DNA Amplification of Methanogenic Bacteria from The Sewage of Coconut Water by Specific Primer 16S rRNA and Specific Methanogenic Gene

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Abstract. This study aims to transform metanogenic bacterial plasmid into E. coli C600 and to identify the products of methane from E. coli transformants. Isolate of methanogenic bacteria from cow’s feces as a positive control, named A and B. The transformation of isolate from the sewage of coconut water (P and M) plasmid to E.coli C600 using “Blind Test” method. Fermentation of coconut water by E.coli transformant that yield gas was quality-analyzed by burning and quantity-analyzed by GC. The result of gen 16S rRNA analysis by PCR, there is a similarity of nucleotide around 1088 bp, which is the same as isolate A and B from cow’s feces. The result of gen mcrA’s PCR on isolate P, M and isolate A and B have the similarity of nucleotide which is around 600 bp. The sequencing of gen 16S rRNA isolate P yielded nucleotide with composition respectively G 31.25%, C 20.58%, A 27.11%, and T 21.04% while the isolate M with composition respectively G 31.25%, C 20.31%, A 27.02, and T 21.32%. The identification of isolate M is similar with Clostridium tyrobutyricum (100%) and isolate P is almost similar with Clostridium tyrobutyricum (99%).

1. Introduction
Microbes or microorganisms can lives in all parts of the world because of the main role in the food cycle of all living things that can be expressed. There are beneficial microbes, harmful (parasitic), and even some unknown purpose. In this study will be identified the bacteria methanogenic which can be grown in coconut water waste[1-11]. In North Sulawesi palm planted area in 2005 was 259 535 ha by the number of trees that produced 29,1764 million trees and production reached 247 156 tons copra. If the average annual yield per tree 30 seeds, each of coconut in North Sulawesi produces nearly 100 million seeds. Furthermore, if each of 1000 seeds produce 140 liters of coconut water, then each year generated approximately 140 million liters of coconut water. The coconut water is used as a raw material of nata de coco, making ketchup, soft drinks, in addition to the growing animal feed and tissue culture media. However, coconut water is still largely untapped as a waste and often led to its exile ground wet, muddy and smelly.
The analysis results of the coconut water contain the following ingredients: sucrose, sorbitol, amino acids, organic acids, vitamins, plant growth substance and inorganic elements such as potassium, sodium, calcium, magnesium, iron, copper, phosphorus, sulfate and chlorine. The nutrient content of coconut water as above makes it possible to be a growing medium or substrate suitable for a variety of specific aerobic and anaerobic bacteria. An important role of coconut water can be converted to methane gas as a source of renewable energy increasingly clear and has good future prospects will come because these bacteria methanogenic track biochemical processes are well known and some of the enzymes produced by certain genes have been identified. Thus, the prospect of engineering can be done in accordance with the needs and specific goals. On the other hand, this bacterium has special properties that are located on the edge of evolution (13) and uncultured, therefore requiring special handling (14).

Isolate of methanogenic bacteria from sewage of coconut water can be a source of gen methanogenic for DNA amplification of methanogenic bacteria, but it is unknown types of bacteria found in sewage methanogenic coconut water. This study aims to transform metanogenic bacterial plasmid into \textit{E. coli} C600 and to identify the products of methane from \textit{E. coli} transformants. After the bacteria known as a methanogenic from coconut water waste and the plasmids can be amplified by \textit{E. coli} transformant and can produce methane gas, it is expected that \textit{E. coli} transformant can be as a biological tool to produce methane gas as one of the alternative renewable fuel.

2. Methodology

Materials

Isolates "P" and "M", which produces methane gas as a source of plasmid DNA, \textit{E. coli} C600 cell, nutrient agar, Ros Bengali modified media, coconut water, primers used in this study consisted of: (1) Specific prokaryotic 16 S-rRNA gene that is 63F (5'-CAG GCC TAA CAC ATG CAA GTC - 3 ') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3 ') (2) Specific gene methanogenic methyl-coenzyme M reductase (mcrA), namely F (5'-GGT GGT TTC ACA GGA GTM TAY CAR GCW ACA GC -3 ') and R (5'-GCR TTC ATT TAG TAG TT TTW GGR - 3 ').

