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Keywords: methanogenic, euryarchaeota, methanosphaerula palustris, terminal restriction fragment length polymorphism (TRFLP), tofu liquid waste.

I. Introduction

The increase demand in the near future caused by population growth and resource depletion of world oil reserves and concerns emissions from fossil fuel requires any country to immediately produce and use renewable energy. Besides, the increase in world oil prices up to 100 U$ per barrel is also a serious reason that afflicts many countries in the world, especially Indonesia. According to data from EMR (2006) Indonesia's oil reserves were only about nine billion barrels. If it continues to be consumed without the efforts to discover of new oil reserves, it is estimated oil reserves will be depleted within the next two decades. As a country that is endowed by abundant energy the renewal energy source. Biogas can be one of the alternative energy sources. This is a kind of gas formed by various organic waste biomasses, such as tofu liquid waste that can be optimized as energy through anaerobic digestion process. This process will result in a massive alternative energy production so that the effect of fossil fuel use will decrease.

At the present time, the organic waste biomass has hardly been studied. The characteristics of chemical, physical, and biological waste biomass of tofu industry are significant to be analyzed. The anaerobic digestion refers to various reactions and interactions that occur between methanogenic and non-methanogenic as well as materials (biomass) which is fed into the digester as inputs. This degradation process is a complex physical-chemical processes and biological processes that involve numerous factors and stages of transformation. Destruction of organic material input is achieved in three stages, namely: (a) hydrolysis, (b) acidification, and (c) the formation of methane (methanization) (de Mezt, *et al*., 2003). According to Raskin *et al*. (2007) the anaerobic fermentation process is divided into 4 stages of decomposition, *i.e.* hydrolysis, asidogenesis, asetogenesis and methanogenesis. Each stage will involve different bacterial groups that will work in synergy between one group against the other bacteria to form a consortium of bacteria.

Methanogenics are the Archaea that produce a methane gas in the entire process of its chain in anaerobic way. Information on the characteristics of microbial community during anaerobic fermentation of waste biomass of tofu industry can be a basis in the understanding of this research focusing on the identification and quantification of methanogenic. T-RFLP (Terminal Restriction Fragment Length Polymorphism) method is used to determine the dynamics of bacterial community structure as a whole. This method has been used in the analysis of structural dynamics of lactic acid bacteria Cheese (Bulut Cisem, 2003), agricultural soil bacterial communities in potato (Lukow *et al*., 2000), larvae of *Litopenaeus vannamei* (Pangastuti, 2008), and plankton bacteria on the Black Sea (Stoica, 2009).

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II. Material and Methods

The laboratory research was done in January 2012 to July 2012. The research sample was taken from one of the tofu industry in Nglorog, Sragen. The molecular research was done in the Faculty of Mathematics and Natural Science laboratory of Sebelas Maret University, Surakarta.

Digester construction: volume of 330 ml bottle, hose diameter of 0.5 and 30 cm long, hose diameter of 1 cm and 30 cm long, *Alteco* glue. Physics analysis (pH and temperature): pH meter and thermometer. DNA Extraction: sterile micro tube, micro pastel, UltraClean Soil DNA Kit MoBio as the manufacturers recommended procedures, along with the micropipette tip, centrifuges, vortex. Electrophoresis: electrophoresis apparatus, the micropipette along with tip, gel dock. Gene amplification of 16S sRNA: *Mung Bean Nuclease* (NEB, MA), QIAquick Gel Extraction Kit (Qiagen, Germany), micropipette, vortex, centrifuge, microtube, miniprep spin coloum. Digestion of amplicons: QIAquick Gel Extraction Kit (Qiagen, Germany), micropipette, vortex, centrifuge, micro tube, miniprep spin coloum.

T-RFLP analysis: Heating, 96-well plate, capillary electrophoresis system ABIprism™3100 Automated DNA Squencer (PE), labeled fragment length determined by program GeneScan ® (Perkin Elmer). The TRF size known is matched with a database on the *Ribosomal Database Project II* website (Marsh *et al*., 2000). Fragsort program (www.oardc.ohio-state.edu/trflpfragsort) is used to confirm the results of TRF cutting with restriction enzyme.

