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The diversity of non-methanogenic bacteria involved in biogas production from tofu-processing wastewater

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Abstract. *Sunarto, Tsaaqifah H, Purwoko C, Pangastuti A. 2014. The diversity of non-methanogenic bacteria involved in biogas production from tofu-processing wastewater. Biodiversitas 16: 62-68.* The energy crisis is an important current issue, so diversification of energy is needed to solve the problem. Biogas from tofu-processing wastewater is one potential alternative energy to be developed because it has high organic matter content. The amount of methane formed by methanogens is also influenced by the presence of bacteria, so it is necessary to do research on the diversity of non-methanogenic bacterial communities. This study aimed to determine the diversity of non-methanogenic bacteria in anaerobic fermentation using ARDRA (Amplified Ribosomal DNA Restriction Analysis) method. Tofu-processing wastewater was anaerobically fermented, followed by DNA extraction, amplification of 16S rRNA marker gene, and cloning. Then, restriction was done using *Msp*I and *Alu*I restriction enzymes, followed by sequencing of different patterns. The data were analyzed to know the diversity of non-methanogenic bacteria and the relative importance of each phylotype in the community using Shannon-Wiener index of diversity (H') and evenness (E).The results showed that there were 38 positive clones of 16S rRNA genes clones. The sequencing using BLASTn analysis showed the presence of 10 species of non-methanogenic bacteria. There were four sequences with ≥97% similarity, indicating that the bacteria belong to species from GeneBank data. There were 6 sequences that had ≤96% similarity with the closest relative in the database, indicating that these bacteria are new species. The Shanon-Wiener index was 1.291, categorized as low and the evenness was 0.561, categorized as low because there was dominance of a species.

Keywords: ARDRA, biogas, non-methanogenic bacteria, wastewater, 16S rRNA.

INTRODUCTION

Enery is essential for every living creature. Global energy demand increases along with the increasing human population and activities. The Ministry of Energy and Mineral Resources of Indonesia reported that energy consumption per capita in Indonesia increases with the growing rate of more than 5% (MEMR 2009). Ironically, the increasing demand for energy is not balanced with its supply, so the energy stocks, especially the fossil fuels are depleted. According to Sun et al. (2013), 88% of global enery consumption comes from fossil fuels, which lead to energy crisis, because fossil fuels are non renewable, which may be gone within 50-100 years.

Many governments in the world, including Indonesian government, have realized the threat of energy crisis. The utilization of alternative energy sources is the appropriate measure to overcome the energy problem. Biogas is one the most considered alternative sources of energy. Biogass refers to the mixture of methane, carbondioxide and other gasses produced by decomposition of organic waste, such as domestic waste and manure by methanogenic bacteria (Ntengwe et al. 2010).

In Indonesia one potential source of organic waste for biogas is tofu-processing wastewater. There are 84.000 tofu production units in Indonesia with a total production of 2.56 million tons per day (Sadzali 2010). Tofu-processing wastewater can be processed to make biogas through anaerobic fermentation, consisting of four stages of decomposition, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Briones et al. 2007). Each stage involves a group of different microbes which work synergically with each other, so they form microbial consortia (Griffin et al. 1998). The consortia are classified as non-methanogenic and archaic bacteria. The non-methanogenic bacteria play important roles in organic waste degradation, namely in liquefaction or hydrolysis stage and the production of acid which provides substrate for the archaic bacteria (Madigan et al. 2009).

Because the information of the diversity of non-methanogenic bacteria involved in the processing of biogas from tofu-processing wastewater is limited, studies are needed. In general, studies on morphology, cell structure and biochemistry of bacteria are conducted through isolation and characterization using culture (Reeve 1994). However, there is a problem because not all microbes, including the major bacteria group, can be cultured (Suwanto 1994). Therefore, it is necessary to choose a method which can be used to study the structure of all bacteria, namely ARDRA (*Amplified Ribosomal DNA Restriction Analysis*). This method can be used to analyze population of bacteria and the predicted genetic changes within a period of time. The objective of this study was to know the diversity of non-methanogenic bacteria involved in the processing of biogas from tofu-processing wastewater.