Method

Total DNA isolation according to Maniatis et al. 1982 as follows; 200 ml of medium containing bacteria methanogenic (marked with a gas production rate) input in sterile polypropylene tubes Sorvall 500 ml. Media were then centrifuged using a Sorvall centrifuge with a speed of 5000 rpm at 4 \( ^\circ \)C for 20 minutes.

The supernatant was discarded then resuspension the obtained bacterial pellet in 20 ml SET buffer (Tris EDTA Sucrose). The suspension was then shaken with a vortex until homogeneous. Bacterial suspension was centrifuged again with the Sorvall centrifuges at a speed of 7000 rpm at 4 \( ^\circ \)C for 15 minutes. Supernatant was discarded. Frozen bacterial pellet obtained in the freezer - 20 \( ^\circ \)C for 10 hours. The pellets are melted again by deeping in water then resuspention the bacteria in 10 ml SET buffer. The reaction mixture was incubated for 25 min in the shaker incubator at 37 \( ^\circ \)C at 100 rpm. Then add 1 ml 20\% Sodium dodecyl sulfate (SDS) solution and the mixture was incubated again at 37 \( ^\circ \)C for 2.5 hours while shaken at 100 rpm.

Once the process is completed into the mixture were added 2 ml of a mixture of chloroform: isoamylalkohol (24:1) and then incubated at 37 \( ^\circ \)C while shaken at 100 rpm on a shaker for one night. To the mixture were then added 2 ml of sterile distilled water and 20 ml mixture of chloroform: isoamylalkohol (24:1). Further shaken gently for about 5 minutes until the solution became milky white. Milky white solution was put into sterile polypropylene tubes Sorvall 30 ml volume. Further centrifuged at 5000 rpm at 4 \( ^\circ \)C for 15 minutes. Upper phase (water phase) was taken with a sterile pipette drops a big mouth and transferred into another sterile tube centrifuges. The supernatant was added 20 ml of chloroform: isoamylalkohol (24: 1) then shake gently about 5 minutes until the solution became milky white. Aqueous phase obtained was measured with a measuring cup sterile. Added a solution of 2.5 m Na-acetate pH 6 with 0.2 fold volume of water phase is
obtained. Into the water phase is added to cold ethanol dropwise while the formed DNA is wrapped in a sterile glass rod. The addition of ethanol made up to 2 fold volume originally obtained.

**PCR amplification**
Total DNA extraction and purification of DNA is used as a template. Primers used in this study consisted of: (1) Specific prokaryotic 16 S-rRNA gene that is 63F (5'-CAG GCC TAA CAC ATG CAA GTC - 3') and 1387R (5'-GGG CGG WGT GTA CAA GG C-3') which will amplification 1300 base pairs (bp)(21). (2) Specific gene methanogenic methyl-coenzyme M reductase (mcrA), namely F (5'-GGT GGT TTC ACA GGA GTM TAY CAR GCW ACA GC -3') and R (5'-GCR TTC ATT TAG TT TTW GGR - 3') which will amplification 450-496 base pairs (bp) (15).

Amplification of 16S rRNA fragments using Gene AmpRPCR engine system 2400 (Perkin Elmer) with the following conditions: initial denaturation for 4 minutes at 94 °C, 35 cycles of amplification are denaturation 30 seconds at 94 °C, anning for 30 seconds at temperature of 56 °C, elongation for 30 seconds at 72 °C; then terminated by the addition (extension) for 10 minutes at 72 °C. PCR products were visualized by agarose gel electrophoresis.

To identify the presence of methane-producing gene amplification is carried out "mcrA gene fragments" using machines Gene AmpRPCR system 2400 (Perkin Elmer) with the following conditions: initial denaturation for 3 minutes at a temperature of 94 °C, 35 amplification cycles of denaturation 45 seconds at the temperature of 94 °C, annealing for 45 seconds at a temperature of 64 °C, elongation for 60 seconds at 72 °C; then terminated by the extension for 5 min at 72 °C. PCR products were visualized by agarose gel electrophoresis.

**Nucleotide sequencing**
Sequencing of 16S rRNA and mcrA gene using the ABI Prism automated sequencing machine version 3.4.1 (USA). Sequencing results were analyzed using BioEdit Sequence Alignment 7.0 c 1997-2004 Tom Hall Isis Pharmaceuticals. Inc.(20) Phylogeny analysis carried out using the program MEGA version 3.1 using the Neighbor-joining bootstrapped 1000 times repetition.

