MICROBIAL DIVERSITY IN TWO-STAGE ANAEROBIC DIGESTION SYSTEM USING TWO DIFFERENT SUBSTRATES: ORGANIC WASTE AND BIODIESEL WASTEWATER

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ความหลากหลายของประชาคมจุลินทรีย์ในระบบหมักไร้ออกซิเจนแบบสองขั้นตอนที่ใช้สารตั้งต้นแตกต่างกันสองชนิด: ขยะอินทรีย์และน้ำเสียไบโอดีเซล

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Thesis Title	MICROBIAL DIVERSITY IN TWO-STAGE ANAEROBIC DIGESTION SYSTEM USING TWO DIFFERENT SUBSTRATES: ORGANIC WASTE AND BIODIESEL WASTEWATER
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Field of Study	Environmental Management
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ชนกภรณ์ เมืองจินดา : ความหลากหลายของประชาคมจุลินทรีย์ในระบบหมักไร้ออกซิเจนแบบสองขั้นตอนที่ใช้สารตั้งต้นแตกต่างกันสองชนิด: ขยะอินทรีย์และน้ำเสียไบโอดีเซล (MICROBIAL DIVERSITY IN TWO-STAGE ANAEROBIC DIGESTION SYSTEM USING TWO DIFFERENT SUBSTRATE: ORGANIC WASTE AND BIODIESEL WASTEWATER) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. อรทัย ชวาลภาฤทธิ์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. อรฤทัย ภิญญาคง, 152 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อวิเคราะห์ความหลากหลายของประชาคมจุลินทรีย์ในระบบหมักไร้ออกซิเจนแบบสองขั้นตอนที่ใช้สารตั้งต้นแตกต่างกันสองชนิด คือ ขยะอินทรีย์และน้ำเสียไบโอดีเซล ความหลากหลายของประชาคมจุลินทรีย์ถูกวิเคราะห์โดยใช้เทคนิค PCR-DGGE และห้องสมุดดีเอ็นเอบริเวณ 16S rDNA จากผลการทดลองพบว่าโครงสร้างประชาคมของแบคทีเรียมีความแตกต่างกันระหว่างถังหมักกรดและมีเทน และระหว่างสารตั้งต้นขยะอินทรีย์และน้ำเสียไบโอดีเซล ในขณะที่โครงสร้างประชาคมของอาร์เคียแตกต่างกันเพียงเล็กน้อยทั้งในระหว่างถังหมักกรดและมีเทน และระหว่างสารตั้งต้นขยะอินทรีย์และน้ำเสียไบโอดีเซล แสดงให้เห็นว่าสารตั้งต้นที่แตกต่างกันมีผลต่อชนิดของจุลินทรีย์ จากผลการทดลองห้องสมุดดีเอ็นเอของแบคทีเรีย พบว่า กลุ่มประชากรหลักในถังหมักกรดที่ใช้ขยะอินทรีย์เป็นสารตั้งต้น คือ *Pseudomonas acephalitica* และ uncultured Firmicutes bacterium ส่วนในถังหมักมีเทนสามารถพบได้หลายกลุ่ม ได้แก่ *Weissella cibaria*, *Clostridium jejuense*, uncultured bacterium, *Sedimentibacter* sp., *Clostridium* sp., uncultured Firmicutes bacterium และ *Tissierella praeacuta* สำหรับถังหมักกรดที่ใช้น้ำเสียไบโอดีเซลเป็นสารตั้งต้น กลุ่มประชากรหลัก ได้แก่ *Klebsiella* sp. และ *Sphingomonas* sp. ในขณะที่ถังหมักมีเทนพบ Chloroflexi bacterium, uncultured bacterium และ *Pseudomonas putida* นอกจากนี้ได้ตรวจหายีนที่เกี่ยวข้องกับการผลิตก๊าซชีวภาพ คือ ยีนไฮโดรจีเนสและยีนเมทิลโคเอนไซม์เอ็มรีดักเตส ซึ่งสามารถตรวจพบทั้งสองยีนได้ทั้งในถังหมักกรดและมีเทน และในสารตั้งต้นขยะอินทรีย์และน้ำเสียไบโอดีเซล สุดท้ายได้ศึกษาหาปริมาณยีนเมทิลโคเอนไซม์เอ็มรีดักเตส โดยวิธี Real-time PCR จากผลการทดลองพบว่า ในถังหมักมีเทนปริมาณยีนมีแนวโน้มเพิ่มขึ้นเรื่อยๆ ในทุกๆ สัปดาห์ แต่ปริมาณยีนได้ลดลงในสัปดาห์สุดท้าย ซึ่งปริมาณยีนในระบบนั้นเป็นไปในทำนองเดียวกับการปริมาณก๊าซชีวภาพสะสมที่เกิดขึ้นในระบบ สำหรับในถังหมักกรดปริมาณยีนในสัปดาห์ที่ 0 และ 1 มีค่าใกล้เคียงกัน ในสัปดาห์ที่ 2 ปริมาณยีนมีค่าลดลง เนื่องจากในระบบมีค่าพีเอชลดลง ซึ่งมีผลยับยั้งการทำงานของเมทาโนเจน เมื่อค่าพีเอชสูงขึ้น ปริมาณยีนจึงค่อยๆ เพิ่มขึ้น ในสัปดาห์ที่ 3 จนกระทั่งสัปดาห์สุดท้าย จากข้อมูลที่ได้นั้นมีประโยชน์ในการเริ่มต้นและควบคุมการทำงานของระบบผลิตก๊าซชีวภาพเพื่อเพิ่มประสิทธิภาพในการผลิตก๊าซชีวภาพต่อไปในอนาคต

สาขาวิชา การจัดการสิ่งแวดล้อม.....ลายมือชื่อนิสิต.....

ปีการศึกษา 2553……...ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORD: BIOGAS PRODUCTION / TWO-STAGE ANAEROBIC DIGESTION / MICROBIAL COMMUNITY/ CLONE LIBRARY/ REAL-TIME PCR

CHANOKPORN MUANGCHINDA: MICROBIAL DIVERSITY IN TWO-STAGE ANAEROBIC DIGESTION SYSTEM USING TWO DIFFERENT SUBSTRATES: ORGANIC WASTE AND BIODIESEL WASTEWATER. THESIS ADVISOR: ASSOC. PROF. ORATHAI CHAVALAPARIT, Ph.D., THESIS CO-ADVISOR: ASST. PROF. ONRUTHAI PINYAKONG, Ph.D., 152 pp.

This study aimed to analyze microbial diversity of biogas production in two-stage anaerobic digestion system using two different substrates: organic waste and biodiesel wastewater. The microbial diversity was analyzed by using polymerase chain reaction-denaturant gradient gel electrophoresis (PCR-DGGE) and 16S rDNA clone library. For bacterial communities, DGGE profiles of biodiesel wastewater-feeding reactor indicated that the band from acid tank was different from the band from methane tank. It is known that conditions can affect the species of microbial community. In comparison between using organic waste and biodiesel wastewater as substrate, the DGGE profiles obtained were different. It is known that type of substrate can affect the species of microbial community. While the profile of archaeal community showed a little bit different in each tank and each substrate. Major bacterial groups represented in the clone library of acid tank using organic waste as substrate were *Pseudomonas acephalitica* (94%) and uncultured Firmicutes bacterium (6%). In methane tank using organic waste as substrate, major bacterial groups represented *Weissella cibaria* (28%), *Clostridium jejuense* (18%), uncultured bacterium (15%), *Sedimentibacter* sp. (15%), *Clostridium* sp. (11%), uncultured Firmicutes bacterium (8%) and *Tissierella praeacuta* (5%). In the clone library of acid tank using biodiesel wastewater as substrate were assigned to *Klebsiella* sp. (69%) and *Sphingomonas* sp. (31%) while the clone library of methane tank were assigned to uncultured Chloroflexi bacterium (76%), uncultured bacterium (22%) and *Pseudomonas putida* (2%). In addition, this study assessed the genes involved in biogas production: hydrogenase genes and methyl-coenzyme M reductase genes. These genes could be detected in acid tank and methane tank of two reactors. Finally, the real-time PCR was carried out to quantify the *mcrA* genes from reactor using biodiesel wastewater as substrate. For the result, the amount of *mcrA* gene in methane tank tended to increase. On the contrary, the amount of *mcrA* gene was decrease in the last week. For production of biogas in methane tank tended to increase in every week and dropped in the last week. The quantification of *mcrA* genes in the system revealed a similar pattern as accumulated biogas production. For acid tank, the amount of *mcrA* genes in week 0 and week 1 was similar. For week 2, the amount of *mcrA* genes in sludge was decrease because pH in the system was decreased which can inhibit the activity of metanogens. On the contrary, the amount of *mcrA* gene in week 3 to last week tended to increase because pH was higher. Based on the data obtained, it was useful for the startup and control of biogas digesters to increased ability of biogas production.

Field of Study: Environmental Management….....Student's signature…….…...
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CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Pollution problems that occur today such as global warming and ozone depletion are caused by use of energy from coal, petroleum and natural gas is connected with emissions of the green house gases. In addition, the demand for energy has become increasingly resulting the resources are decreased and the price has become very high. For these reasons, biogas production from renewable resources or organic waste is a promising alternative to fossil fuels. It is a clean and environmentally friendly fuel, which reduces green house gas emissions (Krober, *et al*., 2009).

Biogas is produced by anaerobic digestion or fermentation of biodegradable materials such as animal manures, organic wastes, sewage sludges and crops by specific microbial communities. Biogas consists of methane, carbon dioxide, hydrogen and low amounts of other gases depending on the feedstock type (Jingura and Matengaifa, 2009).

Since the organic waste is a major component of municipal solid waste in country and has the high humidity which can causes problems in storage, transportation and disposal. However, organic content in solid waste are easy to degrade and suitable to produce biogas. In addition, some organic wastes have enough nutrients for the growth of microorganism. For biodiesel wastewater, it has high concentration of organic compound, it is used as substrate to produce biogas by anaerobic digestion.

In the digestion, microbial composition of the bioreactor is an important factor, especially for the sake of process stability. Such imbalances are reflected by reduce efficiency of the biogas production and may lead to process failure or at least require long recovery periods. Therefore, it is better to understand the functions of the microbial community in the process. The understanding of microbial communities is

essential to effectively control the start up and operation of anaerobic digester for increase process stability and more efficiently of biogas production (Rastogi, *et al*., 2008).

The molecular biological techniques have been used for the detection, quantification and identification of the diversity and structure of microbial community. For example, construction of 16S rDNA clone libraries and subsequent sequencing of individual 16S rDNA clone were used to study the microbial communities (Krober, *et al*., 2009). In addition, fingerprinting techniques like denaturant gradient gel electrophoresis (DGGE), Quantitative Real-time PCR, or terminal restriction fragment length polymorphisms (T-RFLP) were used to analyze microbial communities (Klocke, *et al*., 2007, Tolvanen, *et al*., 2008). However, the knowledge about the composition of the microbial community in the degradation process of biogas production is not well understood.

The aim of this study was to analyze the microbial diversity in two-stage anaerobic digestion system fed with either organic waste or biodiesel wastewater as substrate. Based on the data presented was give a greater understanding of groups of microorganism prevalent in biogas reactor. This was increase ability of biogas production by providing the preferred environments for microorganisms in the system. In addition, this study assessed the gene involved in biogas production. The gene quantification and population density of microbial community during operation of this system could facilitate the development of better process performance monitoring and more economic biogas reactors.

1.2 Objectives

1.2.1. To determine the microbial diversity in two-stage continuous stirred tank reactor using either organic waste or biodiesel wastewater as substrate.

1.2.2. To compare microbial diversity in two-stage anaerobic digestion system using different substrates.

1.2.3. To detect genes involved in biogas production by using real-time PCR in two-stage anaerobic digestion system.

1.3 Hypothesis

1.3.1. The diversity and structure of microbial community in two-stage continuous stirred tank reactor are different depended on type of feed substrate.

1.3.2. The real-time PCR methods can be used to assess genes involved in biogas production in two-stage anaerobic digestion system.

1.4 Scope of Study

1.4.1 Characteristic of the two stage continuous stirred tank reactor

Bioreactor used in this study was a lab bench scale two-stage anaerobic digestion consisted of acid tank and methane tank. The reactor was fed with two different substrates; organic waste and biodiesel wastewater.

1.4.2 Sample collection

The samples used in this study were taken from both of acid tank and methane tank of the reactor. For bioreactor which using organic waste as substrate, samples were collected at steady state. For bioreactor which using biodiesel wastewater as substrate, samples were collected during operation in every week for 6 weeks since the start up state until steady state.

1.4.3 Microbial community structure analysis

Microbial community structure analyses were including:

- PCR denaturant gradient gel electrophoresis (PCR-DGGE)
- 16S rDNA clone libraries

1.4.4 Detection of genes involved in biogas production

Hydrogenase genes (*hydA*) and Methyl-coenzyme M reductase genes (*mcrA*) were detected in this study by PCR amplification and Real-Time PCR.

CHAPTER II

LITERATURE REVIEW

2.1 Anaerobic digestion

Anaerobic digestion is the degradation and stabilization of biodegradable materials by microorganisms in the absence of oxygen. Anaerobic processes produce biogas (a mixture of carbon dioxide and methane, a renewable energy source) and microbial biomass (Chen, *et al*., 2008). There are four basic steps of anaerobic digestion process as shown in Figure 2.1.