MATERIALS AND METHODS

The materials for biogas production were substrate namely tofu-processing wastewater and inoculum in the form of active sludge of tofu. The substrate and inoculum were then fermented in anaerobic condition for 20 days in a digester. Then, the molecular research was started with the extraction of digester samples using UltraClean Soil DNA Kit (MoBio, Carlsbad, CA, USA). The result of extraction was genomic DNA of non-methanogenic bacteria. Then the 16S rRNA gene was amplified using PCR cycle, using primers 63F and 1387R. The result of amplification was subsequently purified with GeneJet™ Gel Extraction Kit (Fermentas c.q. Thermo Fisher Scientific Inc., Waltham, MA, USA). The product of purification was litigated with vector pTZ57R/T (Fermentas, MA, USA) which was then cloned using InsTAclone™ PCR Cloning Kit (Fermentas, Waltham, MA, USA). The transformant cell culture was grown in Luria Bertani (LB) agar medium with the addition of ampicillin, X-gal and IPTG. Then, Blue- white selection was conducted. The positive clones were digested using restriction enzymes, *Alu*I and *Msp*I.

The making of biogas

First, inoculum was put into digester with concentration of 20% of 264 mL of the digester working volume or equivalent to 52.8 mL. Then, 211.2 mL (or 80% of 264 mL) of the substrate was poured into the digester. Then the digester was closed tight. The fermentation took place for 20 days until biogas was produced, which was subsequently channeled through a small tube into a water-filled gas collecting tank (a drinking water bottle, 330 mL). The gas pushed the water out of the bottle. The volume of water spilled out the bottle was equal to the volume of gas filling the bottle.

The samples for molecular study were taken on the 20th day. According to Sunarto et al. (2013), usually the production of biogas has taken place optimally on the 20th day, so all stages of anaerobic fermentation have occurred.

Extraction of DNA samples

After 20 days of incubation, the sludge from the digester was sampled for DNA extraction using UltraClean Soil DNA kit (MoBio, CA, USA), following the procedure recommended by the manufacturer. The result of extraction was genomic DNA of non methanogenic bacteria as the DNA templates in PCR process.

Amplification of 16S rRNA Gene for ARDRA

The genomic DNA of non-methanogenic bacteria was then amplified. PCR reaction was made by mixing 0.5 µL of DNA samples, 17.5 µL of ddH2O, 2.5 µL of dNTP, 2.5 mM 2.5 µL of Buffer Taq, 0.2 µL of DNA Taq Polimerase enzyme, and 1 µL of primer. The DNA amplification used primer 63F (5' CAG GCC TAA CAC ATG CAA GTC 3') 5 pmol pairing with 1387R (5' GGG CGG *W*GT GTA CAA GGC 3') 5 pmol (Marchesi et al. 1998). The PCR program consisted of 1 cycle of pre denaturation at a temperature of 95^{o}C for 5 minutes, followed by denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 72^{o}C for 30 seconds (35 cycles each), post extension 1 cycle at 72^{o}C for 7 minutes, and it was ended with storing at 4^{o}C. Then, the PCR product was processed in electrophoresis at 0.8% agarose gel; then it was purified with GeneJET™ Gel Extraction Kit (Fermentas, MA, USA).

Cloning of 16S rRNA of PCR Product

The PCR products were cloned in accordance with procedures recommended in InsTAclone™ PCR Cloning Kit (Fermentas, Waltham, MA, USA). Clone selection was done in LB agar medium added with 50 mg/mL Ampicillin and X-Gal substrate. Then, the white clone was picked and grown in LB agar added with 50 mg/mL Ampisilin at 37 °C, shaken for 3-5 hours. This cell-containing medium was used as a template for insert amplification using primer M13 with mixed reactant composed of 10% culture, 0.8 mM dNTP, 1µM of each primer, 0.1 U/µL Taq DNA Polymerase, bufferred 1x. The PCR condition consisted of 3 cycles at 94 °C for 70 seconds, at 56 °C for 45 seconds, at 72 °C for 90 seconds; 23 cycles at 90 °C for 15 seconds, 56 °C for 30 seconds, 72°C for 1 second; 1 cycle at 72 °C for 10 minutes, and it was ended at 4 °C. Fifty clones which positively contained insert were randomly selected to be used for the subsequent analyses.