3. Result and Discussion

**Total DNA isolation**
Total DNA P and M isolate were isolated from the fermentation media which produce the most methane. Total DNA profiles are presented in Figure 1. Total DNA obtained is used as a template for 16S rRNA gene and mcrA amplification.

**Amplifikasi 16S rRNA gene**

![Figure 1](image)
16S rRNA gene PCR products using forward primers 63F and 1387R reverse primer either from DNA P isolate and isolates M are the same size as the 1300 bp with the concentration of each product by 61 ng / mL and 51 ng / mL.

Nucleotide Sequencing
16S rRNA gene sequencing using the ABI Prism automated sequencing machine version 3.4.1 (USA). The results obtained with both primer sequencing in both forward and reverse completely. However, after the alignment process using Sequence Alignment bioedit 7.0 c 1997-2004 Tom Hall Isis Pharmaceuticals. Inc. of the PCR product of 1300 bp of 1088 bp only showed traces are good and very accurate, the P isolate and M. From the size of the whole 1088 bp nucleotide composition were obtained for P isolate is G: 31.25%, C: 20:58%, A: 27.11% and T: 21:32%, while for isolates M is M: 31.34%, C: 20:31%, A: 27.02% and T: 21:32%.

The results of 16S rRNA sequencing analysis of the P isolate were aligned using the program after FASTAC with data from the Gene Bank obtained the results that P isolate has a very close similarity (99%) with the 16S rRNA gene of Clostridium tyrobutyricum. Similarly, isolates M of 16S rRNA gene sequences it has 100% similarity with the 16S rRNA gene of Clostridium tyrobutyricum. This is thought to occur because of the movement of methanogenic genes into cells of Clostridium tyrobutyricum.

If the sequence aligned with 16S rRNA gene methanogenic bacteria such as Mbsp.Ps2 = Methanobacterium sp. Ps21 (17) with access code 181 817 AB; Ampb.tr = Amphibacillus tropicus (16) with access code AF418602; Mbsp.Tc3 = Methanobacterium sp.Tc3 (17) with access code AB181819; M ther = Methanothermobacter thermautotrophicus (18) with access code X68717, and non-methanogenic bacteria E. coli (19) with the access code 157 425 AM phylogram obtained as shown in figure 3. P Isolate and M isolate included in the cluster closest to Methanobacterium sp. Ps2 and Amphibacillus tropicus. Methanothermobacter thermautotrophicus and Methanobacterium sp.Tc3 equal to the methanogenic bacteria.

Figure 2. Phylogram base on the neighbor joining method with a bootstrap value of 1000X and genetic distance based "p distance"

McrA gene fragment amplification results of P isolate can be seen in Figure 4. The fragments were successfully amplified with specific primers was successful getting around 600 bp PCR product, it is in accordance with that proposed by Luton et al. (2002).
Figure 3. McrA gene PCR products from isolates of P, M & P and Plasmids 16S rRNA genes derived from waste coconut water. Mr = Marker, column 1 = mcrA P, column 2 = mcrA M, column 3 = mcrA P column 4 = mcrA P plasmid, column 5 = 16S rRNA A, column 6 = 16S rRNA B (A & B from cow feses)

4. Conclusion
Based on the identification by 16S rRNA, P isolates is almost the same (99%) with Clostridium tyrobutyricum, and M isolate is the same (100%) with Clostridium tyrobutyricum. Base on the identification of the genes mcrA, isolates P and M derived from waste coconut water has the same number of base pairs (about 600 bp) with A and B isolates derived from cow feses as a positive control.

References

[1] Cuccioloni M, Bonfili L, Cecarini V, Cocchioni F, Petrelli D, Crotti E, et al. Structure/activity virtual screening and in vitro testing of small molecule inhibitors of 8-hydroxy-5-deazaflavin:NADPH oxidoreductase from gut methanogenic bacteria. Scientific reports 2020;10:13150.

[2] Chen YT, Zeng Y, Li J, Zhao XY, Yi Y, Gou M, et al. Novel Syntrophic Isovalerate-Degrading Bacteria and Their Energetic Cooperation with Methanogens in Methanogenic Chemostats. Environmental science & technology 2020;54:9618-28.

[3] Shi X, Gao G, Tian J, Wang XC, Jin X, Jin P. Symbiosis of sulfate-reducing bacteria and methanogenic archaea in sewer systems. Environment international 2020;143:105923.