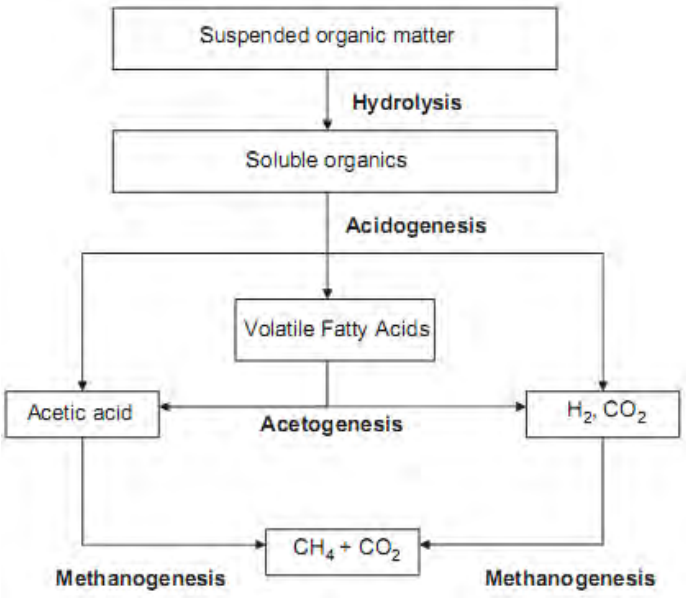

Figure 2.1 Step in anaerobic digestion process (Appels, *et al*., 2008)

The digestion process starts with hydrolysis step break down insoluble organic material and high molecular weight compounds such as lipids, polysaccharides, proteins and nucleic acids organic into soluble organic substances (e.g. amino acids and fatty acids). The second step, components formed during hydrolysis is further split during acidogenesis. Acidogenic bacteria produce volatile fatty acids along with

ammonia, CO_2, H_2S and other by-products. Acetogenesis is the third step of anaerobic digestion, which the higher organic acids and alcohols produced by acidogenesis are further digested by acetogenic bacteria to produce mainly acetic acid as well as CO_2 and H_2. This conversion is controlled to a large extent by the partial pressure of H_2 in the mixture. The final step is methanogenesis which produces methane by two groups of methanogen: the first group converts acetate into methane and carbon dioxide and the second group uses hydrogen as electron donor and carbon dioxide as acceptor to produce methane (Appels, *et al*., 2008).

The advantages and disadvantages of the anaerobic processes can be summarized as follows (United-Tech, Inc., 2009, Usanee, 2008)

Advantages:

- Anaerobic digestion uses readily available CO_2 as an electron acceptor as its oxygen source. It does not require oxygen, the supply of which adds substantially to the cost of wastewater treatment.
- Anaerobic digestion produces lower amounts of sludge because the energy yields of anaerobic bacteria are relatively low. Most of the energy derived from substrate breakdown is found in the final product as methane.
- Anaerobic digestion produces a valuable gas, methane. This gas contains about 90% of the energy and can be burned on site to provide heat for digesters or to generate electricity.
- The demand of energy for wastewater treatment is reduced.
- Anaerobic digestion is proper for high-strength industrial wastes.
- It can be applied high loading rates to the digester.
- The digestion sludge can apply the stabilized residue on the soil as a fertilizer.
- Anaerobic systems can biodegraded xenobiotic compounds such as chlorinated aliphatic hydrocarbons and recalcitrant natural compounds such as lignin.

Disadvantages:

- Anaerobic digestion is slower process than aerobic digestion.
- It is more sensitive to upsets by toxicants.
- Start-up of the process uses long periods of time.
- It cannot eliminate nitrogen and phosphorus.
- It is more sensitive to pH.

2.2 Biogas production

Biogas is a product of anaerobic degradation of organic substrates in anaerobic condition. Biogas composes of methane (50-60%), carbon dioxide (30-40%), hydrogen (5-10%), H_2S and nitrogen depending on the feedstock type (Jingura and Matengaifa, 2009).

Feedstock for biogas fermentation is biodegradable materials such as biomass, manure or sewage, municipal waste, green waste and energy crops. The digester used for biogas production is called a biogas plant. The gas can be used as substitute fuel for firewood, dung, agricultural residues, petrol, diesel, and electricity for any heating purpose, such as cooking and lighting (Mwakaje, 2008). Biogas is a renewable fuel, so it qualifies for renewable energy subsidies in some parts of the world.

2.3 Anaerobic digesters

The anaerobic digesters can be designed and engineered to operate using a number of different process configurations:

- Batch or continuous
- Temperature: Mesophilic or Therphilic
- Solid content: High solids or low solids
- Complexity: Single or multistage

For levels of complexity, digestion systems can be divided into single stage and two stage digestion.

2.3.1 Single stage digesters

A single stage digestion system is one in which all of the biological reactions occur within a single reactor. The rate of feeding is fed continuously for maximum efficiency. Acidogenic bacteria and methanogen are occurred in the single reactor and in direct competition with each other. Example of single stage digesters can be summarized as follows:

2.3.1.1 Continuous stirred tank reactor (CSTR)

This reactor consists of a well-stirred tank into which there is a steady flow of reacting materials and from which the reacted material passes continuously (Denbigh and Turner, 1971). The digester is maintained constantly at mesophilic or thermophilic temperature (Gunaseelan, 1997).

2.3.1.2 Plug-flow digester

In tubular plug-flow digester, a volume of the medium with suitable inoculums enters at one end of the tube and, if the rate of passage of the medium is correct, by the time the medium reaches the other end the digester is completed. For continuous operation, some of the digested effluent flowing from the end of the tube is separated and returned to the influent substrate (Gunaseelan, 1997).

2.3.1.3 Anaerobic Filter

This is primarily meant for digestion of easily fermentable factory waste waters produced in large quantities. Even a 6-day retention time would mean an impossibly large digester. Hence, in order to prevent washout, the bacteria are allowed to attach to a solid support, such as stones packed inside a tank and the waste water flows upward through the tank. This process requires a retention time of only a few hours and the gas is collected from the top (Gunaseelan, 1997).

2.3.1.4 Anaerobic Contact

This process can be considered as an anaerobic activated sludge because sludge is recycled from a clarifier or separator to the reactor. Since the material leaving the reactor is a gas-liquid-solid mixture, a vacuum degasifier is required to separate the gas and avoid floating sludge in the clarifier (Biomine, 2009).

2.3.1.5 Fluidized Bed

In a fluidized-bed digester, a modified form of anaerobic filter, the bacteria are attached to small glass spheres which are freely suspended in the up-flowing feed (Gunaseelan, 1997).

2.3.1.6 Upflow Anaerobic Sludge Blanket (UASB)

Under proper conditions anaerobic sludge will develop as high density granules. These will form a sludge blanket in the reactor. The wastewater is passed upward through the blanket. Because of its density, a high concentration of biomass can be developed in the blanket (Biomine, 2009).

2.3.2 **Two-stage digesters** (Ince, 1998)

A two-stage anaerobic digester is based upon the hypothesis that the environmental conditions relating in most anaerobic wastewater digesters are not optimal for both fermentative and methanogenic microorganisms. Since their differing growth characteristics, it is hard to select a single set of digester operating conditions which can maximize both acid and methane-forming bacterial growth as shown in Figure 2.2. Conditions such as short hydraulic retention time (HRT) and low pH that are suitable to the growth of the acid formers are inhibitory to the methane formers. The system separates the two main groups of microorganisms; acid and methane-forming microorganisms physically into serial reactors to make use of the differences

in their growth kinetics. This system operates conditions which can maximize both acid and methane-forming bacterial growth.

The two-stage process has several advantages:

- The basic concept of two stage digestion is to optimize the conditions for the hydrolytic acidogenic group of bacteria and for the acetogenic-methanogenic group, leading to the production of the most suitable acid metabolites for the methanogens and increase in the rate of substrate turnover. A two-stage system may allow a reduction in total reactor volume.
- By proper control of acidification, increased stability due to the more heterogeneous nature of the bacterial population should result because the process would insure against organic and hydraulic over loadings and fluctuations, with the first-stage acting as a metabolic buffer. Materials toxic to methanogenic bacteria may also be removed in the first stage.
- Fast growing, acidogenic biomass/sludge may be disposed of without the loss of methanogenic bacteria.

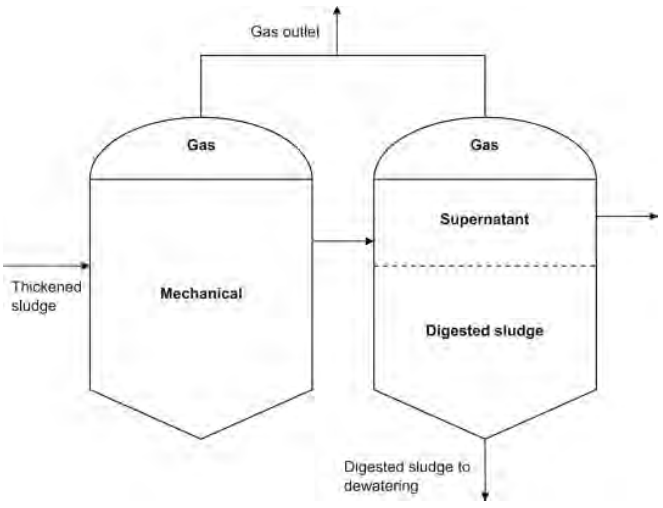

Figure 2.2 Two-stage digesters (Appels, *et al*., 2008)

2.4 Affecting parameters on anaerobic digestion

2.4.1 pH, alkalinity and volatile fatty acid/alkalinity ratio

Each group of microorganisms has a different optimum pH. Methanogenic bacteria are extremely sensitive to pH with an optimum between 6.5 and 7.2. The acidogenic bacteria are less sensitive and can function in a wider range of pH optimum between 4.0 and 8.5. The volatile fatty acid (VFA) produced during anaerobic digestion tend to reduce the pH. This reduction is countered by the activity of the methanogenic bacteria, which also produce alkalinity in the form of carbon dioxide, ammonia and bicarbonate. The system pH is controlled by the CO_2 concentration in the gas phase and the HCO_3-alkalinity of the liquid phase. If the CO_2 concentration in the gas phase remains constant, the possible addition of HCO_3-alkalinity can increase the digester pH (Appels, *et al*., 2008).

2.4.2 Organic load variations

Organic loading variations can directly affect the anaerobic digestion and the reactor performance. For example, over loading due to dissolved degradable compounds can lead to an accumulation of volatile fatty acid (VFA), a drop in pH values, and possibly an inhibition of methanogenic activity (Leitao, *et al*., 2006).

2.4.3 Temperature

Variations in temperature can affect the performance of anaerobic reactors because it also influences the growth rate and metabolism of microorganisms and hence the population dynamics in the anaerobic reactor. The activity of methanogens drop can occurs at temperatures lower than 16˚C and lead to an accumulation of volatile fatty acid (VFA) and a drop in pH (Leitao, *et al*., 2006). A high temperature has several benefits such as increasing solubility of the organic substances and enhancing biological and chemical reaction rates. However, the high temperature

has counteracting effects. For example, there will be increase the fraction of free ammonia, which can inhibit microorganism activities (Appels, *et al*., 2008).

2.4.4 Solid and hydraulic retention time

The solids retention time (SRT) is the average time the solids spend in the digester, while the hydraulic retention time (HRT) is the average time the liquid sludge is held in the digester. The subsequent steps of the digestion process are directly related to SRT. A decrease in the SRT decreases the extent of the reactions and vice versa. Each time, sludge is withdrawn, a fraction of the bacterial population is removed thus implying that the cell growth must at least compensate the cell removal to ensure steady state and avoid process failure (Appels, *et al*., 2008).

2.5 Type of substrates

Anaerobic digestion is a well established process for treating many types of organic materials, both solid and liquid. Biomass and water can be used as renewable resources for biogas production. The major criteria of the selection of materials to be used in biogas production are the availability, cost, carbohydrate content and biodegradability. Several studies have examined the effect of substrate on the anaerobic digestion performance. For example, the anaerobic degradation of cellulose-poor waste like fruit and vegetable waste is limited by methanogenesis. A major limitation of anaerobic digestion of fruit and vegetable waste is a rapid acidification of this waste decreasing pH in the bioreactor and a large volatile fatty acid production, which stress and inhibit the activity of methanogen (Bouallagui, *et al*., 2009). Ginkel, *et al*. (2005) studied biogas production from confectioners, apple and potato processor industrial effluents. The highest production yield was obtained from potato processing wastewater, apple and confectioners processing wastewater, respectively. From this result, it revealed that the wastewater which different composition can affect rate of biogas production. Moreover, Fukuzaki, *et al*. (1995) tested four different substrates; starch, sucrose, ethanol and butyrate, to assess the long-term effect of distinct wastewater composition on UASB stability. Their research

demonstrated that variations in the carbon source present in the wastewater caused changes in the physical structures, chemical contents and bacterial distribution. Based on the data obtained, the microbial capability usually relate to type of the feed substrate. Example of microorganisms in different substrates is shown in Table 2.1. Major materials which use for biogas production can be summarized as follows;

2.5.1 Animal manure

The animals such as cattle, pigs, sheep and goats produce large amounts of manure, which are suitable substrates for anaerobic digestion. Animal manure has been the most common substrate for biogas production by anaerobic digestion (Jingura and Matengaifa, 2009).