ARDRA (Amplified Ribosomal DNA Restriction Analysis)

Inserts were digested using restriction enzymes, *Msp*I and *Alu*I, in a mixture reaction with the following composition: 0.2 µL of enzymes, 2 µL of tango buffer, 10 µL of DNA, and 7.8 µL of ddH_2O. The mixture was then incubated at 37 °C for one night. Then, the DNA resulted from the digestion was processed in electrophoresis at 2% agarose gel, at a voltage of 70 V, for 2 hours. The number of inserts having different restriction patterns was counted in percentage of the total analyzed clones. Then, the 16S rRNA marker genes of the clones were sequenced for identification.

Sequencing and identification of the16S rRNA marker genes

The resulted clones were run in PCR again for sequencing by 1st Base Sequencing Services (Axil Scientific Pte. Ltd., Singapore through PT. Genetika Science Indonesia, Jakarta). Identification was done by comparing the sequences of the16S rRNA marker gene, about 1300 bp in size, with the database using the Program BLAST (Basic Local Alignment Search Tool) in the web site of National Center for Biotechnology Information (http://www.ncbi.nlm.gov/BLAST).

Data analyses

The diversity index of Shannon-Wiener (H') and the evenness index (E) were determined to show the diversity of bacteria community and the relative importance value of each phylotype in the community.

The diversity index:

$$H' = -\sum_{i=1}^{S} (Pi \log_2 Pi)$$

H' = Diversity index
S = number of species
Pi = Ni/N = proportion of Species i
Ni = number individuals of species i
N = number of individuals of all species

The criteria used to interpret the diversity index:
H' < 1: low diversity
1-3: medium diversity
>3: high diversity

Relative importance value:

$$E = \frac{H'}{Hmax}$$

E = Evenness; H' = Diversity index; Hmax = ln S

RESULTS AND DISCUSSION

The production of biogas from tofu-processing wastewater

The sludge from tofu-processing wastewater digestion, after 20 days of anaerobic fermentation, was sampled for molecular analyses. The measurements of pH and temperature were done in day 0 and day 20. The pH in day 0 was 7, and in day 20 was 7.87. The temperature in day 0 and day 20 was the same, which was 30 ^{o}C. The increase of pH in day 20 was influenced by the archaic methanogenic bacteria activities. According to Fulford (1988), at the early phase of biogas processing, the acid-producing non-methanogenic bacteria are active, so the pH of digester becomes low. Then, the archaic methanogenic bacteria use the acid as substrate, so the pH increases. The pH in the 20th day in this study was 7,87 which is tolerable by archaic methanogenic and non-methanogenic bacteria (NAS 1981).

Concentration of methane measured using chromatography was 655.82 ppm (R. Setioningsih, pers. comm.). The flame test showed that the production of gas was not sufficient to be lit. The biogas production refers to the reaction and interaction between the methanogenic microbes and the non-methanogenic ones (Gunnerson and Stuckey 1986). The non-methanogenic bacteria are categorized into three groups, namely hydrolitic, acidogenic, and acetogenic. These non-methanogenic bacteria groups play roles in fermentation of acid, in which they convert complex compounds into simple ones. The compounds resulted from fermentation are then used by archaic methanogenic bacteria in methanogenic fermentation and converted into methane (Sunarto et al. 2013). Generally, biogas is composed of methane 55-65%, carbon dioxide 30-40%, H_2O 2-7%, H_2S 2-3%, NH_3 0-0.05%, Nitrogen 0-2%, oxygen 0-2%, and 0-1% hydrogen (Yimer and Sahu 2014).