The substrates are derived from tofu industry waste; the inoculums are obtained from industrial waste that is fermented for about 2 weeks with a concentration of 20% of the digester working volume (330 ml), and water. DNA extracted using the UltraClean Soil DNA Kit MoBio as the manufacturers recommended procedure, electrophoresis using TAE buffer 10 times, distilled water, 0.8% of agarosa gel, Ethidium Bromide, loading dye. 16S sRNA gene amplification using forward primer 5'-(Ar109f) ACK GCT CAG TAA CAC GT -3 'and reverse primer 5'-(Ar 915r) GTG CTC CCC CGC CAA TTC CT-3', buffer (NEB, MA), dNTP Mix, U Taq DNA Polymerase (NEB, MA), and ddH2O. T-RFLP analysis using DNA isolates digestion, the HD-400 [ROX], and ice. Materials for the sequencing of genes encoding 16S rRNA: Ar109f primary and Ar 912r.

a) Sampling

This research uses tofu waste as substrate and the tofu industry waste that has been fermented out in a long period of time to form sludge (activated sludge) as the inoculums. In this research, the digester with a volume of 330 ml, which is 80% of the volume of the digester is used as the volume of work, while the rest (20%) as an air space. 80% (330 ml) of digester working volume filled by the source the inoculums with a concentration of 20%, 80% then the remaining volume is used for the substrate.

b) Biogas Production

Inoculums are poured first into the digester with a certain concentration (the research used a concentration of 20 % of the working volume of 264 ml, equivalent to 52.8 digester ml), then the substrate into the digester as the remaining volume of the digester working volume is 80 % of 264 ml, or approximately 211.2 ml), then the digester should be closed tightly. The fermentation process runs for 20 days, until the biogas formed. Once it is formed, the biogas will be supplied from the biogas digester tank (jerry cans) into the gas collection tank (330 ml volume bottle) through a small hose. Previously, the gas collection tank is full filled with water (330 ml). So when the gas flows into the gas collection tank, then the water will be pushed out and biogas will flow into the tank (replacing water). Thus, it can be seen that the volume of gas that flows into the gas collection tank equals to the volume of water coming out of the gas collection bottle. Agitation is done once a day. Sampling for measurement of the temperature, pH and molecular research conducted once every five days (days 0, 5, 10, 15, 20).

c) The Measurement of pH, temperature, and CH_4

Put the electrode pH meter in distilled water, wipe with a tissue and then put in a buffer solution of pH: 4, rinse with water, wipe with a tissue and put in Buffer pH: 7. Electrodes then inserted into 25 ml of sample in a beaker and the read pH meter. A thermometer is used to measure the temperature. CH_4 was measured by gas kromatrografi

i. *DNA Extraction Sample*

DNA extraction uses *UltraClean Soil DNA kit* (MoBio, CA).

ii. *Gen 16S rRNA Amplification*

Genes encoding 16S rRNA amplification performed with the forward (Ar109f) 5'-ACK GCT CAG TAA CAC GT -3' primer and reverse fam(Ar 915r) 5'-GTC CTC CCC CGC CAA TTC CT -3': 100 ng of DNA primer, 1x buffer (NEB, MA), 2 µl of 10 mM dNTP Mix, 2 U Taq DNA Polymerase (NEB, MA), 5 pmol of each primer, ddH2O to 50 µl. The PCR program consists of 1 cycle at 94°C for 3 min, 30 cycles at 94°C for 1 min, 48°C for 1 min, 72°C for 1 min, 1 cycle at 72°C for 7 min; ends with storage at 4°C. Part of the single-stranded DNA amplicons digested with Mung Bean Nuclease (NEB, MA) and then purified with QIAquick Gel Extraction Kit (Qiagen, Germany).

iii. *The PCR Digest Product of DNA Gen 16S rRNA Amplification Result*

The PCR product as a result of 16S rRNA gene DNA amplification was digested with restriction enzymes

that cut the high frequencies i.e. Alu1 (NEB, MA) separately to the reaction conditions: 5U enzyme, 1x buffer, 100-200ng of DNA, ddH2O to 20µl, and incubated at 37°C overnight. Desalting then performed with QIAquick Nucleotide Removal Kit (Qiagen, Germany). DNA digestion was dissolved in 30µl of elution buffer.