2.5.2 Municipality solid waste

Various organic wastes from households and municipal authorities provide municipality solid wastes, a potential feedstock for anaerobic digestion. Anaerobic digestion is one of the most effective processes for getting rid of organic waste material. Anaerobic digestion not only provides pollution prevention but also allows for energy, compost and nutrient recovery. Worldwide there are approximately 150 anaerobic digestion plants in operation using municipality solid wastes or organic industrial waste as their principal feedstock (Jingura and Matengaifa, 2009).

2.5.3 Sewage sludge

Worldwide the anaerobic stabilization of sewage sludge is the most important anaerobic digestion process. In Europe, typically between 30% and 70% of sewage sludge is treated by anaerobic digestion. In developing countries, anaerobic digestion is in most cases the treatment of wastewater. The anaerobic digestion of sewage sludge provides significant benefits as it leads to the production of energy in the form of biogas (Jingura and Matengaifa, 2009).

2.5.4 Crops

In some studies, these have been estimated at over 10 million tones of agricultural crop residues and are disposed through different ways. These crop residues have a high potential as a bioenergy resource and can provide over 123 pJ of energy per year (Hemstock and Hall, 1995). A number of crops demonstrate good biogas potentials. In fact, all C_4 plants have very good growth yields and produce large amounts of biomass. Several crop residues have been shown to be suitable for anaerobic digestion such as cotton waste (Isci and Demir, 2007), maize and rice residues (El-Shinnawi, *et al*., 1989).

Table 2.1 Example of microorganisms in different substrates

Substrates	**Microorganisms**	**References**
Cattle dung	*Methanomicrobiales* *Methanosarcinales* *Methanococcales* *Methanobacteriales*	Rastogi, *et al*., 2008
Swine manure	*Methanobacteriales* *Methanomicrobiales* *Methanosarcinales*	Zhu, *et al*., 2010
Starch	*Clostridium* sp. *Bifidobacterium* sp.	Cheng, *et al.*, 2008
Fodder beet silage	Firmicutes Proteobacteria Bacteroidetes	Klocke, *et al*. 2007
Cassava watewater	*Methanosaeta* *Methanosarcina*	Boonapatcharoen, *et al.*, 2006

Table 2.1 Example of microorganisms in different substrates (continued)

Substrates	Microorganisms	References
Domestic wastewater	Actinobacteria Firmicutes Bacteroidetes Chloroflexi Proteobacteria Methanosaeta Methanospirillum	Ariesyady, *et al*., 2007

2.6 Group of microorganisms involved in anaerobic digestion process

Consortia of microorganisms, mostly bacteria, are involved in the transformation of complex high molecular weight organic compounds to methane. Furthermore, there are synergistic interactions between the various groups of microorganisms implicated in anaerobic digestion of wastes. Each of microorganism groups has their own optimum working conditions such as pH and temperature. Example of hydrogen and methane producing microorganisms are shown in Table 2.2 and Table 2.3. Therefore, for the sake of process stability, it is better to understand the function of the microbial community. The knowledge about microbial community is useful for the start up and control of biogas digesters. For example, the performance of biogas reactors can be controlled by studying and monitoring the variation in parameters like pH, temperature, feedstock type and loading rate to suitable for microorganism involved in the system (Santosh, *et al*., 2004). Different microorganisms are recognized to be involved in the anaerobic fermentation of organic matter to methane can be summarized as follows:

2.6.1 Hydrolytic Bacteria

Consortia of anaerobic bacteria break down complex organic molecules (proteins, cellulose, lignin, and lipids) into soluble monomer molecules such as amino acids, glucose, and fatty acids (Zheng, *et al*., 2009). The monomers are directly available to the next group of bacteria. Hydrolysis of the complex molecules is catalyzed by extracellular enzymes such as cellulases, proteases, and lipases. However, the hydrolytic phase is relatively slow and can be limiting in anaerobic digestion of waste such as raw cellulolytic wastes, which contain lignin (United-Tech, Inc., 2009).

2.6.2 Acidogenic Bacteria

The hydrogen producing, acidogenic bacteria which convert sugars, amino acids, and fatty acids to organic acids, alcohols, ketones, acetate, CO_2, and H_2. Acetate is the main product of carbohydrate fermentation. The products formed vary with the type of bacteria as well as with culture conditions (temperature, pH, redox potential) (United-Tech, Inc., 2009).

Table 2.2 Example of hydrogen producing bacteria and archaea (Tuksadon, 2006)

Broad classification	Name of the microorganism
Fermentative bacteria	*Enterobacter aerogenes*
	E. cloacae
	Clostridium butyricum
	C. pasteurianum
	Desulfovibrio vulgaris
	Magashaera elsdenii
	Citrobacter intermedius
	Escherichia coli

2.6.3 Acetogenic Bacteria

Acetogenic bacteria convert fatty acids (e.g., propionic acid, butyric acid) and alcohols into acetate, hydrogen, and carbon dioxide, which are used by the methanogens (Zheng, *et al*., 2009). This group requires low hydrogen tensions for fatty acid conversion; and therefore a close monitoring of hydrogen concentrations is necessary. Under relatively high H_2 partial pressure, acetate formation is reduced and the substrate is converted to propionic acid, butyric acid and ethanol rather than methane (United-Tech, Inc., 2009).

2.6.4 Methanogen

The methanogen are microorganisms that produce methane as a metabolic by product in anoxic conditions. They were one classified as archaebacteria but archaebacteria have now been reclassified as Archaea, a group quite distinct from bacteria. Methanogen utilize acetate, CO_2 and H_2 to produce methane. The methanogenic phase is strict anaerobic. These microorganisms are sensitive to pH and the optimal pH for methane producing is 6.8-7.2.

Archaea species synthesize methane as an end product of their energy metabolism by utilizing various substrates can be summarized as follows: (1) species exclusively utilization acetate (acetotrophic or acetoclastic methanogens); (2) species using H_2/CO_2 or formate (hydrogenotrophic methanogens); (3) species catabolizing methyl compound; and (4) generalists that form methane from all theses substrates.

Hydrogenotrophic methanogenesis is the mean energy producing pathway of most methanogenic Archaea and it is found in all known genera of Methanobacteriales and Methanomicrobiales. The catabolization of methyl compounds is found within genera of the order Methanococcales, as well the order Methasarcinales. Acetotrophic methanoenesis is known for species of the genus *Methanosaeta* and also for the genus *Methanosarcina* (Klocke, *et al*., 2008).

Table 2.3 Taxonomy of methane producing archaea (Tuksadon, 2006)

Order of methane producing archaea	Family of methane producing archaea	Genus of methane producing archaea	Species of methane producing archaea
Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	*M. formicicum* *M. bryanri* *M. thermoautotrophicum* *M. ruminantium*
		Methanobrevibacter	*M. arboriphilus* *M. smihii* *M. vannielli*
Methanococcales	Methanococcaceae	*Methanococcus*	*M. voltae* *M. mobile*
Methanomicrobiales	Methanomicrobiceae	*Methanogenium*	*M. cariaci* *M. marisnigri*
		Methanosprillum	*M. hungatei* *M. barkeri*
	Methanosarcinaceae	*Methanosarcina*	*M. mazei*

2.7 Genes involved in biogas production

2.7.1 Hydrogenase genes

Hydrogenase (*hydA*) gene codes hydrogenases enzyme which play a central role in hydrogen metabolism in many microorganisms such as sulfate-reducing, photosynthetic, methanogenic, nitrogen-fixing, enteric, and acetogenic prokaryotes. Hydrogenases can be classified to three groups according to their metal content in the H_2-activating sites: [FeFe]-hydrogenases, [NiFe]-hydrogenases and [Fe]-hydrogenases. The [NiFe]-hydrogenases are most often involved in the oxidation of

hydrogen and the [FeFe]-hydrogenases catalyze the reduction of protons as a means of disposal of electrons, according to $2H^+ + 2e^- \longleftrightarrow H_2$, whereas the [Fe]-hydrogenases have been found in some methanogens and catalyze an intermediary step in CO_2 reduction with H_2 to methane. This gene can be found in *Clostridium* species (Tolvanen, *et al*., 2008).

2.7.2 Methyl-coenzyme M reductase genes

Methyl coenzyme M reductase (*mcrA*) gene codes for Methyl-coenzyme M reductase which is the key enzyme of methanogenesis. The presence of the *mcrA* gene is restricted to methanogenic archeae (Thauer, 1998) and is involved in the final stage of methanogenesis causing reduction of methyl group attached to coenzyme M. This enzyme catalyses the reduction of methyl-coenzyme M leading to the release of methane (Ellermann *et al*., 1988). This gene can be found in *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales* and *Methanococcales* (Rastogi, *et al*., 2008).

2.8 Molecular biology techniques for determining microbial diversity

The molecular biology techniques have been used widely to identification of microorganisms. It is a promising alternative to the conventional microbiological techniques, based on the isolation of pure cultures and morphological, metabolic, biochemical and genetic assays, have provided large information that inadequate for study of microbial communities in natural or engineering systems. The molecular biology techniques are based on the RNA of the small ribosomal subunit or their corresponding genes. This molecule was chosen because it is universal and abundant in all living beings. The main molecular biology techniques used to identify and quantify microbial diversity can be summarized as follows:

2.8.1 **Cloning of 16S rDNA** (Sanz and Kochling, 2007)

Cloning and sequencing of 16S rRNA is widely used in the field of microbial ecology. This approach involves of nucleic acids extraction, amplification, 16S rRNA genes cloning, sequencing and finally identification and affiliation of the isolated clone with phylogenetic software. The advantages of the approach can be summarized as follows:

Advantages:

- Covers most microorganisms, including minority groups, which would be hard to detect with genetic fingerprinting methods.
- Identification of microorganisms that have not been yet cultured or identified.

2.8.2 **Denaturant gradient gel electrophoresis (DGGE)** (Sanz and Kochling, 2007)

It is based on the differing mobility on a gel of denatured DNA-fragments of the same size but with different nucleic acid sequences. The number of bands corresponds to the number of dominant species. The most important application of DGGE is monitoring dynamic change in microbial communities, especially when many samples have to be processed. The advantages of the approach can be summarized as follows:

Advantages:

- Rapid and simple monitoring of the microbial populations.
- Easy to obtain an overview of the dominant species of an ecosystem.
- Suitable for analysis of a large number of samples

2.8.3 **Quantitative Real-time PCR** (Hoffmann, *et al*., 2009)

Quantitative Real-Time PCR (qPCR) is based on detection of a fluorescent signal produced proportionally during the amplification of PCR product. This approach is a highly sensitive technique enabling simultaneous amplification and

quantification of specific nucleic acid sequences. qPCR is suitable for a wide range of applications, such as gene quantification and population density of microbial community. The detection is determined by identifying the cycle number at which the reporter dye emission intensities rises above ground noise; this cycle number is called the threshold cycle (C_t). The C_t is inversely proportional to the copy number of the target template. If the template concentration is high, the threshold cycle measured is low. A standard curve can be plotted as C_t value and log concentration of known amounts of DNA or plasmid to find out levels of unknown samples. The advantages of the approach can be summarized as follows:

Advantages:

- Accurate
- Sensitive
- Without labor-intensive post amplification analysis
- Increase dynamic range of detection

2.8.4 Alternatives and new methods

As an alternative to DGGE as a community profiling method, terminal restriction fragment length polymorphism (tRFLP) can be applied when treating complex, species rich samples. This technique is based on the position of a restriction site closest to a labeled end of an amplified gene. In tRFLP the 16S DNA gene is amplified with universal primers, one of them being fluorescently labelled, and the product is digested with frequently cutting restriction enzymes. Given that each species in the sample has differences in the amplified gene sequence, the terminal restriction fragment will differ in size, so can be separated electrophoretically. Furthermore, it is possible to sequence and identify the generated fragments via comparison with a sequence database. The strength of the fluorescent signal yields additional information on the abundance of the different species, though this feature should be regarded with caution, just like the band intensity in patterns of a DGGE gel.

2.9 Relevant studies on microbial diversity of biogas production

Klocke, *et al*. (2007) studied the diversity of microorganisms involved in the biogas process within a completely stirred tank reactor fed continuously with fodder beet silage as mono-substrate. A 16S rDNA library was constructed by PCR amplification and analyzed by amplified rDNA restriction analysis. Major bacteria groups were the class Clostridia, Deltaproteobacteria, Bacilli and members of the phylum Bacteroidetes.

Cheng, *et al*. (2008) explored the bacteria composition in a starch-feeding fermentative hydrogen production reactor. The microorganism community structure from samples was analyzed and quantified using DGGE and FISH. The sequencing 16S rDNA approach was used for bacterial species identification. A more complex Clostridia community and other bacterial species including *Streptococcus* sp., *Pseudomonas* sp. and *Dialister* sp. were found in the system.