Extraction of DNA and amplification of 16S rRNA genes

Extraction of non-methanogenic bacteria DNA was done to get genomic DNA as a template for PCR process. UltraClean Soil DNA Kit (MoBio, CA, USA) was used for the extraction of genomic DNA of non-methanogenic bacteria from the tofu sludge, following the procedures in the manual kit. The genomic DNA of non-methanogenic bacteria resulted from the extraction was then amplified. The amplification of 16S rRNA genes used the PCR cycle which was the result of optimizing in the research of Sunarto et al. (2013). The primers used were 63F and 1387R, the universal primers for amplification of 16S rRNA gene of bacteria with an amplicon target of 1.300 bp (Marchesi et al. 1998). Primer is an important component in PCR reaction because it will determine the region of targeted genome which will be amplified (Rafsanjani 2011). The amplified DNA was separated with 0.8% agarose gel electrophoresis and then visualized using coloring agent of ethidium bromide and detected with UV light. The result of electrophoresis of amplification of PCR product of non-methanogenic bacteria 16S rRNA is shown in Figure 1.

The electropherogram shows that the size of region of 16S rRNA of non-methanogenic bacteria was 1300 bp, and it also shows single band indicating that the primer pair used was specific and attached to targeted area. The single band contained collection of 16S rRNA genes from many bacteria present in tofu waste sludge from the digester. The thickness of the band indicates the quantity of the amplified DNA templates. The electropherogram in this study was clearly visible and thick, indicating that the concentration of molecules separated in this region was high. The amplicon was then amplified using GeneJet^{TM} Gel Extraction Kit (Fermentas, MA, USA). Purification is needed because the cloning stage requires clean gene inserts, free from contaminant such as oligonucleotide which is not used in PCR process, the residues of buffers, and dNTP. The contaminant must be removed, because if the residue of dNTP combines with residue of DNA polymerase, it will disturb the methods designed for amplified DNA during cloning stage (Sanger et al. 1977).

Cloning of 16S rRNA genes

The product of purification was litigated with plasmid vector pTZ57R/T (Fermentas, MA, USA), catalyzed with T4 ligase enzyme to get plasmid recombinant. The litigation product was then transformed into *E. coli* JM107, using InsTAclone^{TM} PCR Cloning Kit (Fermentas, Waltham, MA, USA) following the procedures recommended by the manufacturer. Transformant cell culture was grown in LB agar medium with the addition of X-gal by spreading it using L rod and then incubated for 16 hours at 37°C. The result of *E. coli* JM 107 transformation showed the formation of blue-white colonies. The white colonies were estimated to contain fragments of insert genes, whereas blue colonies were estimated not to have fragments of insert genes.

The formation of blue white colonies was caused by the presence of selection marker at vector pTZ57R. The selection marker in vector pTZ57R was gene resistant to ampicillin and additional selection marker was LacZ gene. This ampicillin-resistant gene is the marker of β-lactamase enzyme, which will degrade the ampicillin, so when the cells carrying recombinant plasmid are grown in ampicillin-containing media, the cells will grow while the cells which do not carry recombinant plasmid will die. Lac Z gene is the marker for β-galactosidase enzyme which will hydrolyze X-Gal into galactoside and its derivative compounds, namely the blue 5-bromo-4-chloro-indoxyl (Susanti and Ariani 2003). If there is a fragment of inserted gene in the plasmid, the synthesis of α-peptide which will serve as an activator for β-galactosidase enzyme will be impeded, so the blue color will not appear (Brown and Brown 1991). The white colonies formed in selection media were then amplified with PCR and confirmed with electrophoresis. The PCR of colonies was started with the selection of separated colonies in selection media. Previously, a number was assigned to each colony. Then, with sterile toothpicks the colonies were duplicated in selection media which had been divided into several regions. The positive white clones which were estimated to contain fragments of inserted genes were subsequently grown in LB media added with 50 mg/mL of ampicillin and X-Gal substrate. The clones were used as templates for amplification of inserts using primer M13. The electropherogram of insert amplicon from cloning is presented in Figure 2.

The insert amplicon was digested with *Msp*I and *Alu*I restriction enzymes which cut with high frequency, which resulted in 10 different restriction patterns. The restriction patterns of DNA of non-methanogenic bacteria 16S rRNA marker genes are presented in Figure 3.