d) T-RFLP Analysis

DNA digestion was mixed with 1µl HD-400 [ROX] as an internal size standard. DNA was denatured at 95°C heating for 5 minutes and then immediately placed on ice for 5 minutes. Subsequently the mixture was inserted in a 96-well plate and inserted in the capillary electrophoresis system ABIprism™3100 Automated DNA Sequencer (PE Applied Bio systems). Labeled fragment length determined by Gene Scan® program (Perkin Elmer). TRF sizes known were matched with a database on the Ribosomal Database Project II website (Marsh et al., 2000). For the TRF identification compared with MICA database (http://mica.ibest.uidaho.edu).

e) Analisis data

Phylotype richness (S) is the total peak of different TRF/distinct restriction types found in each sample. Shannon-Wiener index (H') and evenness (E) was calculated to describe the diversity of the community in different instars and the relative importance of each filotipe in the whole community. H' is calculated by the following formula: $H' = -\sum pi \ln pi$ to discover the extent of each individual genus dominating the population used Eveness same index as follows: $E = \frac{H'}{H' \, maks}$ (Krebs, 1972)

III. Result and Discussion

The total 16SrRNA gene of the community was amplified with Ar915r primer labeled with 6-FAM and 109f primer that is not labeled. The DNA band as a result of PCR amplification of 16SrRNA encoding genes was obvious, namely a single band of DNA that measures approximately 750bp (Figure 1).

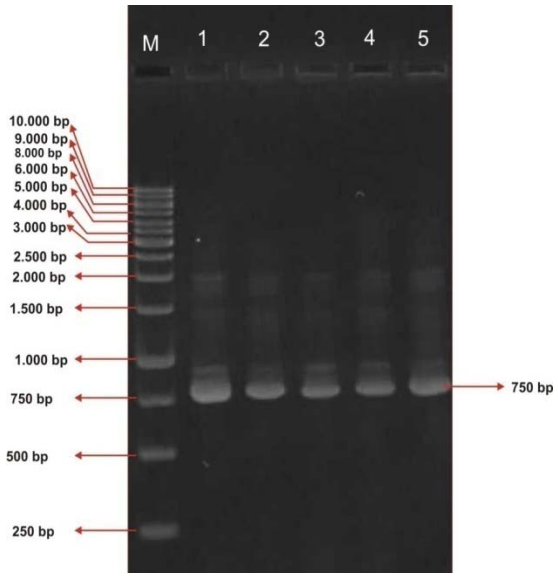

Figure 1 : The product of PCR amplification result of 16SrRNA gene DNA were 750bp sized at 0.8% Agarosa

Description: (M) DNA marker, Fermentation day (A) zero, (B) five, (C) ten, (D) fifteen, and (E) twenty.

T-RFLP profile of methanogenic community in this research showed a range of TRF sizes from 60-171bp, representing many different methanogenic populations (Figure 2). There were three fragments, namely 62bp, 136bp, and 167bp detected as a peak of TRF in the result on the electropherogram. Furthermore, the size of TRF on electropherogram was identified using the Microbial Community program analysis III (MICA) (http://mica.ibest.uidaho.edu/trflp.php).

MiCA was developed based on the Ribosomal Data Project II (RDP II) (Cole *et al*., 2003). TRF size details on each of the three filotipe are TRF peak size 62 bp, filotipe identified and classified from the Euryarchaeota phylum; TRF peak size 136bp, identified methanogenic belongs to Crenarchaeota phylum, whereas the TRF peak size 167bp, identified filotipe was closely related to Methanosphaerula palustris. The three filotipe detected entered in the Domain Archaea.

The three filotipe's population dynamics can be seen in Figure 2. The composition of the methanogenic filotipe detected based on the RDP database for each day observation is relatively stable, Euryarchaeote and *Methanosphaerula palustris* consistently detected from the beginning to the end of fermentation. This is presumably due to the biogas producer medium in a biodigester is highly influenced by the type of filotipe that has grown in the inoculums as a mud tofu waste. The consistency of those two filotipe growth shows that the digester used was able to support the growth of two filotipe.