Keyser, *et al*. (2006) identified the methanogens in three different types of UASB granules that had been used to treat brewery, winery and peach-lye canning effluents. This study was performed by using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE). The DGGE profiles of the *Archaea* in UASB granules were compared with the DGGE fingerprints of the methanogen reference cultures which included *Methanosaeta Concilii*, *Methanosaeta thermophila, Methanosarcina barkeri*, *Methanosarcina mazeii* and *Methanobacterium formicicum.* In this study, *Methanosaeta Concilii* was found to be detected in the fingerprints of the winery and brewery granules, while *Methanosaeta thermophila* was detected in the fingerprints of the brewery granules. *Methanosarcina mazeii* was only detected in the fingerprints of the winery granules, while *Methanobacterium formicicum* was only detected in the fingerprints of the brewery granules. Identification of the methanogenic Archaea in UASB granules lead to a better understanding of the population shift which can improve the anaerobic process stability.

Tolvanen, *et al*. (2008) examined the hydrogenase (*hydA*) gene and *hydA* transcript level of *Clostridium butyricum* in hydrogen-fermenting bioreactor by a quantitative real-time PCR (qrt-PCR). The detection limit of the qrt-PCR was 3.9 x 10^2 *hydA* copies and the linear range 3.9 x 10^2 - 3.9 x 10^7 *hydA* copies. After a re-inoculation of the bioreactor on day 120, the *hydA* gene number increased and stabilized after day 127. The *hydA* transcript gene number continued to rise until day 142. The results demonstrate that this method is suitable for detecting the *hydA* gene and gene transcript levels of *C. butyricum* in bioreactor samples.

Rastogi, *et al*. (2008) investigated the methanogen community structure in biogas reactor fed with cattle dung in two different seasons; summer and winter by phylogenetic analyses *mcrA* clone libraries. The phylogeny of methanogen based on *mcrA* closely resembles the 16S rDNA therefore *mcrA* was used as a suitable target for PCR-based detection in many molecular ecological studied. In summer month's library, 41.7% clones were to *Methanomicrobiales*, 30% to *Methanosarcinales*, 19% to *Methanobacteriales*, 5% to *Methanococcales* and 4.3% clones belonged to unclassied euryarchaeotal lineages. In winter month's library, 98.6% clones were to *Methanomicrobiales* and 1.4% to *Methanobacteriales*. The *mcrA* gene survey of biogas plant represented a highly diverse methanogenic community. Based on the data obtained, showed the effect of lowering in ambient temperature on the methanogen community structure.

CHAPTER III

METHODOLOGY

3.1 Experimental framework

The main focus of this study concerns to the microbial diversity in two-stage continuous stirred tank reactor using organic waste and biodiesel wastewater as substrate. The experiments are divided into 5 steps (Figure 3.1);

Step1: Sample collection

In this study, two lab bench scale two-stage anaerobic digestion consists of acid tank and methane tank fed with organic waste and biodiesel wastewater as substrate. The samples were collected from both of acid and methane tank of the reactors. For the reactor fed with organic waste, biosludge samples were collected at the steady state condition. While the reactors fed with biodiesel wastewater, samples were collected every week for 6 weeks since the start up state until steady state.

Step2: Microbial community structure analyses by PCR-DGGE

PCR-DGGE used to analysis of bacterial and archaea community.

Step3: Microbial community structure analyses by 16S rDNA clone libraries

A 16s rDNA clone libraries were constructed to analyze microbial community structure.

Step4: Detection of gene involved in biogas production by PCR amplification

Hydrogenase gene (*hydA*) and Methyl-coenzyme M reductase gene (*mcrA*) were selected in this study because they encode a key enzyme of biogas production. The *hydA* gene encodes hydrogenase and the *mcrA* gene encodes methyl-coenzyme M reductase.

Step5: Quantitative Real-time PCR assay for determination of *mcrA* gene copy number

A Qrt-PCR used to quantify the *mcrA* genes during biogas production from samples which taken from reactor using biodiesel wastewater as substrate.

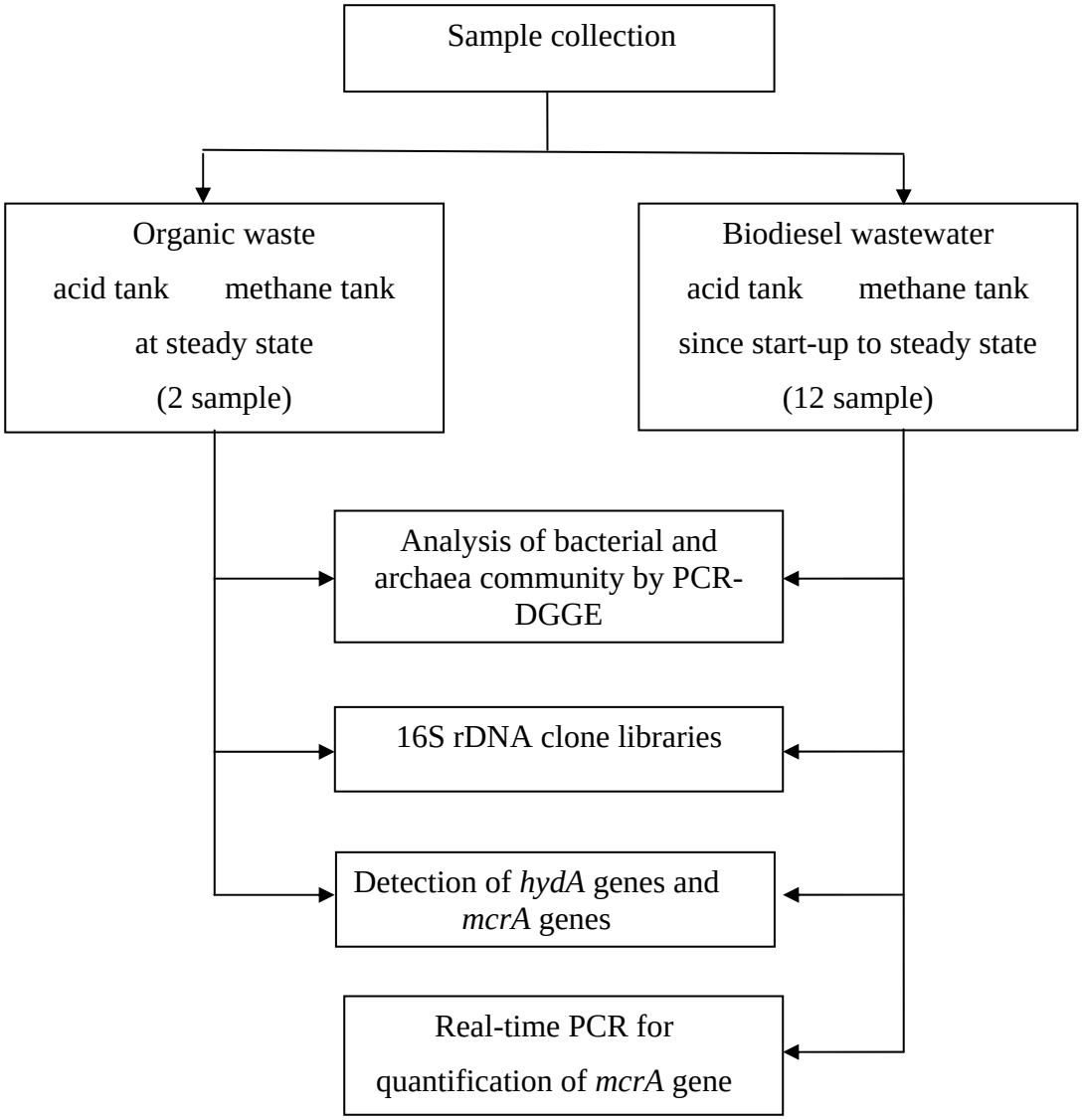

Figure 3.1 Flow chart of research method

3.2 Chemicals and equipments

3.2.1 Chemicals

1. Agarose gel was obtained from IUAI, Japan
2. Ampicillin was obtained from Nacalal tesque, Japan
3. Calcium chloride ($CaCl_2.2H_2O$) was obtained from Merck, Germany
4. Chloroform was obtained from Lab-Scan, Ireland

5. EDTA (ethylenediaminetetraacetic acid), ($C_{10}H_{14}N_2O_8Na_22H_2O$) was obtained from Sigma, USA
6. Glacial acetic acid (CH_3COOH) was obtained from Merck, Germany
7. Glass powder for Recovery of DNA EASYTRAP^{TM} Ver.2 was obtained from TAKARA, Japan
8. Hydrochloric acid (HCl) was obtained from BDH Chemicals, Australia
9. IPTG (Isopropyl thio-β-D-galactoside) was obtained from BIO BASIC INC., Canada
10. Isoamylalcohol was obtained from Sigma, USA
11. Lambda *Hin*dIII was obtained from New England Biolabs, USA
12. Magnesium sulfate ($MgSO_4.7H_2O$) was obtained from Carlo ERBA, France
13. Maxima ^{TM} SYBR Green qPCR Master Mix was obtained from Fermentas, USA
14. PCR purification kit QIAquick PCR purification kit was obtained from Qiagen, Germany
15. Phenol was obtained from Merck, Germany
16. Potassium chloride (KCl) was obtained from Merck, Germany
17. Proteinase K was obtained from US. Biological, USA
18. QIAprep Spin Miniprep Kit was obtained from Qiagen, Germany
19. Restriction enzymes were obtained from Promega, USA and Fermentas, USA
20. Rubidium chloride (RbCl) was obtained from Sigma, USA
21. SDS (sodium dodecyl sulfate), ($C_{12}H_{25}OSO_3$) was obtained from Nacalal tesque, Japan
22. Sodium chloride (NaCl) was obtained from Merck, Germany
23. *Taq* DNA polymerase was obtained from New England Biolabs, USA
24. Trizma base (tris [hydroxymethyl] aminomethane), ($C_4H_{11}NO_3$) was obtained from Sigma, USA
25. Tryptone was obtained from Difco Laboratories, USA
26. X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) was obtained from BIO BASIC INC., Canada

27. Yeast extract was obtained from Difco Laboratories, USA
28. 100 base pair DNA ladder plus was obtained from New England Biolabs, and Fermentas, USA
29. Chemicals used in DGGE were obtained from Bio-Rad Laboratories Inc., USA

    Formamide (Deionized)

    40% Acrylamide/Bis solution, 37.5:1 (2.6% C)

    Urea

    Ammonium persulfate

    TEMED (N,N,N′,N′-Tetra-methyl-ethylenediamine)

    50xTAE

    Dye solution

    Ethidium bromide solution 10 mg/mL

3.2.2 Equipments

1. Autoclave, Kakusan, Japan
2. Balance, model P2002-S and AG285, Mettler Toledo, Switzerland
3. Bench-top centrifuge, model Mikro20, Hettich zentrifugen Inc., USA
4. Deep freezer (-70°C), model ULT 1786, Forma Scientific, Japan
5. Deep freezer (-20°C), model MDF-U332, Sanyo Electronic, Japan
6. DGGE equipments, Bio-Rad Laboratories Inc., USA
7. Digital Dry Bath, model D1100,Labnet International, Inc., USA
8. DNA Thermal Cycler, model 2400, Perkin Elmer, USA and model MJ Mini™ Personal Thermal Cycler, Biorad,USA
9. Gel documentation system, model Gel DOC 2000™, Bio-Rad Laboratories Inc., USA.
10. Hot air oven, model D06063, Memmert, Germany
11. Incubator (37°C), New Brunswick Scientific, Edison NJ., USA
12. ISSCO laminar flow, International Scientific Supply, Japan
13. Micropipette (2, 10, 20, 200, 1,000 and 5,000 µl), Gilson, France

14. Mini Gel migration trough, Cosmo Bio, Japan
15. MiniOpticon Real-Time PCR detector, Bio-Rad Laboratories Inc., USA
16. Oven, Contherm Scientific, New Zealand
17. pH meter, model 240, Corning, USA
18. Qubit fluorometer, Invitrogen, USA
19. Spectrophotometer, model UV-160A, Shimadzu, Japan
20. UV transilluminater, Fotodyne Co., Inc., USA
21. Vortex mixer, model Genie 2, Scientific Industries, USA
22. Water bath, model digital water bath SB-100, EYELA, Japan

3.2.3 Nucleotide sequences of primers

Table 3.1 Nucleotide sequences of primers used in this study

Primer name	Nucleotide sequence (5'-3')	Reference
341F	CCT ACG GGA GGC AGC AG	Muyzer, *et al*, 1993
520R	GTA TTA CCG CGG CGG CTG	Ohkuma, *et al*., 2002
350F	TAC GGG AGG CAG CAG	Yu,*et al*., 2006
1400R	ACGGGCGGTGTGTAC	Kudo, *et al*, 1997
PRA46F	C/TTA AGC CAT GCG/A AGT	Ovreas, *et al*., 1997
PREA1100R	T/CGG GTC TCG CTC GTT G/ACC	Ovreas, *et al*., 1997
PARCH340F	CCCTACGGGGC/TGCAG/CCAG	Ovreas, *et al*., 1997
PARCH519R	TTA CCG CGG CG/TG CTG	Ovreas, *et al*., 1997
933F	GCACAAGCGGTGGAGCATGTGG	Iwamoto, *et.al*., 2000
1387R	GCC CGG GAA CGT ATT CAC CG	Iwamoto, *et.al.*, 2000
GC clamp	CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCG	Kim, *et al*., 2002

Table 3.2 Nucleotide sequences of primers used for detection of genes

Genes	Primer name	Nucleotide sequence (5'-3')	Reference
mcrA genes	*mcrA* F	GGTGGTGTMGGATTCACACARTAYGCWACAGC	Luton, *et al*., 2002
	mcrA R	TTCATTGCRTAGTTWGGRTAGTT	
hydA genes	*hydA* F	TCACCACAACAAATATTTGGT	Fang, *et al*., 2006
	hydA R	GCTGCTTCCATAACTCC	

3.3 Sample collection

In this study, the bioreactor consisting of acid tank and methane tank fed with organic waste and biodiesel wastewater as substrate were used as models for anaerobic treatment system. Biosludge samples were collected from both of acid and methane tanks of the reactors. For the reactor fed with organic waste, biosludge samples were collected at the steady state condition. While the other reactor, sample were collected every week for 6 weeks since the start up state until steady state.