Sequencing and identification of 16S rRNA marker genes

The results of PCR amplification were sent to 1st Base Sequencing Services Singapore (via PT. Genetika Science Indonesia, Jakarta) to be sequenced using ABI Big Dye Terminator Kit (Applied Biosystems® c.q. Thermo Fisher Scientific Inc., Waltham, MA, USA). The results of sequencing were then compared with the sequences in the GeneBank database, using Basic Local Alignment Search Tool (BLAST) on-line in website: www.ncbi.nlm.nih.gov. All ten phylotypes were identified based on the 16S rRNA marker gene and compared with the database and using BLASTn analyses.

Based on the results of BLASTn analyses, there were 4 phylotype sequences of non-methanogenic bacteria from the fermentation of tofu-processing wastewater which had partial sequences similar to those in the database of the GeneBank with percent similarity ≥97%. This indicated that the bacteria were of the same species with the isolates which had been identified (Larsen et al. 1993).

There were 6 phylotype sequences with percent similarity <97%, meaning that those were new species whose data were not available in the GeneBank. The high percentage of <97% similarity between 16S rRNA gene fragments from the 6 phylotype sequences and those in the GeneBank indicated that the identified bacteria had close kinship, but not from the same species, with those in the GeneBank. The absence of ≥97% similarity between 16S rRNA fragments and those registered in the GeneBank indicated that the identified bacteria were partial sequences of the new strains of those in the GeneBank.

The compared sequences were partial sequences resulted from the sequencing and each restriction pattern produced different sequences. The results of phylotype identification indicated that there were at least 10 species of non-methanogenic bacteria, the composition of which is presented in Table 2.

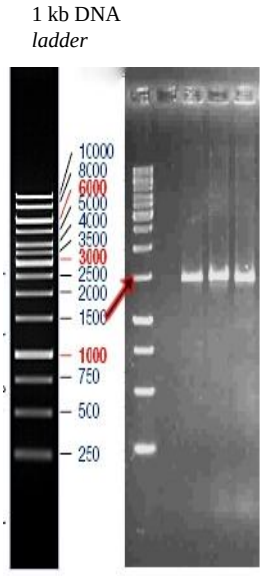


Figure 1. Electropherogram of amplicon of genomic DNA of non-methanogenic bacteria 16S rRNA marker gene.