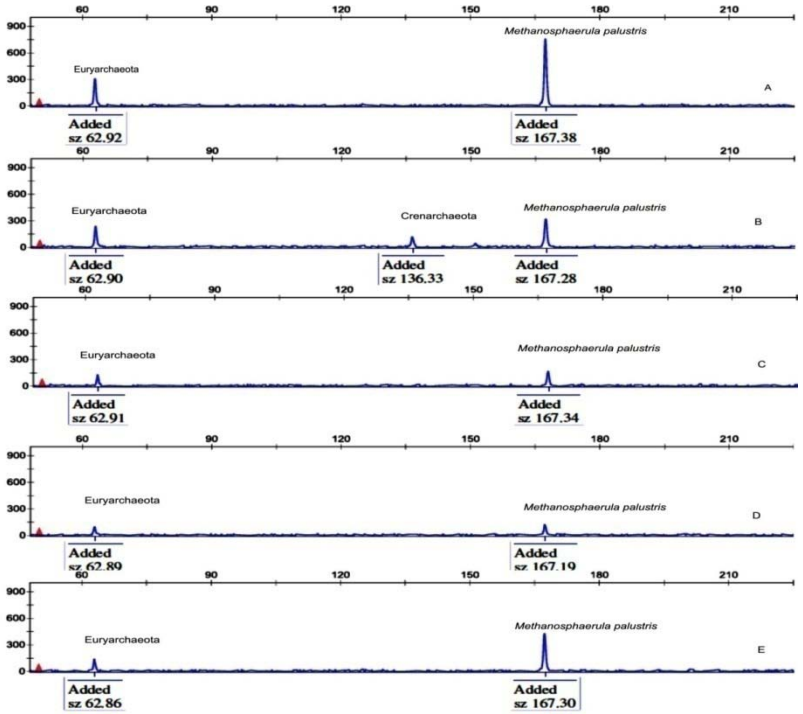

Figure 2 : Terminal Retriction Pryment of methanogenic archaea community uses restriction enzyme Alu1 in fermented Tofu liquid waste

Description: Fermentation day (A) zero, (B) five, (C) ten,(D) fifteen, and (E) twenty.

Methanospaerula palustris dominated in every observation and consistently grew from the beginning to the end of the fermentation process. The observation result in day 5 to day 15 showed that the abundant amount of *Methanospaerula palustris* was not stable, We assume that the changing condition of the digester was due to the additional liquid tofu waste which cause the minimum capability of methanogenic to grow the. Implying that on day 0 (zero) today 15 the growth of Methanospaerula palustris was still in lag phase. Liu *et al*., (2011) observed the amount of organic could carbon influence the population of methanogenic in a bog, if the amount of the organic carbon is available, it means that the population of the methanogenic will increase. However, they did not measure the amount of organic carbon and organic matter content (COD) contained in the digester. In this research, *Methanospaerula palustris* has the highest abundance on day 20 and we assume *Methanospaerula palustris* has stabilized and start with exponential growth.

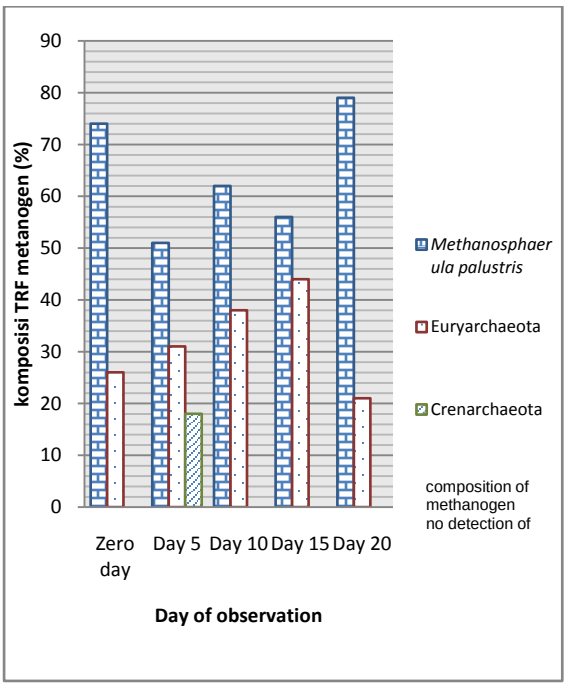

Figure 6 : TRF Metanogen Composition Using Restriction Enzyme *Alu1* during the Tofu Waste Anaerobic Fermentation

The Euryarchaeote abundance has increased significantly from day 0 to day 15, it is estimated that the Euryarchaeote easy to adapt medium in digester and used for growth medium. Euryarchaeote decreased on day 20, it could be due to growth competition with