3.4 Analysis of bacterial and archaeal community in two-stage anaerobic digestion system

3.4.1 DNA extraction and purification

For the extraction of DNA from sludge, 1.5 ml of sample from bioreactor was added into tube, centrifuged at 8000 rpm for 5 minutes and discarded supernatant. Nine hundred microliters of DNA extraction buffer (1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, 1 M Na_2PO_4) was added in the sample tube and mixed by vortex. Then 20 μl of 20 mg/ml protenase K solution and 20 μl of 60 mg/ml lysozyme solution were added and mixed by invention. After incubation at 37˚C for 30 minutes, three freeze-thaw steps were performed through freezing at -80˚C and thawing at 65˚C, and then 100 μl of a 20% SDS was added into the tube. The samples were incubated at 65˚C for 2

hours. After that samples were centrifuged at 10,000 rpm for 10 minutes and upper phase was collected. Two extraction steps were performed by adding equal volume of phenol:chloroform and mixed by inventing and then centrifuged at 12,000 rpm for 10 minutes and collected upper phase. Equal volume of chloroform:isoamylalcohol (24:1) was then added and mixed by inventing then centrifuged at 12,000 rpm for 10 minutes and collected upper phase. The DNA was precipitated by adding 0.8 volume of isopropanol and mixed by inventing then centrifuged at 12,000 rpm for 15 minutes and discarded supernatant. The DNA was washed with 70% ethanol then centrifuged at 13,000 rpm for 10 minutes and the pellet was dried at 37˚C for 30 minutes. Then TE buffer was added and the DNA solution was stored at -20˚C.

Extracted DNA was separated by electrophoresis in a 0.9% agarose gel in 1x TAE buffer and visualized under UV light through staining with ethidium bromide. Then the genomic DNA was purified by using Glass powder for Recovery of DNA EASYTRAP^{TM}Ver.2 (TAKARA BIO INC, JAPAN), according to the manufacturer's instructions. The band in gel was cut and put in sterilized microtube. NaI 3 volumes of gel weight was added and the tube was incubated at 55˚C until the gel was completely melted. Glass powder 5 µl per 1 µg of DNA was added, mixed well and let it settle down for 5 minutes then centrifuged at 10,000 rpm for 5-10 seconds and discarded supernatant. Washing buffer 5 volumes of applied glass powder was added and mixed well then centrifuged at 10,000 rpm for 5-10 seconds, discarded supernatant and air dried washing buffer completely. TE buffer 1-2 volumes of applied glass powder was added and incubated at 55°C for 2-5 minutes then centrifuged at 10,000 rpm for 5-10 seconds. DNA solution was transferred to new sterilized microtube. DNA solution was stored at -20˚C prior to PCR reactions.

3.4.2 PCR amplification

The PCR amplification targeting bacterial 16S rDNA was performed by using a touchdown PCR. The PCR was carried out with the forward primer 341F containing a GC-clamp and the reverse primer 520R to generate a product of 200 bp. Primers used in this study are shown in Table 3.1. The PCR mixture contains: 100 ng extracted DNA, 0.2 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, 20 pmol of

each primer and was filled up to the final volume of reaction of 30 µl with distilled water. The touchdown PCR was carried out under the following condition:

1. Initial denaturation step at 94°C for 5 min
2. Touchdown program for 20 cycles
   2.1 Denaturation step at 94°C for 1 min
   2.2 Annealing step at 65°C for 1 min
   (decreasing annealing temperatures in decrements of 0.5˚C per cycle)
   2.3 Extension step at 72°C for 2 min
3. Denaturation step at 94°C for 1 min
4. Annealing step at 55°C for 1 min
5. Extension step at 72°C for 2 min
6. Go to step 3-5 for 30 cycles
7. Final extension at 72°C for 10 min

The PCR amplification targeting archaea was carried out with the forward primer PRA46F and the reverse primer PREA1100R as shown in Table 3.1 to generate a product of 1072 bp. This PCR product was then used as a template for the PCR amplification of 179 bp using the forward primer PARCH340F containing a GC-clamp and the reverse primer PARCH519R as shown in Table 3.1. The PCR mixture contains: 100 ng extracted DNA, 0.2 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, 20 pmol of each primer and was filled up to the final volume of reaction of 30 µl with distilled water.The PCR amplification condition for the first primer sets was as follows:

1. Initial denaturation step at 94°C for 5 min
2. Denaturation step at 94°C for 1 min
3. Annealing step at 53.5°C for 1 min
4. Extension step at 72°C for 2.23 min
5. Go to step 2-4 for 35 cycles
6. Final extension at 72°C for 7 min

The PCR amplification condition for the second primer sets was as follows:

1. Initial denaturation step at 94°C for 5 min
2. Denaturation step at 94°C for 45 sec
3. Annealing step at 52°C for 45 sec
4. Extension step at 72°C for 45 sec
5. Go to step 2-4 for 30 cycles
6. Final extension at 72°C for 10 min

The PCR products were checked by electrophoresis in a 2% agarose gel in 1x TAE buffer through staining with ethidium bromide and visualized under UV light.

3.4.3 DGGE

DGGEs were carried out using the DCode^{TM} system (Bio-Rad Laboratories Inc., USA) PCR products were loaded onto 8% polyacrylamide gel with a 30% to 70% denaturant gradient (100% denaturant was defined as 7 M urea and 40% formamide). Electrophoresis was performed at a constant condition of 60˚C and 130 V for 5 hours in 7 liters of 1x TAE buffer. After electrophoresis, the gel was stained with ethidium bromide for 20 minutes and visualized under UV light.

3.4.4 Sequencing of DGGE bands

3.4.4.1 Amplification of DNA and purification of PCR product

Bands excised from the DGGE gels were eluted in 30 μl of distilled water over night at 4˚C. Eluted DNA 1 μl was used as the PCR template with the primer 350F and 520R for bacterial 16S rDNA and the primer PARCH340F and PARCH519R for archaea. Primers used in this study are shown in Table 3.1. The PCR amplification condition was as follows:

1. Initial denaturation step at 94°C for 5 min
2. Denaturation step at 94°C for 30 sec
3. Annealing step at 50°C for 30 sec
4. Extension step at 72°C for 1 min
5. Go to step 2-4 for 30 cycles
6. Final extension at 72°C for 7 min

The PCR products were checked by electrophoresis in a 2% agarose gel in 1x TAE buffer through staining with ethidium bromide and visualized under UV light. Then the PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Germany), according to the manufacturer's instructions. PB buffer 5 volume of PCR product was added, mixed and transferred to QIAprep spin column then centrifuged at 13,000 rpm for 1 minute and discarded flow-through solution. Added 750 µl of PE buffer into column then centrifuged at 13,000 rpm for 1 minute, discarded flow-through solution and centrifuged again. The column was transferred to new sterilized microtube. Deionized water or EB buffer for 30-50 µl was added to the center of column and let the column stand for 1 minute then centrifuged at 13,000 rpm for 1 minute. Purified PCR products were stored at -20°C.

3.4.4.2 Cloning of PCR product

The purified PCR products were ligated through pGEM-T Easy Vector (Promega, USA) of which the reaction is described as below:

2X ligation buffer	5	µl
pGEM-T Easy Vector (50 ng)	1	µl
The purified PCR product (100 ng)	1	µl
T4 DNA Ligase (3 U)	1	µl
Deionized water	2	µl

The ligase reaction was incubated overnight at 4 ºC. Then, the ligation mixture was transformed into the competent *E.coli* JM109 cell which was prepared by calcium chloride method (Sambrook and Russell, 2001). The competent cell was prepared by streaked *E.coli* JM 109 on Ψb agar and incubated at 37°C for 16 – 18

hours. The single colony of the strain was transferred to 5 ml of Ψb broth and shaken for 4 hours until OD_{600} was 0.3-0.5. Then 5 ml of cell suspension was transferred into arm flask containing 100 ml of Ψb broth then it was shaken at 37°C until OD_{600} reached 0.5. Cell suspension was transferred into sterilized centrifuged tube and stored in ice for 5 minutes and centrifuged at 3,000 rpm, 4°C for 5 minutes. Supernatant was discarded then 40 ml of TfbI solution was added and mixed by hand. Centrifuge tube contained cell suspension was stored in ice for 5 minutes then centrifuged at 3,000 rpm, 4°C for 5 minutes and discarded supernatant. Four milliters of cold TfbII solution was added to suspended cell pellet and kept in ice for at least 15 minutes. One hundred microliters of cell suspension was aliquoted into sterilized microtube. Competent cell was stored at -70°C.

Recombinant plasmid was transformed into competent cell by heat shock method (Sambrook and Russell, 2001). Competent cell was thawed in ice. Two microliters of ligated recombinant plasmid was added to 50 µl of competent cell, then mixed and incubated in ice for 20 minutes. Heat shocked the cell by put into heat box at 42°C for 45-50 seconds then put into ice immediately for 2 minutes. Added 950 µl of SOC broth and incubated at 37°C for at least 1 hour.

Then, the transformed solution was spreaded on the LB agar containing 100 µg/ml of amplicilin, 100 µg/ml of X-gal, and 100 µg/ml of IPTG. The plate was incubated at 37 ºC for 16 – 24 hours. The white colonies were picked to check the insert fragment. The white colonies were grown in the LB broth containing 100 µg/ml amplicilin at 37 ºC overnight.

3.4.4.3 Plasmid extraction

The plasmid was extracted by using QIAprep Spin Miniprep Kit (Qiagen, Germany), according to the manufacturer's instructions. The cell was harvested by centrifuged cell suspension at 10,000 rpm, room temperature for 2 minutes and re-suspended cell in 250 µl of P1 buffer. P2 buffer 250 µl was added and mixed by inverting. N3 solution for 350 µl was added, inverted until white pellet was observed then centrifuged at 13,000 rpm for 10 minutes. Supernatant was transferred into QIAprep Spin Column, centrifuged at 13,000 rpm for 1 minute and discarded flow-

through solution. PB buffer for 500 µl was added into column and centrifuged at 13,000 rpm for 1 minute. PE buffer 750 µl was added into column, centrifuged at 13,000 rpm for 1 minute then discarded flow-through solution and centrifuged again. The column was transferred to sterilized microtube. Deionized water or EB buffer for 50-100 µl was added to the center of the column and let the column stand for 1 minute then centrifuged at 13,000 rpm for 1 minute. Plasmid solution was stored at -20°C until being used.

3.4.4.4 Digestion of recombinant plasmid by restriction enzyme

Extracted plasmid was digested with *Eco*RI restriction enzyme to confirm the presence of inserted fragment. The restriction digestion condition was described as below:

Plasmid (pGEM-T Easy Vector)	1	µl
10X Buffer	1	µl
*Eco*RI enzyme (0.5 U)	0.5	µl
Steriled water	7.5	µl

The digest reaction was incubated overnight at 37 ºC. The insert fragment was examined by running in 2% agarose gel electrophoresis.

3.4.4.5 Nucleotide base sequencing

Five clones of each band were sent for sequencing at 1st Base Co., Ltd., Malaysia. The sequence results were analyzed using BLASTn program to identify the bacterial and archaeal species.

3.4.4.6 Phylogenetic analysis

Alignment of clonal sequence and sequence from selected reference species from the NCBI Genbank were performed with the software Clustal X using standard setting. Phylogenetic trees were constructed by the neighbor-joining method.

Bootstrap resamplings analysis for 100 replicates was performed to estimate the confidence of tree topologies.

3.4.5 Statistical analysis

Statistical analysis of the DGGE band was carried out using STATISTICA 7.0 software (StatSoft. Inc. USA). The band patterns were analyzed by cluster analysis using tree clustering with an unweighted pair-group centroid.

3.5 Clone libraries of 16S rDNA

3.5.1 DNA extraction and purification

DNA was extracted from the samples of bioreactor fed with organic waste and biodiesel wastewater as substrate in both of acid and methane tank in the steady state (total 4 samples). Extraction and purification were carried out by using method described in 3.3.1

3.5.2 PCR amplification

The PCR amplification targeting bacterial 16S rDNA was carried out with the forward primer 350F and the reverse primer 1400R. The PCR mixture contains: 100 ng of extracted DNA, 0.2 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, 20 pmol of each primer and was filled up to the final volume of reaction of 30 µl with distilled water. The PCR amplification condition was as follows:

1. Initial denaturation step at 94°C for 5 min
2. Denaturation step at 94°C for 30 sec
3. Annealing step at 55°C for 1 min
4. Extension step at 72°C for 1 min
5. Go to step 2-4 for 30 cycles
6. Final extension at 72°C for 6 min

The PCR products were checked by electrophoresis in a 2% agarose gel in 1x TAE buffer through staining with ethidium bromide and visualized under UV light.