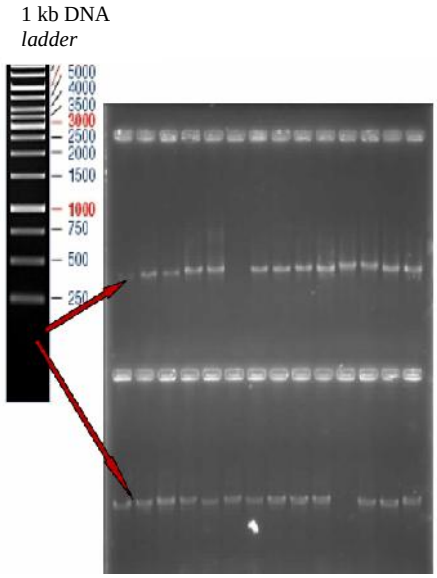
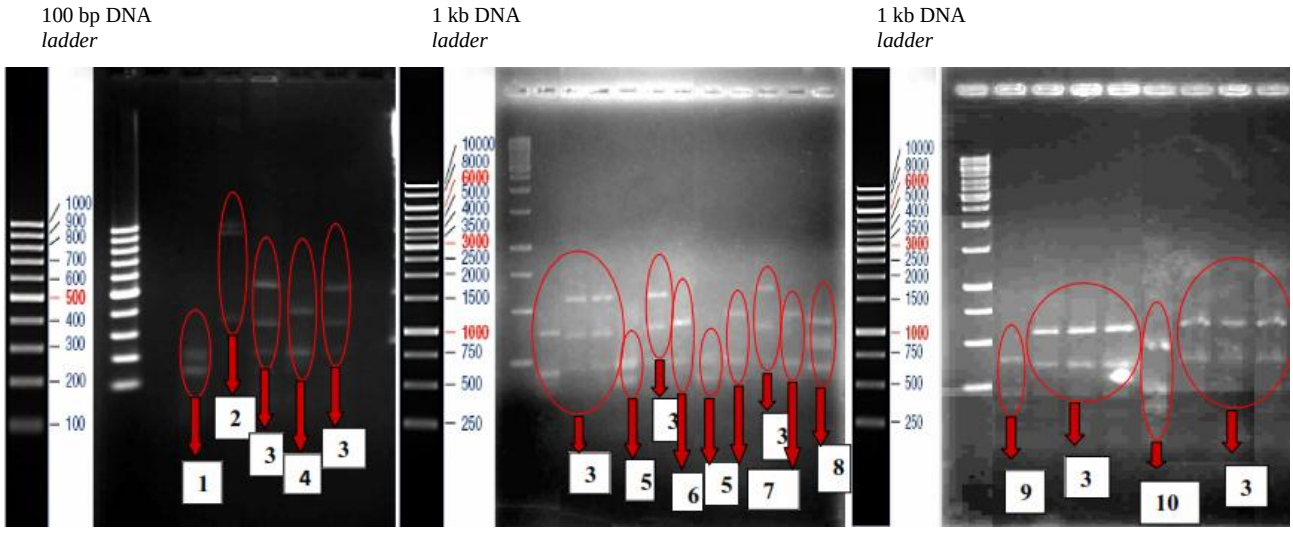
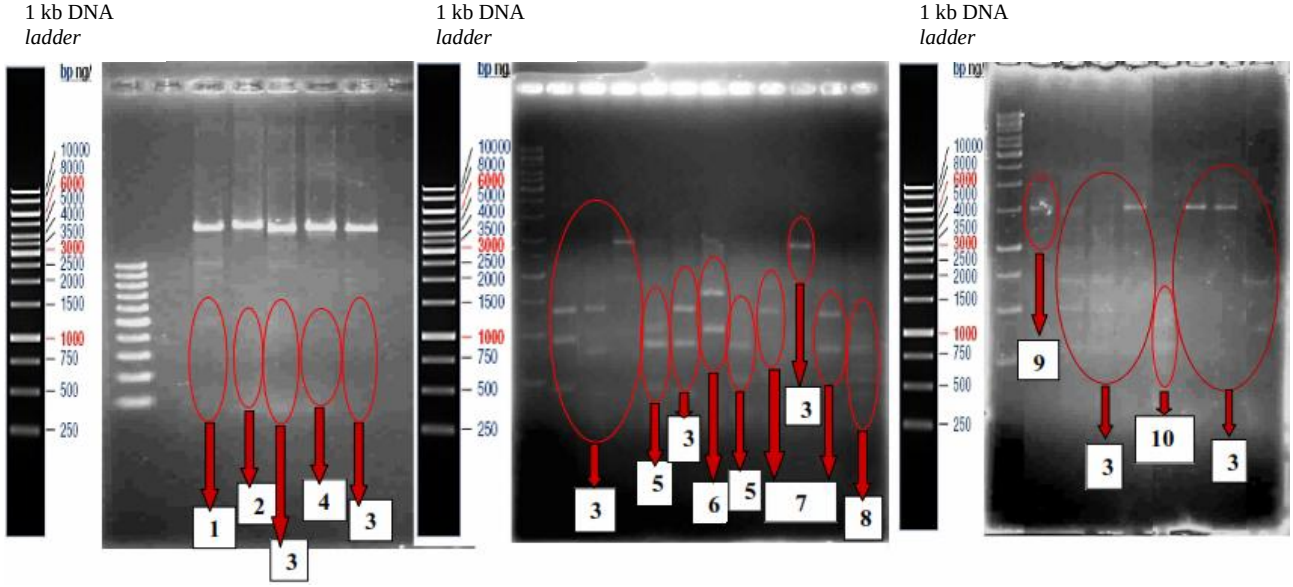


Figure 2. The electropherogram of insert amplicon resulted from cloning of 16S rRNA gene from tofu-processing wastewater samples.