[4] Ta DT, Lin CY, Ta TMN, Chu CY. Biohythane production via single-stage anaerobic fermentation using entrapped hydrogenic and methanogenic bacteria. Bioresource technology 2020;300:122702.

[5] Bao QL, Wang FH, Bao WK, Huang YZ. [Effects of Rice Straw Addition on Methanogenic Archaea and Bacteria in Two Paddy Soils]. Huan jing ke xue = Huanjing kexue 2019;40:4202-12.

[6] Zheng D, Wang HZ, Gou M, Nobu MK, Narihiro T, Hu B, et al. Identification of novel potential acetate-oxidizing bacteria in thermophilic methanogenic chemostats by DNA stable isotope probing. Applied microbiology and biotechnology 2019;103:8631-45.

[7] Jiang L, Hu Z, Wang Y, Ru D, Li J, Fan J. Effect of trace elements on the development of cocultured nitrite-dependent anaerobic methane oxidation and methanogenic bacteria consortium. Bioresource technology 2018;268:190-6.

[8] Wang HZ, Gou M, Yi Y, Xia ZY, Tang YQ. Identification of novel potential acetate-oxidizing bacteria in an acetate-fed methanogenic chemostat based on DNA stable isotope probing. The Journal of general and applied microbiology 2018;64:221-31.
[9] Dykstra CM, Pavlostathis SG. Methanogenic Biocathode Microbial Community Development and the Role of Bacteria. Environmental science & technology 2017;51:5306-16.

[10] Esquivel-Elizondo S, Parameswaran P, Delgado AG, Maldonado J, Rittmann BE, Krajmalnik-Brown R. Archaea and Bacteria Acclimate to High Total Ammonia in a Methanogenic Reactor Treating Swine Waste. Archaea 2016;2016:4089684.

[11] Ney B, Ahmed FH, Carere CR, Biswas A, Warden AC, Morales SE, et al. The methanogenic redox cofactor F420 is widely synthesized by aerobic soil bacteria. The ISME journal 2017;11:125-37.

[12] Azhar, M., Ahda, Y., Ihsanawati, I., Puspasari, F., Mawarni, S., Risa, B. and Natalia, D. (2017) “Skining Bakteri Pendegradasi Inulin dari Rizosfer Umbi Dahlia Menggunakan Inulin Umbi Dahlia”, Eksakta : Berkala Ilmiah Bidang MIPA (E-ISSN : 2549-7464), 18(02), pp. 13-20. doi: 10.24036/eksakta/vol18-iss02/44

[13] White D. 2000. The Physiology and Biochemistry of Prokaryotes. Ed. Ke 2. Oxford University Press. London. England

[14] Galand PE. 2002. Uncultured methanogenic archaeon partial mcrA gene for methyl-Coenzyme M reductase subunitA, clone FenB-MCR,. http://www/query.fcgi/ncbi (29/10/2002).

[15] Luton PE, Wayne JM, Sharp RJ, Riley PW. 2002. The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen population in Landfill. Microbiology, 148:3521 – 3530.

[16] Zhilina TN, Gernova ES, Tourova TP, Kostrikina NA and Zavarzin GA. 2001. Amphibacillus tropicus sp. New acaliphilc and facultatively anaerobic saccharolytic Bacilli from the lake Magadi. www.ncbi.com (12/8/2005).

[17] Deevong P, Hattori S, Yamada A, Trakulnaleamsai S, Ohkuma M, Noparatnaraporn N, and Kudo T. 2004. Isolation and detection of Methanogens from the Gut of Higher Termites. www.ncbi.com (12/8/2005).

[18] Nolling J, Hahd D, Ludwig W and de Vos MM. 1993. Phylogenetic analysis of thermophilic Methanobacterium spec., evidence for a formate-utilizing ancestor. www.ncbi.com (9/26/2005).

[19] Martin R, Heilig HG, Zoetendal EG, Jimenes E, Fernandes L, Smidt H and Rodrigues J. 2005. Cultivation-independent assessment of bacterial diversity of breast milk of healthy women. www.ncbi.com (12/7/2005).

[20] Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. Molecular evolutionary genetics analysis version 2.0, Pennsylvania State Univ.: Inst of Molecular Evolutionary Genetics.