTU-A10	*Methanosarcina mazei* (92%)	KP231476.1	0.47 - 3.20	CO_2, H_2, Acetic acid/ CH_4	(Yang et al., 2019)	*Methanosarcina/* Euryarchaeota
OTU-A11	*Methanosphaera cuniculi* (93%)	NR_104874.1	0.28 - 1.98	Methanol, H_2 / CH_4	(Yang et al., 2019)	*Methanosphaera/* Euryarchaeota

The Correlation of OTU Bacteria and Archaea in Fermentation Metabolism

There was a significant positive correlation between OTU-B1 (cellulose and hemicellulose decomposers) and OTU-B12 as a protein decomposer (0.811**), starch-degrading bacteria OTU-B3 (0.754*) and OTU-B4 (0.705*), and fat-degrading bacteria OTU-B25 (0.654*). The dominant methanogens OTU-A1 and OTU-A2 were also significantly positively correlated ($p< 0.05$) with bacteria producing CH_3COOH, H_2, and CO_2. This condition illustrates the close relationship between bacteria and Archaea in determining the low temperature biogas metabolism pathway.

DISCUSSION

The role of bacterial and archaea species in the biogas fermentation pathway

The well-established theory regarding the stages of the biogas fermentation process has not been fully recognized by all researchers. The process of forming biogas goes through two stages (McKinney, 1962), three stages (Lawrence and McCarty, 1969) and four stages (Eckenfelder and O'Connor (2013). This study wanted to determine the stages based on the metabolic function of bacterial and archaea species, as well as determine the metabolic pathway if the fermentation was carried out at low temperatures (12 °C).

The hydrolysis process is the main stage in this research. Complex organic materials, insoluble in water, are hydrolyzed by extracellular hydrolases, so that they become water-soluble organic molecules (Reintjes et al., 2020). Substrate components in the form of cellulose and hemicellulose were hydrolyzed by the bacteria *Clostridium cellulovorans, Clostridium Saudi, Clostridium lavalense* (Domingo et al., 2009)*Clostridium celerecrescens* (Chamkha et al., 2001). and *Cellulosilyticum lentocellum* (Murray et al., 1986). Meanwhile starch-based substrates which were hydrolyzed by the bacteria *Clostridium butyricum*, and *Clostridium beijerinckii* (Sakamoto and Ohkuma, 2011). The hydrolysis of these substrates xylase, glucose, fructose, and maltose. Proteins are converted into amino acids involving *Pseudomonas gallolyticus*, and long chain fatty acids are the result of fat hydrolysis with *Pseudomonas caeni* (Xiao et al., 2009). Hydrolyzing bacteria play a major role in initiating metabolic pathways.

The second process is a fermentation process which includes the formation of short chain fatty acids (butyric acid, pentanoic acid, propanoic acid) from the decomposition of long chain fatty acids by *Pseudomonas caeni* bacteria. Xylose, maltose, fructose, glucose are fermented by bacteria *Terrisporobacter petrolearius*, *Turicibacter sanguinis* (Bosshard et al., 2002), *Romboutsia timonensis* (Ricaboni et al., 2016), *Bacteroides graminisolvens* (Nishiyama et al., 2009), *Clostridium lavalense, Atopostipes suicloacalis* (Cotta et al., 2004), *Lactobacillus reuteri,* and *Lactobacillus amylovorus* (Nakamura, 1981). Amino acids are fermented by *Clostridium moniliforme* (Lawson & Rainey, 2016)