A



B

Figure 3. Restriction patterns of DNA of non-methanogenic bacteria 16S rRNA marker gene with *Msp*I phylotype 1-10 restriction enzymes. A. 100 bp DNA ladder, B. 1 kb DNA ladder.

Table 1. The persentage of similarity between non-methanogenic bacteria DNA in the biogas production process from tofu-processing wastewater and the sequences in the GeneBank.

Restriction pattern codes	Closest kin	No Access	% Similarity
Type 1	*Chelatococcus daeguensis* strain TAD1	HM000004.1	94
Type 2	Uncultured bacterium clone NBBP10309_58	JQ072591.1	98
Type 3	*Brevundimonas diminuta* strain R057	KC252887.1	99
Type 4	*Ochrobactrum anthropi* strain YZ-1	JN248785.1	94
Type 5	*Devosia albogilva* strain IPL15	NR_044212.1	96
Type 6	*Acinetobacter junii* strain J14N	JX490072.1	95
Type 7	*Ochrobactrum oryzae* strain CK1711	KC955129.1	97
Type 8	*Olsenella umbonata*	FN178463.2	94
Type 9	*Defluvibacter lusatiensis* strain S1	NR_025312.1	97
Type 10	*Rhizobium borbori* strain DN365	GU356639.1	94

Table 2. The composition persentage of non-methanogenic bacteria of biogas production from tofu-processing wastewater.

Restriction pattern codes	Closest kin	% Composition of non-methanogenic bacteria
Type 1	*Chelatococcus daeguensis* strain TAD1	5.26%
Type 2	Uncultured bacterium clone NBBP10309_58	2.63%
Type 3	*Brevundimonas diminuta* strain R057	68.42%
Type 4	*Ochrobactrum anthropi* strain YZ-1	2.63%
Type 5	*Devosia albogilva* strain IPL15	5.26%
Type 6	*Acinetobacter junii* strain J14N	2.63%
Type 7	*Ochrobactrum oryzae* strain CK1711	5.26%
Type 8	*Olsenella umbonata*	2.63%
Type 9	*Defluvibacter lusatiensis* strain S1	2.63%
Type 10	*Rhizobium borbori* strain DN365	2.63%

The non-methanogenic bacterium with the highest percentage in this study was *Brevundimonas diminuta*, which was 68.42% with 26 clones out of the total of 38 clones. *Chelatococcus daeguensis*, *Devosia albogilva*, and *Ochrobactrum oryzae* had the same percentage, namely 5.26%, each having two clones. Several bacteria which had the smallest percentage, only 2.63%, were uncultured bacterium, *Ochrobactrum anthropi*, *Acinetobacter junii*, *Olsenella umbonata*, *Defluvibacter lusatiensis*, and *Rhizobium borbori*, each having only 1 clone.

Uncultured bacterium clone NBBP10309_58 referred to bacterium that had not been cultured and identified because it had such low metabolism activities that its similarity to or difference from other bacteria was not known. Based on the results of BLASTn, this phylotype was considered to have close kinship with phylum Firmicutes they had 92% similarity. According to Sun et al. (2013) the important role of phylum Firmicutes in biogas production is its ability to hydrolyze organic substrate.

So far there has been no sufficient information about non-methanogenic bacteria which play role in the hydrolysis and fermentation of substrate from tofu-processing wastewater. The only available information is limited to general species of non-methanogenic bacteria responsible for hydrolysis and fermentation processes, including facultative and obligate anaerobic bacteria such as *Actinomyces*, *Bacteroides*, *Bifidobacterium* spp., *Clostridium* spp, *Corynebacterium* spp., *Desulfovibrio* spp., *Escherichia coli*, *Lactobacillus*, *Peptococcus anaerobius*, *Staphylococcus* and *Streptococcus* (Tchobanoglous et al. 2002; Madigan et al. 2009).