Methanospaerula palustris as shown, by increase population of *Methanospaerula palustris*, while decreased of Euryarchaeote population (Cadillo et al., 2009). The filotipe at peak TRF 136, belonging to the Crenarchaeota phylum detected only on day 5 and was not successfully detected on day 10 until the end of fermentation, it may be influenced by the temperature of the fermenter's temperature that ranges between 30-31 °C and a pH in the range 7-8. That was the temperature for Archea mesofolik and pH that suit the methanogenic, where as Crenarchaeota phylum is hipertermofilik Archaea. According to Todar (2009) most of the Phylum Crenarchaeota composed of Archaea hipertermofilik. Phylum Crenarchaeota hipertermofilik requires a specific growth temperatures in range 80-105°C, grew in a habitat with a high sulfur content such as volcano area, geysers, and hot springs as in Yellowstone National Park area, United States of America. The pH of hipertermofilik Crenarchaeota growth is in the range of less than 2. This indicates that the tofu waste digester used in this research is not suitable for the growth of the Crenarchaeota phylum. Table 2 shows that the highest relative abundance from the beginning until the end of fermentation is *Methanosphaerula palustri*.

Table 2 : The Relative Abundance (%) of Methanogen Community during the Anaerobic Fermentation of Tofu Liquid Waste Using TRFLP Technique

Methanogen Community	Relative Abundance (%)				
	Zero day	Day 5	Day 10	Day 15	Day 20
Euryarchaeota	25.5	30.8	38.3	43.8	21.4
Crenarchaeota	0	18.4	0	0	0
Methanosphaerula palustris	74.5	50.8	61.7	56.2	78.6

The number of filotipe or Species Richness (S) detected in the methanogenic communities from all observations were two to three filotipe Alu1 using restriction enzymes (Table 3). Two filotipe were found from the beginning until the end of fermentation, except on the 5th day of observation there is an addition filotipe. The diversity index analysis (indicated by the Shannon-Weiner index/H') was the highest on day 5 of observation (1.02) and the lowest index values are shown on day 20 (0.52). The higher Methanogenic diversity on treatment day 5 showed that on day 5 there was a diverse methanogenic communities with equitable filotipe distribution, although evenness is not the highest value. This means that there was no filotipe relatively dominant on day 5. In contrast, based on the result of broad peak areas of T-RFLP, the estimation of the total number of methanogenic was not the highest. The lowest diversity found on day 20 (0.52), the value of evenness day 20 was also low, indicating that there was a very dominant filotipe. Based on the broad peak area of T-RFLP results, the estimation of the total number of methanogenic on the 20th day is not the highest or the lowest.

Table 3 : Diversity of methanogen community on the anaerobic fermentation of Tofu liquid waste by TRFLP Technic.

The day of Observation	The amount of Filotipe Richness	Shannon Weiner(H') Indeks	Evenness/E
0	2	0.57	0.82
5	3	1.02	0.93
10	2	0.67	0.96
15	2	0.69	0.99
20	2	0.52	0.75

The physic-chemical characteristics analysis as a concentration of CH_4, pH and temperature is intended to indicate the presence of methanogenic in accordance with the factors of physical-chemical in its growth. CH_4 concentration measurement was not performed on days 0, it is assumed that on 0 day, the CH_4 has not been formed yet for it has not been fermented. The CH_4 production on day 5, day 10, day 15, day 20, respectively for 1092.6 ppm, 570.2 ppm, 712.66 ppm, and 655.82 ppm, so the highest CH_4 production found on day 5 in the amount of 1092.61 ppm, while the population of *Methanospaerula palustris* on day 5 is not the highest abundance (Figure 7). It shows that the CH_4 production was not significantly associated or correlated with methanogen population abundance.

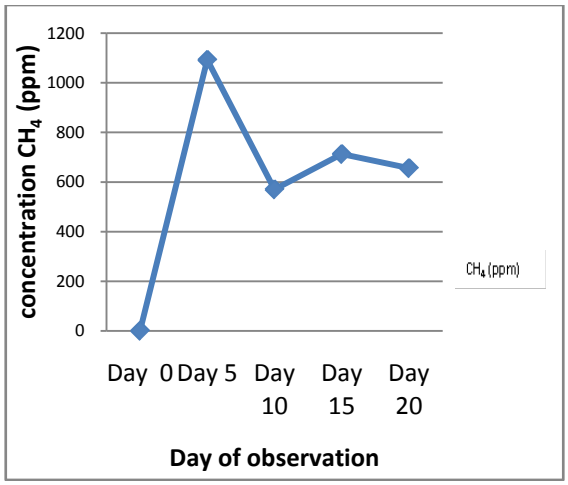

Figure 7 : The Graphic of CH4 concentration during the anaerobic fermentation of Tofu liquid waste