3.5.3 Purification of PCR product

PCR products were purified using method described in 3.4.4.1.

3.5.4 Cloning of PCR product

PCR products were cloned using method described in 3.4.4.2.

3.5.5 Plasmid extraction

The plasmids were extracted using method described in 3.4.4.3.

3.5.6 Screening of clone libraries by PCR-DGGE analysis

3.5.6.1 PCR amplification

The PCR amplification was carried out with the forward primer 933F containing a GC-clamp and the reverse primer 1387R to generate a product of 454 bp. Primers used in this study are shown in Table 3.1. The PCR mixture contains: 100 ng of plasmid, 0.2 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, 20 pmol of each primer and was filled up to the final volume of reaction of 30 µl with distilled H_2O. The PCR amplification condition was as follows:

1. Initial denaturation step at 95°C for 5 min
2. Denaturation step at 94°C for 1 min
3. Annealing step at 55°C for 1 min
4. Extension step at 72°C for 2 min
5. Go to step 2-4 for 30 cycles
6. Final extension at 72°C for 10 min

The PCR products were checked by electrophoresis in a 2% agarose gel in 1x TAE buffer through staining with ethidium bromide and visualized under UV light

3.5.6.2 DGGE

PCR products were loaded using method described in 3.4.3.

3.5.7 Sequencing

Selected plasmids were sent for sequencing at 1st Base Co., Ltd., Malaysia. The sequence results were analyzed using and BLASTn programs.

3.6 Detection of genes involved in biogas production by PCR amplification

3.6.1 DNA extraction and purification

DNA was extracted from the samples of bioreactor fed with organic waste and biodiesel wastewater as substrate in both of acid and methane tank in the steady state (total 4 samples). Extraction and purification were carried out by using method described in 3.3.1

3.6.2. PCR amplification

The PCR amplification of methyl-coenzyme M reductase gene (*mcrA*) that codes for Methyl-coenzyme M reductase which is the key enzyme of methanogenesis *mcrA* gene was carried out with the forward primer *mcrA* F and the reverse primer *mcrA* R to generate a product of 464-491 bp. Primers used in this study are shown in Table 3.2. The PCR mixture contains: 100 ng of extracted DNA, 0.2 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, 20 pmol of each primer and was filled up to

the final volume of reaction of 30 µl with distilled H_2O. The PCR amplification condition was as follows:

1. Initial denaturation step at 95°C for 5 min
2. Denaturation step at 95°C for 1 min
3. Annealing step at 55°C for 1 min
4. Extension step at 72°C for 1 min
5. Go to step 2-4 for 25 cycles
6. Final extension at 72°C for 10 min

The PCR amplification of hydrogenase genes (*hydA*) which play a central role in hydrogen metabolism was carried out with the forward primer *hydA* F and the reverse primer *hydA* R to generate a product of 300 bp. Primers used in this study are shown in Table 3.2. The PCR mixture contain: 100 ng of extracted DNA, 0.2 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, 20 pmol of each primer and was filled up to the final volume of reaction of 30 µl with distilled H_2O. The PCR amplification condition was as follows:

1. Initial denaturation step at 94°C for 7 min
2. Denaturation step at 92°C for 30 sec
3. Annealing step at 52°C for 30 sec
4. Extension step at 72°C for 30 sec
5. Go to step 2-4 for 35 cycles
6. Final extension at 72°C for 10 min

The PCR products were checked by electrophoresis in a 2% agarose gel in 1x TAE buffer through staining with ethidium bromide and visualized under UV light.

3.6.3 Purification of PCR product

PCR products were purified using method described in 3.4.4.1.

3.6.4 Cloning of PCR product

PCR products were cloned using method in 3.4.4.2.

3.6.5 Plasmid extraction

The plasmids were extracted using method in 3.4.4.3.

3.6.6 Digestion of recombinant plasmid by restriction enzyme

Extracted plasmids were digested using method in 3.4.4.4.

3.6.7 Restriction Fragment Length Polymorphisms (RFLPs)

Recombinant plasmids which have the correct DNA insert fragment were analyzed by using Restriction Fragment Length Polymorphisms (RFLPs) to group the plasmids that have the same pattern of DNA arrangement. The order of usage for restriction enzymes for RFLPs was *BSu*RI, *Hin*FI and then *Rsa*I. The condition of RFLPs was the same as described for *Eco*RI in 3.3.4.4.

3.6.8 Sequencing

Selected plasmids were sent for sequencing at 1st Base Co., Ltd., Malaysia. The sequence results were analyzed using and BLASTx programs.

3.7 Quantitative real-time PCR assay for determination of gene copy number

3.7.1 DNA extraction and purification

For the extraction of DNA, duplicate samples of bioreactor fed with biodiesel wastewater as substrate from both acid and methane tank were taken during the

operation. The samples were collected in every week for 6 weeks since the start up state until steady state (total 12 samples). DNA was extracted and purified using method described in 3.3.1.

3.7.2 Standard for real-time PCR calibration

External standards used to determine gene copy number of *mcrA* genes. The plasmid concentration was measured by using Quant-iT™ dsDNA BR Assay Kits with Qubit fluorometer. The plasmid copy number was calculated using the equation as follows:

$$\text{Number of copies per microliter} = \frac{(6 \times 10^{23})(\text{DNA concentration})}{\text{molecular weight of one genome}}$$

The molecular weight of 1 bp is 660 g/mol. The number of copies per mole is 6×10^{23}. The DNA concentration is given in grams per microliter. The molecular weight of one genome is given in grams per mole. Series of 10-fold dilutions of the plasmid were prepared, and these dilutions of the plasmid were amplified with DNA samples. Linear regression equation for obtained cycle threshold values (Ct) was calculated as a function of known plasmid copy number.

3.7.3 Real-time PCR for quantification of *mcrA* genes

The primer set which acts as marker gene was used for quantify amount of selected genes in bioreactor by real-time PCR assay. Real-time PCR experiments were performed in a MiniOpitcon Real-Time PCR detector associated with MJ Opticon Monitor Analysis Software (version 3.1, Bio-Rad). The reaction was performed in 0.2 ml thin wall, clear PCR strip tubes which have 25 µl reaction volumes containing Maxima ^{TM} SYBR Green qPCR Master Mix (Fermentas), 0.3 µM of primers and 2 µl of template DNA (10 times dilution series of plasmid standard and DNA samples). The amplifications were carried out with following program:

1.	Initial denaturation step	at 95ºC	for 10 min
2.	Denaturing step	at 94ºC	for 1 min
3.	Annealing step	as 57 ºC	for 1 min
4.	Extension step	at 72ºC	for 1 min
5.	Go to step 2 for 39 times		
6.	Final extention step	at 72˚C	for 10 min

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Sample collection

Bioreactor used in this study was a lab bench scale two-stage anaerobic digestion fed with two different substrate; organic waste and biodiesel wastewater. The reactor consisted of acid tank and methane tank. For bioreactor which using organic waste as substrate, the total volume of each tank was 1.45 liters. The reactor was carried out continuously, under the room temperature, with a hydraulic retention time (HRT) of 2 days and 15 days, respectively and pH 5.5 and 7.48, respectively. The samples were taken from both of acid tank and methane tank of the reactor during the 43 days steady state operation. For bioreactor which using biodiesel wastewater as substrate, the total volume of each tank was 0.5 liters and 5 liters, respectively. The reactor was carried out continuously, under the room temperature, with a hydraulic retention time (HRT) of 1 day and 10 days, respectively. The samples were taken from both of acid tank and methane tank of the reactor during operation in every week for 6 weeks since the start up state until steady state.

Performance of bioreactor fed with organic waste and biodiesel wastewater as substrate were summarized in Table 4.1 and Table 4.2.

Table 4.1 Performance of bioreactor fed with organic waste as substrate (Lanna, 2009)

Parameters	**Organic waste**	
	acid tank	**methane tank**
Biogas yield production (l/day)	18.25	7.15
CH_4%	26.23	66.38
$pH_{influent}$	5.50	7.48
$pH_{effluent}$	5.49	7.50
Temperature (˚C)	31.37	31.63
Hydraulic retention time (day)	2	15
$COD_{influent}$ (mg/l)	33,153	28,196
$COD_{effluent}$ (mg/l)	28,196	6,252
% Reduction in COD	14.95	77.83
Soluble $COD_{influent}$ (mg/l)	22,969	20,556
Soluble $COD_{effluent}$ (mg/l)	20,556	782
% Reduction in Soluble COD	10.51	96.18
$TS_{influent}$ (g/l)	35.68	21.92
$TS_{effluent}$ (g/l)	21.92	12.80
% Reduction in TS	38.57	41.59
$VS_{influent}$ (g/l)	30.29	15.59
$VS_{effluent}$ (g/l)	15.59	7.24
% Reduction in VS	48.53	53.56
Volatile fatty acid (mg/l)	8,793	785
Alkalinity (mg/l)	5,747	6,427
Volatile fatty acid/alkalinity ratio	1.53	0.12

Table 4.2 Performance of bioreactor fed with biodiesel wastewater as substrate (Panadda, 2009)

Parameters	**Biodiesel wastewater**	
	acid tank	**methane tank**
Biogas yield production (l/day)	5.86	6.99
CH_4%	60.48	63.60
pH	7.74	8.75
Temperature (˚C)	31.61	31.16
Hydraulic retention time (day)	1	10
Organic loading rate (kgCOD/ m^3-d)	1.50	1.50
Soluble $COD_{influent}$ (mg/l)	15,696	6,617
Soluble $COD_{effluent}$ (mg/l)	6,617	833
% Reduction in Soluble COD	57.84	87.40
Methanol $_{influent}$ (mg/l)	2,767	1,298
Methanol $_{effluent}$ (mg/l)	1,298	332
% Reduction in Soluble Methanol	53.10	74.43
Glycerol $_{influent}$ (mg/l)	2,028	1,089
Glycerol $_{effluent}$ (mg/l)	1,089	669
% Reduction in Soluble Glycerol	46.30	38.54
Volatile fatty acid (mg/l)	4,978	784
Alkalinity (mg/l)	6,380	7,643
Volatile fatty acid/alkalinity ratio	0.78	0.11

4.2 Analysis of microbial communities in two-stage anaerobic digestion system

4.2.1 Analysis of bacterial communities in two-stage anaerobic digestion system using biodiesel wastewater as substrate

4.2.1.1 DGGE analysis

The analysis of bacterial communities was conducted using DGGE technique. Samples were taken from bioreactors fed with biodiesel wastewater in both of acid and methane tank after 30 weeks of operation. Week 0 was the first week that the system changed the organic loading rate (OLR) to 1.50 kgCOD/ m^3-d then samples were collected in every week for 6 weeks since the start up state until steady state of this OLR. DNA was extracted and 16S rDNA were amplified by PCR using primers 341F with GC clamp and 520R. The amplified fragments were run in DGGE. The changes of bacterial populations are shown in Figure 4.1.

From the result, the profiles of bacterial communities in acid tank and methane tank were different. In acid tank, DGGE profiles of week 0 to week 3 (a) were almost similar. Bands A1, A2, A3, A4 and A5 always presented in all weeks. While, band A6 was detected since week 3 and still presented until week 5. Band A7 was only detected in the last week. For the profile of bacterial community in methane tank (b), the bands C1, C2, C3, C4 and C5 were observed in every week. While, band C6 was detected since week 0 until week 3 and disappeared in week 4.

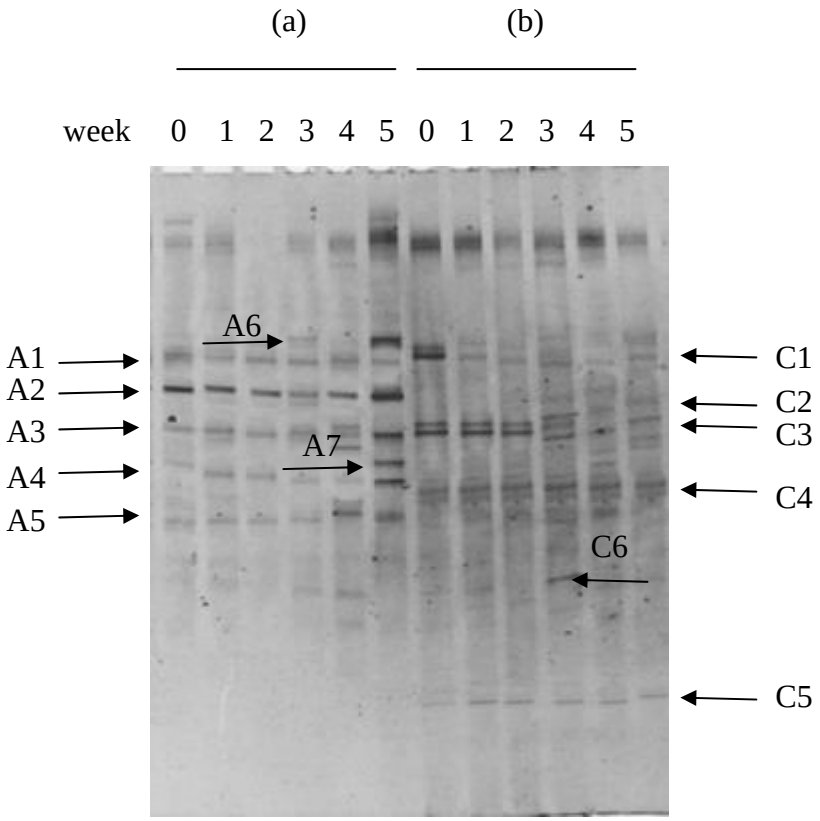

Figure 4.1 DGGE profile of bacterial communities from bioreactor using biodiesel wastewater as substrate; condition: 30-70% denaturant gradient (a): biosludges from acid tank weeks 0-5, (b): biosludge from methane tank weeks 0-5

Dominant DNA bands (A1, A2, A3, A4, A5, A6, A7, C1, C2, C3, C4, C5 and C6) were cut and eluted in distilled water and re-amplified. PCR products were ligated to pGEM-T Easy Vecter and transformed into competent *E.coli* JM109. Required colonies were picked and extracted plasmid that contain inserted PCR products. Extracted plasmids were subjected to sequence analysis and the sequences of PCR products were compared to GenBank database using BLASTn software (http://www.ncbi.nlm.nih.gov/). The results of 16S rDNA comparison of bacterial communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software are shown in Table 4.3.