Based on the available information, there are 6 phylotypes of non-methanogenic bacteria considered to play role in biogas production. Uncultured bacterium clone considered to have close kinship with Firmicutes plays role in hydrolysis stage because it is able to hydrolyze organic substrate (Sun et al. 2013). *Brevundimonas diminuta* plays role in acidogenesis stage because it is able to produce acetic acid (Kohyama et al. 2006). *Olsenella umbonata* also plays role in acidogenesis stage because it can convert glucose into acetic acid and formic acid (Isolauri et al. 2004; Wilson 2005). *Acinetobacter junii*, *Ochrobactrum anthropi* and *Defluvibacter lusatiensis* are reported to be able to reduce nitrate into nitrite which in turn is used by other bacteria as an acceptor of electron until it becomes nitrogen. According to Sterling et al. (2001) nitrogen sources are important as nutrient for microbes to produce biogas.

In this study, seven out of ten phylotypes were aerobic which had close kinship with *Ochrobactrum oryzae*, *Defluvibacter lusatiensis*, *Devosia albogilva*, *Rhizobium borbori*, *Chelatococcus daeguensis*, *Brevundimonas diminuta* and *Ochrobactrum anthropi*. The presence of aerobic bacteria in the fermented sludge indicated the presence of oxygen in the digester, so the biogas production was not maximal. The high concentration of oxygen in the digester impedes archaic methanogenic bacteria in producing methane gases.

The presence of oxygen in the digester in this study might be caused by the use of simple, laboratory-scale digester. The initial condition of digester was not anaerobic, so the aerobic bacteria still survived. This condition was correlated with the low biogas production, especially the CH_4.

Analyses of non-methanogenic bacteria

The analyses of non-methanogenic bacteria were based on the calculation of the number of clones. Species richness (S) was the number of clones recorded, which was 10. The Shannon-Wiener index was 1.291, indicating the low diversity in the tofu-processing wastewater processed into biogas. According to Krebs (1989), the classification of H' are as follows: low diversity (H' = 0-2.302), medium diversity (H' = 2.302-6.907), and high diversity (H' > 6.09).

The Evenness index (E) indicates evenness of a community. The evenness index in the tofu-processing wastewater processed into biogas was 0.561, categorized as low. According to Krebs (1985), the value of E ranges from 0 to 1. If E approaches 0, the evenness of community is low and the abundance of species is not uniform, and the community is dominated by certain species. If E approaches 1, the evenness is high, meaning that there is no dominant species, or the abundance of species is uniform.

The low diversity in this study was caused by the dominance of *Brevundimonas diminuta*. The plausible reason for the dominance of this species was that the high protein content in the tofu-processing wastewater provided nutrient for the growth of this bacterium, as Chaia et al. (2000) states that *Brevundimonas diminuta* uses protein as

source of carbon in the form of amino acid.

In this study ten bacteria were found, namely *Brevundimonas diminuta*, uncultured bacterium clone, *Defluvibacter lusatiensis*, *Ochrobactrum oryzae*, *Devosia albogilva*, *Acinetobacter junii*, *Chelatococcus daeguensis*, *Ochrobactrum anthropi*, *Olsenella umbonata*, and *Rhizobium borbori*. These bacteria are able to survive in mesophylic condition, namely at temperature of 30-40°C and pH of 5-8.5.

It can be concluded that 10 phylotypes were found after anaerobic fermentation of tofu-processing wastewater which had been incubated for 20 days. There were 6 phylotypes which were estimated to play role in biogas production, namely *Brevundimonas diminuta, Olsenella umbonata*, uncultured bacterium (considered to have close kinship with phylum *Firmicutes*), *Acinetobacter junii*, *Ochrobactrum anthropi* and *Defluvibacter lusatiensis*. The Shannon-Wiener Index was 1.291, categorized as low. The evenness index was 0.561, categorized as low due to the dominance of *Brevundimonas diminuta*. Further study is needed to optimize the anaerobic fermentation of tofu-processing wastewater to get higher methane gas concentration and sampling should be done in various periods of fermentation in order to find more non-methanogenic bacteria.

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