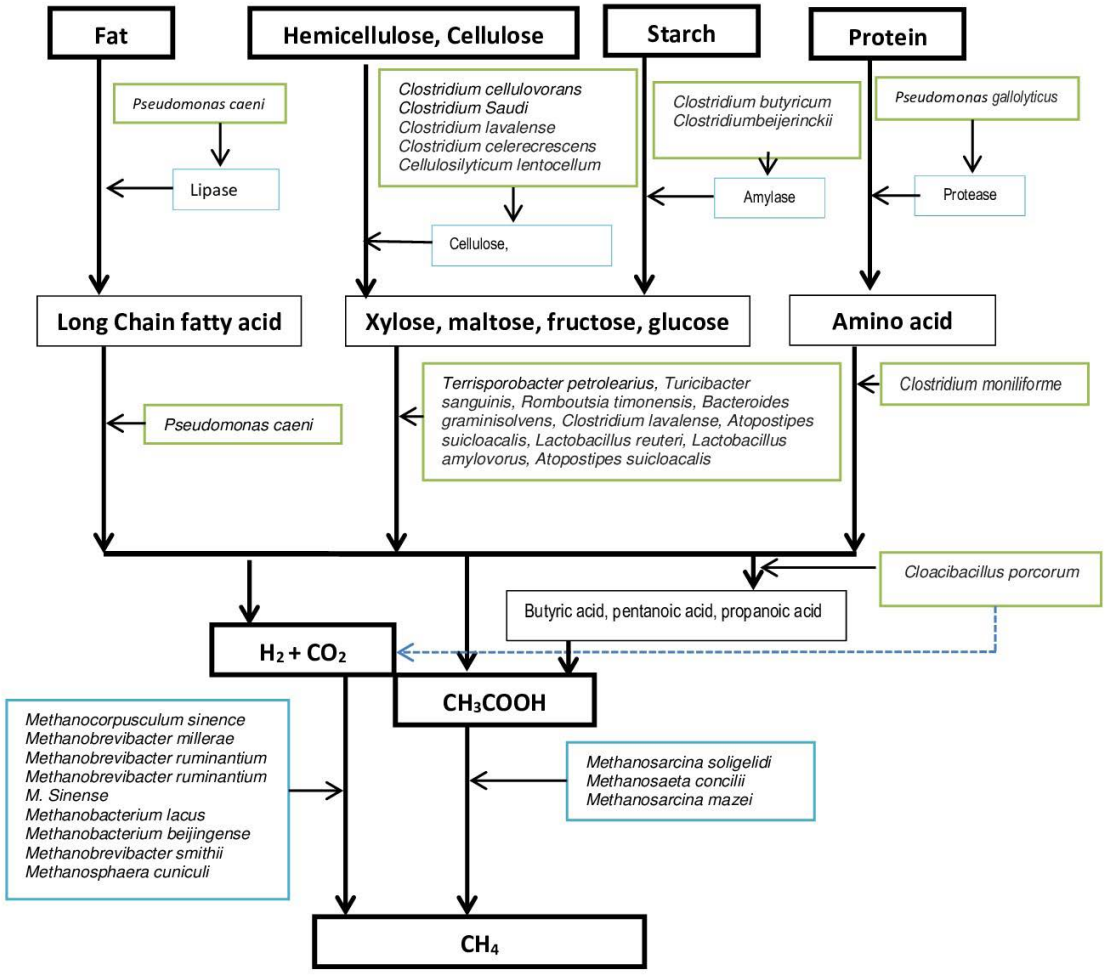

Figure 3. Biogas metabolism pathway based on bacterial and archaea species at low temperature fermentation (12°C)

The formation of hydrogen and acetic acid was the third stage. The degradation process of short chain fatty acids was through OTU-B21 into H_2 and CH_3COOH. Although the *ra* of OTU-B21 was relatively low (0.40 - 1.90 %), the formation of CH_3COOH can occur through OTU-B23, OTU-B18, OTU-B15, OTU-B14 and OTU-B9. Conversion of butyric acid, pentanoic acid, propanoic acid to CH_3COOH requires acetogen to produce H_2. In this study acetogen was very low in the OTU of bacterial species, although the VFA (except CH_3COOH) varied relatively widely.

VFA variation was not linear with acetogen abundance. This is caused by the environmental temperature that affects *Cloacibacillus porcorum* (Looft et al., 2013). The optimal growth of these bacteria ranges from 25-40°C and the strain growth temperature (37°C) is the same as that of *S. zehnderi* as an acetogen producer (Sousa et al., 2007). In this study the growth of the OTU-B21 line was not in the ideal range, so its growth was slow (low *ra*). Despite the low abundance of OTU-B21, the strain still played its role in metabolic function.

The fourth stage is the formation of CH_4 gas by OTU methanogen archaea. In this study, the formation of CH_4 was dominated by the substrate degradation pathway of CO_2, H_2 by OTU-A1, OTU-A2, OTU-A4, OTU-A5, OTU-A6, OTU-A7, and OTU-A8. The dominant *methanocorpusculum sinence* (*ra*= 4.95 - 37.10 %) had a major role in the CH_4 formation stage. OTU-A2 was *Methanobrevibacter millerae* which had an *ra* of 2.00 - 11.20 %.

The formation of CH_4 was through the CH_3COOH degradation pathway carried out by OTU-A3 (*ra*= 0.70 - 3.60 %), OTU-A9 (*ra*= 0.78 - 2.34 %), and OTU-A10 (*ra*= 0.47 - 3.20 %). This pathway only contributed a small amount to the formation of CH_4 (*ra* max 3.60 %). Another possibility was from the methanol degradation pathway played by OTU-A11 which had similarity to *Methanosphaera cuniculi* (93 %).