The DGGE profile of acid tank, band A1 showed 100% sequence similarity to *Megasphaera sueciensis*. *Megasphaera* species are strictly anaerobes and have ability

to ferment carbohydrate, utilize organic acid and produce gas (Juvonen and Suihko, 2006). Band A2 showed 100% sequence similarity to *Pectinatus* sp. C5. *Pectinatus* species are strictly anaerobic mesophiles with fermentative metabolism. Glucose and fructose are mainly metabolized to acetic and propionic acids. H_2S and acetoin and occasionally minor amounts of succinic acid are produced (Lee, *et al*., 1978). Band A3 showed 98% sequence similarity to uncultured *Pseudomonas* sp. that had previously been found in hydrogen production reactor (Cheng, *et al*., 2008). Band A4 showed 100% sequence similarity to *Azospira* sp. that had been isolated from activated sludge derived from municipal wastewater treatment plants, characterizing a denitrifying potential (Heylen *et al*., 2006). Band A5 showed 95% sequence similarity to *Clostridium acetobutylicum* and Band A7 showed 100% sequence similarity to uncultured *Clostridium* sp. In general, *Clostridium* species are well-known hydrogen producing bacteria in anaerobic hydrogen fermentation. Cheng, *et al*. (2008) revealed that *Clostridium* species were determined by DGGE and FISH from a starch-feeding fermentative hydrogen production reactor. Band A6 showed 98% sequence similarity to uncultured Bacteroidetes bacterium that had been recovered from biogas-producing completely stirred tank reactor fed with fodder beet silage as mono-substrate (Klocke, *et al*., 2007).

The DGGE profile of methane tank, band C1 showed 95% sequence similarity to *Clostridium kluyveri*. Band C2 showed 99% sequence similarity to *Propionibacterium* sp. B2M2. It is strictly anaerobic saccharolytic organism and produces acetate and traces of ethanol from glucose. It also ferments casaminoacid, peptone, pepticase, arginine and yeast extract, and it is able to reduce the elemental sulfur to hydrogen sulfide (Diaz, *et al*., 2010). Bands C3 and C4 showed 100% sequence similarity to *Pseudomonas* sp. and uncultured *Pseudomonas* sp. Band C5 showed 98% sequence similarity to uncultured delta proteobacterium that had been recovered from biogas-producing completely stirred tank reactor fed with fodder beet silage as mono-substrate (Klocke, *et al*., 2007).. Band C6 showed 100% sequence similarity to uncultured Bacteroidetes bacterium.

Table 4.3 The result of comparison 16S rDNA of bacterial communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software

DNA band	Clone no.	Bacterial strains	Accession no.	Sequence Identity (%)	References
A1	1	*Megasphaera sueciensis*	DQ223729	188/188 (100%)	Juvonen and Suihko, 2006
	2	*Megasphaera sueciensis*	DQ223729	175/181 (96%)	Juvonen and Suihko, 2006
	3	*Megasphaera sueciensi*	DQ223729	190/190 (100%)	Juvonen and Suihko, 2006
	4	*Megasphaera paucivorans*	DQ223730	187/188 (99%)	Juvonen and Suihko, 2006
	5	*Megasphaera cerevisiae*	EU589448	165/181 (91%)	Juvonen, *et.al.*, 2008
A2	1	*Pectinatus* sp. C5	GU586299	180/180 (100%)	Wenzel, *et.al.*, (unpublished)
	2	*Pectinatus* sp. C5	GU586299	178/178 (100%)	Wenzel, *et.al.*, (unpublished)
	3	*Pectinatus* sp. C5	GU586299	192/192 (100%)	Wenzel, *et.al.*, (unpublished)
	4	*Pectinatus* sp. H2	FJ668029	178/178 (100%)	Castello, *et.al.*, (unpublished)
	5	*Pectinatus frisingensis*	EU589446	192/192 (100%)	Juvonen, *et.al.*, 2008
A3	1	uncultured *Pseudomonas* sp.	FN666225	100/102 (98%)	Sayeh, *et.al.*, (unpublished)
	2	uncultured *Pseudomonas* sp.	FN666225	106/107 (99%)	Sayeh, *et.al.*, (unpublished)

Table 4.3 The result of comparison 16S rDNA of bacterial communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software (continued)

DNA band	Clone no.	Bacterial strains	Accession no.	Sequence Identity (%)	References
	3	*Brevundimonas diminuta*	FN796836	167/167 (100%)	Becerra-Castro, *et.al.*, (unpublished)
	4	*Comamonas* sp. JMC-UBL 19	HM451433	149/150 (99%)	Senthil Kumar, *et.al.*, (unpublished)
	5	uncultured *Brevundimonas* sp.	HQ132463	146/146 (100%)	Zhang, *et.al.*, (unpublished)
A4	1	uncultured *Azospira* sp.	FJ823859	160/160 (100%)	Borole, *et.al*, 2009
	2	*Azospira* sp. ECC1-pb2	GU202937	192/192 (100%)	Sun, *et.al*, (unpublished)
	3	uncultured *Azospira* sp.	GU216627	161/161 (100%)	Steinbusch, *et.al*, (unpublished)
	4	*Azospira* sp. IHBB 2277	HM233970	179/179 (100%)	Gulati, *et.al*, (unpublished)
	5	uncultured bacterium	GU616865	141/145 (97%)	Jeong (unpublished)
A5	1	*Clostridium acetobutylicum*	AM231184	159/167 (95%)	Berezina, *et.al*., 2008
	2	*Clostridium sartagoforme*	FJ384380	158/167 (94%)	Johansson, *et.al.*, (unpublished)
	3	uncultured bacterium	FJ825468	157/163 (96%)	Podmirseg, *et.al.*, (unpublished)

Table 4.3 The result of comparison 16S rDNA of bacterial communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software (continued)

DNA band	Clone no.	Bacterial strains	Accession no.	Sequence Identity (%)	References
	4	uncultured bacterium	EF688246	162/168 (96%)	Roest (unpublished)
	5	uncultured bacterium	FJ825468	159/165 (96%)	Podmirseg, *et.al.*, (unpublished)
A6	1	Uncultured Bacteroidetes bacterium	EU551096	178/181 (98%)	Wang, *et.al.*, 2009
	2	uncultured Bacteroidetes bacterium	CU926845	176/181 (97%)	Riviere, *et.al.*, 2009
	3	*Syntrophomonas palmitatica*	AB274040	169/183 (92%)	Hatamoto, *et.al*, 2007
	4	uncultured Firmicutes bacterium	CU924171	147/161 (91%)	Riviere, *et.al*, 2009
	5	uncultured Firmicutes bacterium	CU920790	160/176 (90%)	Riviere, *et.al*, 2009
A7	1	*Agrobacterium* sp.EC2_3502	EU877077	167/168 (99%)	Gren, *et.al.*, (unpublished)
	2	uncultured *Clostridium* sp.	GU556245	160/160 (100%)	Rotaru, *et.al.*, (unpublished)
	3	uncultured *Dechloromonas* sp.	FJ525535	158/161 (98%)	Li, *et.al.*, (unpublished)
	4	uncultured *Dechloromonas* sp.	FJ525534	172/175 (98%)	Li, *et.al.*, (unpublished)

Table 4.3 The result of comparison 16S rDNA of bacterial communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software (continued)

DNA band	Clone no.	Bacterial strains	Accession no.	Sequence Identity (%)	References
	5	uncultured bacterium	GU591545	153/153 (100%)	Aguirre de Carcer and Chang (unpublished)
C1	1	*Clostridium kluyveri* DSM 555	CP000673	133/140 (95%)	Seedorf, *et.al*, 2008
	2	uncultured *Clostridium* sp.	GU216630	129/136 (94%)	Steinbusch, *et.al.*, (unpublished)
	3	*Clostridium* sp. R9	GU097452	125/133 (93%)	Liu, *et.al.*, (unpublished)
	4	uncultured bacterium	AM921478	125/130 (96%)	Malinowska (unpublished)
	5	uncultured Firmicutes bacterium	FM252564	88/95 (92%)	Ladygina, *et.al*, 2009
C2	1	uncultured bacterium	FM242723	111/113 (98%)	Byrne, *et.al*, 2009
	2	*Propionibacterium* sp. B2M2	EU980607	171/172 (99%)	Diaz, *et.al*, (unpublished)
	3	uncultured bacterium	CU918461	169/169 (100%)	Riviere, *et.al*, 2009
	4	Propionibacteriaceae bacterium WN033	AB377178	159/159 (100%)	Ueki, *et.al*, (unpublished)
	5	*Propionibacterium* sp. B2M2	EU980607	150/150 (100%)	Diaz, *et.al*, (unpublished)

Table 4.3 The result of comparison 16S rDNA of bacterial communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software (continued)

DNA band	**Clone no.**	**Bacterial strains**	**Accession no.**	**Sequence Identity (%)**	**References**
C3	1	uncultured bacterium SJA-88	AJ009479	120/124 (96%)	von Wintzingerode, *et.al*, 1999
	2	uncultured Firmicutes bacterium	GQ483893	108/114 (94%)	Myshrall, *et.al*, 2010
	3	*Pseudomonas* sp. PsS79	HM627629	182/182 (100%)	Mehri, *et.al*, (unpublished)
	4	uncultured gamma proteobacterium	FM252627	195/202 (96%)	Ladygina, *et.al*, 2009
	5	Pseudomonas sp. PsS79	HM627629	184/184 (100%)	Mehri, *et.al*, (unpublished)
C4	1	sulfide-oxidizing bacterium ISW_10	FJ482025	190/190 (100%)	Cardinali-Rezende, *et.al*, (unpublished)
	2	uncultured *Pseudomonas* sp.	HM124797	183/183 (100%)	Lu, *et.al*, (unpublished)
	3	uncultured *Pseudomonas* sp.	HM124797	179/180 (99%)	Lu, *et.al*, (unpublished)
	4	Uncultured Bacteroidales bacterium	HM080219	179/181 (98%)	Frank, *et.al*, (unpublished)
	5	uncultured *Pseudomonas* sp.	HM124797	171/172 (99%)	Lu, *et.al*, (unpublished)

Table 4.3 The result of comparison 16S rDNA of bacterial communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software (continued)

DNA band	Clone no.	Bacterial strains	Accession no.	Sequence Identity (%)	References
C5	1	uncultured bacterium	FM242723	156/199 (78%)	Byrne, *et.al*, 2009
	2	uncultured delta proteobacterium	FM206228	111/113 (98%)	Imfeld and Richnow (unpublished)
	3	uncultured Bacteroidetes bacterium	FM206237	81/81 (100%)	Imfeld and Richnow (unpublished)
	4	uncultured bacterium	FM213511	76/76 (100%)	Malinowska (unpublished)
	5	uncultured delta proteobacterium	FM252761	86/92 (93%)	Ladygina, *et.al*, 2009
C6	1	uncultured Firmicutes bacterium	AM706663	108/109 (99%)	Muhling, *et.al*, 2008
	2	uncultured alpha proteobacterium	FM252847	84/90 (93%)	Ladygina, *et.al*, 2009
	3	uncultured Bacteroidetes bacterium	FM206232	88/88 (100%)	Imfeld and Richnow (unpublished)
	4	uncultured bacterium	EU275375	75/86 (87%)	Moreno, *et.al*, 2009
	5	uncultured Bacteroidetes bacterium	FM206237	72/72 (100%)	Imfeld and Richnow (unpublished)

Based on bacterial community analyses for biodiesel wastewater-feeding reactor, DGGE bands in acid tank and methane tank were different. It is known that the conditions can affect the species of microbial community. In acid tank, fermentative bacteria such as *Pectinatus* sp. were found. Fermentative bacteria can degrade a variety of different sugars and polysaccharides and produce acetate, carbon dioxide and hydrogen (Winter and Zellner, 1990). In methane tank, methanotrophic bacteria such as Proteobacterium were found. Methanotrophic bacteria or methanotrophs are unique in their ability to utilize methane as a sole carbon and energy source (Hanson and Hanson, 1996).

In comparison between bacterial community and biogas production, in acid tank, the number of DGGE bands was increased in week3 and the accumulated biogas production in week3 had highest. In methane tank, the number of DGGE bands had increased in every week and the accumulated biogas production tended to increased in every week. From this result, it is known that the bacterial community can affect the biogas production.