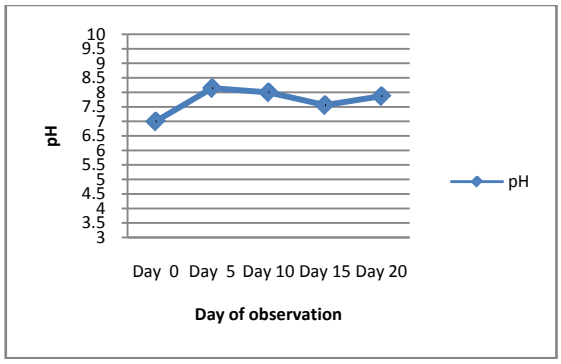

Figure 8 : The Graphic of pH during The Anaerobic Fermentation of Tofu Liquid Waste

Figure 8 shows the results of pH measurements. Measurement of pH on day 0 (zero), at the beginning of fermentation, pH was made to be neutral to 7 with NaOH, it is intended to equate the measurements at the beginning of fermentation. On day 5 was 8.146, day 10 was 8, the 15th day was 7.56 and 20th day was 7.87. The pH changes happened due to the fermented compound as well as the acetogenesis has been converted into H_2, CO_2, H_2O, and CH_4, as well as the breakdown of proteins into NH_4^+ which is then form into an alkaline compounds. The methane consuming acetic acid and convert it into methane and CO_2, so that the concentration of acetic acid in the waste water decrease and the pH increase (Suryandono dan Wagiman, 2004). The continued digestion process causes the concentration of NH_4^+ increased so that pH value increased. The NH_4^+ ions will form the alkaline compound and raise the pH in the digester to neutral. The result of pH measurement that ranging from 7 to 8.1 indicates that the pH produced is accordance with the terms of pH methanogenic growth.

The dominance of *Methanospaerula Palustris* and the diversity of Archaea population during the observation was not affected by temperature, because there was no much differences in the temperature measurement results from day 0 to day 20, ranging from 30°C to 31°C

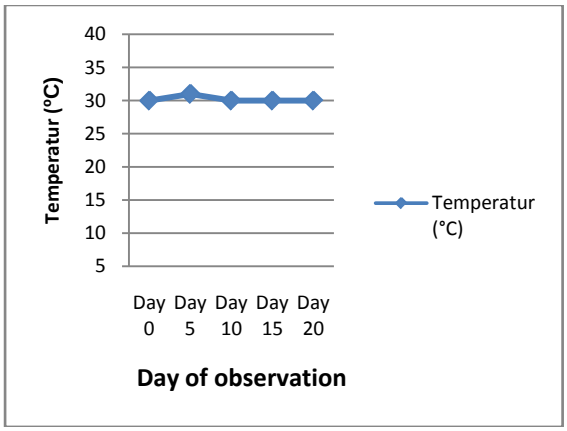

Figure 9 : Temperatures During Anaerobic Fermentation of Tofu Liquid Waste

According to Dubey (2005) most of the methanogens are mesophilic with an optimum temperature range between 20-40°C, but some methanogens can also be found in extreme environments such as hydrothermal vents that have temperatures up to 100°C.

In this research, the results of temperature measurements obtained 30°C and 31°C in the digester, it indicates that the methanogens that are closely related to *Methanospaerula palustris* is able to grow and produce CH_4.

The CH_4 production depends on the temperature of the digester, the research in peat lands, CH_4 production would be maximize dat a temperature between 20-35°C (Svensson, 1984; Segers, 1998; Kotsyurbenko et al., 2004; Metje and Frenzel, 2005).

IV. Conclusion

The methanogenic population in biogas production from tofu liquid waste for twenty days consit of two filotipe namely: methanogenic belongs to the phylum Euryarchaeota and methanogenic closely related to *Methanosphaerula palustris*. The third Filotipe detected is Crenarchaeota that may not belong to methanogens. Euryarchaeota and *Methanosphaerula palustris* found consistently from the beginning until the end of fermentation, whereas Crenarchaeota Phylum only detected on day 5. The methanogens dominated from the beginning to the end of the fermentation process was *Methanosphaerula palustris*.

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