The role of bacteria and archaea species in biogas fermentation pathways under ideal condition

The ideal conditions for the growth of effective biogas fermenting bacterial and archaea strains at high temperatures are in the range of 36-41°C (Tampio et al., 2019). Achinas et al. (2018) used cow manure in research on biogas production. The abundant bacterial phylum was dominated by Firmicutes and Bacteroidetes. With the same sample, Achinas et al. (2018) found the dominance of Archaeas *Methanosaeta* and *Methanosarcina*. Another study found the abundance of Archaea *Methanoculleus*. OTU bacteria genus are *Draconibactericeae, Cloacimonetes* and *Ruminofilibacter*, while OTU Archaea is dominated by *Methanosarcina* and *Bathyarchaeota* (L. Dong et al., 2019)

Samples of cow manure mixed with other materials have also been tried by several researchers. Muratçobanoğlu et al. (2020) tested samples in the form of a mixture of cow manure and food waste with the predominance of *Clostridium* bacteria (18 %), *Actinobaculum* (15 %), and *Aminiphilus* (14 %). The most abundant methanogens were dominated by *Methanosaeta*. Hagen et al. (2014) who mixed cow manure with whey permeate found the dominance of the phylum Bacteroidetes, Firmicutes. Methanogens are dominated by *Methanobacteriales* and *Methanomicrobiales*.

The description above shows differences in results even from the same substrate (cow manure). This is caused by differences in the nutritional composition of feed and beverages that affect the concentration of NH_2-N (Manyi-Loh et al., 2016)

Biogas formation at low temperature

The results of this study are in agree with the findings of Seib et al .(2016) who used samples from wastewater by fermentation at a temperature of 10 °C. The dominant bacterial OTU is the genus *Clostridium*. The same results occurred for livestock waste samples carried out at 10°C (Bialek et al., 2012), wetlands in Qinghai-Tibet (Dai et al., 2016), although different Archaea were found for cow manure samples, namely *Methanosarcina*.

The process of formation of CH_4 through the main pathway (CO_2 + H_2) is in line with previous studies. O'Reilly et al. (2009) found the dominance of *Methanocorpusculum* as hydrogenotrophic in wastewater fermentation at a temperature of 15 °C. Tian et al. (2018) found *Methanobacterium formicicum* for fermenting pig manure at 15 °C. *Methanobacteriaceae* and *Methanomicrobiales* are on algae fermentation. In fermentation at low temperatures, the process of forming CH_4 is more dominant using the H_2 pathway than the CH_3COOH degradation pathway. This is a process of adaptation of hydrogenotropic methanogens to a cold environment. Free Gibbs energy requirements are smaller for the hydrogenotrophic process of methanogenesis at low temperatures (Lettinga et al., 2001). At low temperatures, the solubility of CO_2 and H_2 is higher so that it is easily consumed by Methanogens.

The CH_3COOH pathway will require greater free Gibbs energy because the viscosity of the liquid increases which results in the CH_3COOH diffusion process. An increase in the viscosity of the liquid can affect the diffusion process of CH_3COOH, which in turn affects the level formation of these compounds by microorganisms. The higher the viscosity of the liquid, the slower the diffusion of CH_3COOH molecules, which means that it takes longer for the molecules to reach the microorganisms involved in the formation of CH_3COOH.

When the free Gibbs energy required for the diffusion process increases due to an increase in the viscosity of the liquid, the microorganisms must generate additional energy to overcome this resistance. This can cause a change in the growth rate of the microorganism or affect the capacity of the microorganism to produce CH_3COOH. The effect of specific free Gibbs energy on the enthalpy value and heat

change in the formation of CH_3COOH by microorganisms will depend on the special conditions involved in the reaction (Von Stockar, and Liu,1999).

CONCLUSIONS

The accumulation of relative abundance of 25 OTU bacteria (79.7 % - 99.2 %) was found in the biogas formation process with cow manure as raw material at an operating temperature of 12°C. OTU bacteria are divided into four phylum, namely: 19 Firmicutes, four Bacteroidetes, one Synergistetes, one Proteobacteria. They all play a role in three stages: hydrolysis, fermentation, and the formation of hydrogen and CH_3COOH. Based in 16S RNA sequence analysis we found that the first dominant bacteria OTU, with an ra of 13.9 %, had 98 % similariy to *Clostridium cellulovorans* with an *ra* of 13.9-27 %. The second dominant was *Terrisporobacter petrolarius* (99 %) with *ra* of 16.2- 23 %.

The formation of biogas (CH_4) in this experiment is through the main pathway of H_2. This is evidenced by the dominance of hydrogenotrophic Archaea OTUs. The first dominance based on the 16S rDNA sequence, 98 % similarity was *Methanocorpusculum sinence* with *ra* 4.95 –37.10 %. The second dominant dominant was *Methanobrevibacter millerae* (99 %) with ra of 2.00 -11.20 %.

AUTHORS PARTICIPATION

Budianto: Research conceptualization and design involves forming research questions, selecting methodologies, planning data collection and analysis. Data visualization involves creating visual aids such as figures and tables to effectively present research findings.

Zefki Okta Feri: Translators, writing manuscripts, revising manuscripts.

Rinny Ermiyanti Yasin: Research analysis for manuscript

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CONFLICT OF INTEREST

There is no conflict of interest between the researchers

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