4.2.1.2 Cluster analysis of DGGE banding pattern

DGGE analysis were performed to compare the bacterial composition of the reactor fed with biodiesel wastewater in both of acid and methane tank and DGGE banding patterns were used to construct the dendograms as seen in Figure 4.2. The distribution and compositional changes of the bacteria reflect the clusters from the DGGE band patterns which were divided into two clusters. The first cluster consisted of all samples obtained from acid tank and the second cluster contained all samples from methane tank. These clusters were defined by 40% pattern similarity. For acid tank, the DGGE band patterns of weeks 0-2 were very similar. This result clearly showed a change of bacterial community from acid tank to methane tank. The DGGE band patterns of week 4 had the most different from the other weeks because it had the most number of DGGE bands in this week. For methane tank, the DGGE band patterns were very similar in all weeks.

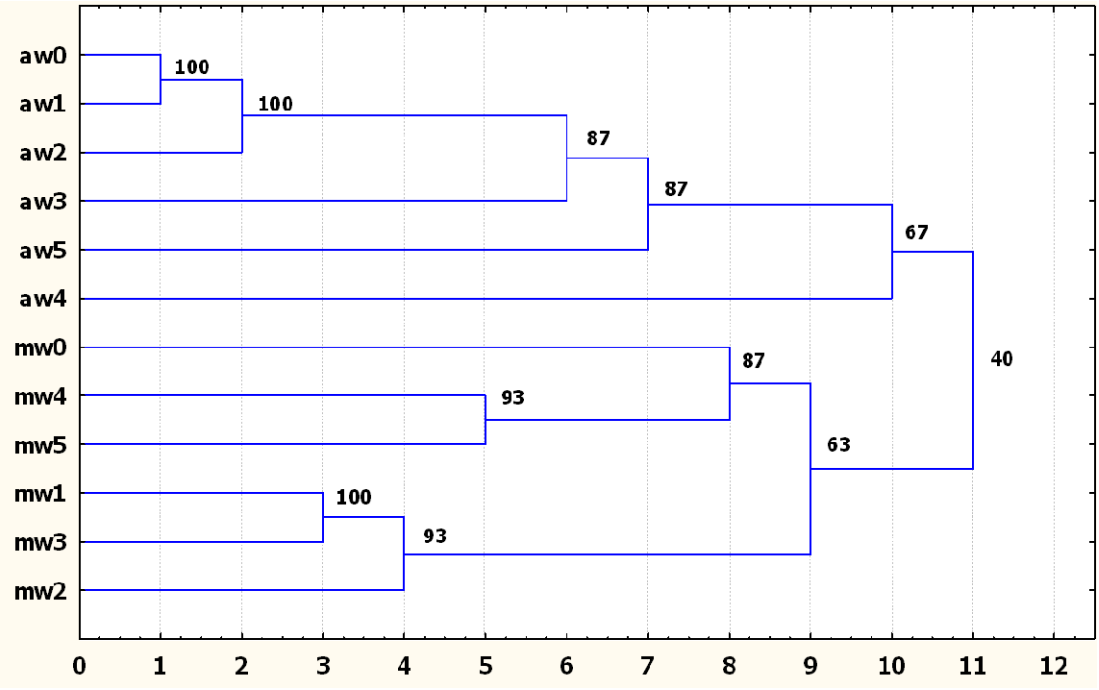

Figure 4.2 Cluster analysis of bacterial DGGE banding patterns; aw0-aw5: biosludges from acid tank weeks 0-5, mw0-mw5: biosludges from methane tank weeks 0-5

4.2.2 Analysis of bacterial communities in two-stage anaerobic digestion system using two different substrates: organic waste and biodiesel wastewater

The analysis of bacterial communities was conducted using DGGE technique. DNA from bioreactors fed with organic waste and biodiesel wastewater in both of acid and methane tank in steady state were extracted and 16S rDNA amplified by PCR using primers 341F with GC clamp and 520R. The DGGE result is shown in Figure 4.3.

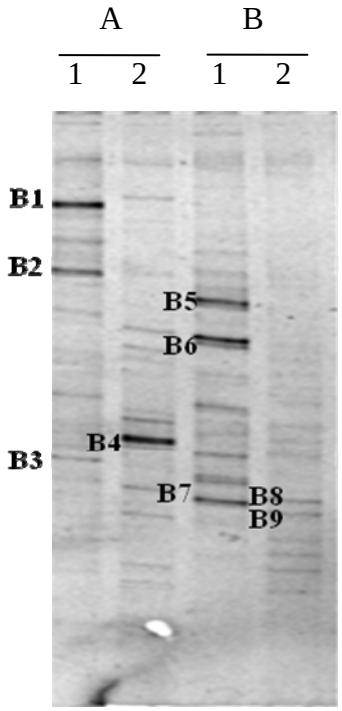

Figure 4.3 DGGE profile of bacterial communities in different substrates; condition: 30-70% denaturant (A): biosludges from acid tank and methane tank of bioreactor using organic waste as substrate, (B): biosludges from acid tank and methane tank of bioreactor using biodiesel wastewater as substrate

In comparison between using organic waste and biodiesel wastewater as substrate, the DGGE profiles obtained were different. It is known that type of substrate can affect the species of microbial community. Dominant DNA bands were excise for sequencing. The results of comparison 16S rDNA of bacterial communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software are shown in Table 4.4.

For bioreactor using organic waste as substrate, band B1 showed 98% sequence similarity to uncultured Bacteroidetes bacterium. Band B2 showed 100% sequence similarity to *Pseudomonas* sp. Band B3 showed 92% sequence similarity to *Syntrophomonas palmitatica* that had been isolated from granular sludge of an upflow anaerobic sludge blanket reactor treating palm oil mill effluent. They are strictly anaerobes and can utilize straight-chain saturated fatty acid (Hatamoto, *et al*., 2007).

Band B4 showed 100% sequence similarity to uncultured *Dialister* sp. that had been found in a starch-feeding dark fermentation agitated granular sludge bed (AGSB) reactor (Cheng, *et al*., 2008).

For bioreactor using biodiesel wastewater as substrate, band B5 showed 100% sequence similarity to *Megasphaera sueciensis*. Band B6 showed 100% sequence similarity to *Pectinatus* sp. Band B7 showed 95% sequence similarity to *Clostridium acetobutylicum*. Band B8 showed 100% sequence similarity to *Klebsiella pneumonia* that had been found in a starch-feeding fermentative hydrogen production reactor (Cheng, *et al*., 2008). Band B9 showed 94% sequence similarity to uncultured *Chloroflexus* sp. that had previously been found in anaerobic sewage digester (Seshadri, *et al*., 2005). They are known to play an important role in organic matter degradation under iron and nitrate reducing conditions in anaoxic microhabitats (Cetecioglu, *et al*., 2009)

Table 4.4 The result of comparison 16S rDNA of bacterial communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software

Band no.	**Clone no.**	**Bacterial stains**	**Access number**	**% similarity**	**References**
B1	1	uncultured Bacteroidetes bacterium	GQ501024	177/180 (98%)	Feng *et.al.*, 2010
	2	uncultured Bacteroidetes bacterium	EF188633	159/162 (98%)	Portillo *et.al.*, 2009
	3	uncultured Bacteroidetes bacterium	EF188796	176/179 (98%)	Portillo *et.al.*, 2009

Table 4.4 The result of comparison 16S rDNA of bacterial communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software (continued)

Band no.	**Clone no.**	**Bacterial stains**	**Access number**	**% similarity**	**References**
	4	uncultured Bacteroidetes bacterium	EF188340	156/159 (98%)	Portillo *et.al.*, 2009
	5	uncultured Bacteroidetes bacterium	GQ501024	151/155 (97%)	Feng *et.al.*, 2010
B2	1	*Pseudomonas* sp. SKU	AY954288	190/190 (100%)	Jin, *et.al.* (unpublished)
	2	sulfide-oxidizing bacterium ISW_10	FJ482025	190/190 (100%)	Cardinali-Rezende, *et.al.* (unpublished)
	3	*Pseudomonas* sp. SKU	AY954288	191/191 (100%)	Jin, *et.al.* (unpublished)
	4	sulfide-oxidizing bacterium ISW_10	FJ482025	190/190 (100%)	Cardinali-Rezende, *et.al.* (unpublished)
	5	*Pseudomonas* sp. SKU	AY954288	186/186 (100%)	Jin, *et.al.* (unpublished)
B3	1	uncultured Bacteroidetes bacterium	EU551096	178/181 (98%)	Wang, *et.al.* 2009
	2	uncultured Bacteroidetes bacterium	CU926845	176/181 (97%)	Riviere, *et.al*, 2009

Table 4.4 The result of comparison 16S rDNA of bacterial communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software (continued)

Band no.	**Clone no.**	**Bacterial stains**	**Access number**	**% similarity**	**References**
	3	*Syntrophomonas palmitatica*	AB274040	169/183 (92%)	Hatamoto ,*et.al*, 2007
	4	uncultured Firmicutes bacterium	CU924171	147/161 (91%)	Riviere, *et.al*, 2009
	5	uncultured Firmicutes bacterium	CU920790	160/176 (90%)	Riviere, *et.al*, 2009
B4	1	uncultured Firmicutes bacterium	GU954613	170/173 (98%)	Patil, *et.al*., (unpublished)
	2	uncultured *Dialister* sp.	GQ332220	161/161 (100%)	Adolphe, *et.al*. (unpublished)
	3	uncultured Firmicutes bacterium	GU954957	150/150 (100%)	Patil, *et.al*. (unpublished)
	4	uncultured *Dialister* sp.	GQ332220	160/160 (100%)	Adolphe, *et.al*. (unpublished)
	5	uncultured *Dialister* sp.	GQ332218	181/181 (100%)	Adolphe, *et.al*. (unpublished)
B5	1	*Megasphaera sueciensis*	DQ223729	188/188 (100%)	Juvonen and Suihko, 2006
	2	*Megasphaera sueciensis*	DQ223729	175/181 (96%)	Juvonen and Suihko, 2006

Table 4.4 The result of comparison 16S rDNA of bacterial communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software (continued)

Band no.	Clone no.	Bacterial stains	Access number	% similarity	References
	3	*Megasphaera sueciensis*	DQ223729	190/190 (100%)	Juvonen and Suihko, 2006
	4	uncultured bacterium	HM820001	191/191 (100%)	Grice, *et.al.*, 2010
	5	proteobacterium ARJR SMBS	HQ148164	174/174 (100%)	Anoop and Muruganandam (unpublished)
B6	1	*Pectinatus* sp. C5	GU586299	180/180 (100%)	Wenzel, *et.al.* (unpublished)
	2	*Pectinatus* sp. C5	GU586299	178/178 (100%)	Wenzel, *et.al.* (unpublished)
	3	*Pectinatus* sp. C5	GU586299	203/211 (96%)	Wenzel, *et.al.* (unpublished)
	4	*Pectinatus* sp. H2	FJ668029	180/180 (100%)	Castello, *et.al.* (unpublished)
	5	*Pectinatus frisingensis*	EU589446	179/179 (100%)	Juvonen, *et.al.*, 2008
B7	1	*Clostridium acetobutylicum*	AM231184	159/167 (95%)	Berezina, *et.al.*, 2008
	2	*Clostridium sartagoforme*	FJ384380	158/167 (94%)	Johansson, *et.al.* (unpublished)
	3	uncultured bacterium	FJ825468	157/163 (96%)	Podmirseg, *et.al.* (unpublished)
	4	uncultured bacterium	EF688246	162/168 (96%)	Roest (unpublished)

Table 4.4 The result of comparison 16S rDNA of bacterial communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software (continued)

Band no.	**Clone no.**	**Bacterial stains**	**Access number**	**% similarity**	**References**
	5	uncultured bacterium	FJ825468	159/165 (96%)	Podmirseg, *et.al.* (unpublished)
B8	1	*Klebsiella pneumoniae*	HM751200	179/179 (100%)	Li, *et.al.* (unpublished)
	2	Klebsiella sp. ICB390	HM748059	176/176 (100%)	Barbosa, *et.al.* (unpublished)
	3	*Klebsiella pneumoniae*	HM751200	184/184 (100%)	Li, *et.al.* (unpublished)
	4	*Klebsiella pneumoniae*	HM751200	181/182 (99%)	Li, *et.al.* (unpublished)
	5	Klebsiella sp. ICB390	HM748059	178/179 (99%)	Barbosa, *et.al.* (unpublished)
B9	1	uncultured *Chloroflexus* sp.	FJ481370	159/168 (94%)	Montalvo and Hill (unpublished)
	2	uncultured Chloroflexi bacterium	GQ337198	159/168 (94%)	Galand, *et.al*., 2010
	3	uncultured Chloroflexi bacterium	GQ337198	156/164 (95%)	Galand, *et.al*., 2010
	4	uncultured bacterium	CU918600	169/169 (100%)	Riviere, *et.al*., 2009
	5	uncultured bacterium	AB363453	153/178 (85%)	Kimura and Kamagata, 2009

4.2.3 Analysis of archaeal communities in two-stage anaerobic digestion system using biodiesel wastewater as substrate

4.2.3.1 DGGE analysis

The analysis of archaea communities was conducted using DGGE technique. DNA from bioreactors fed with biodiesel wastewater in both of acid and methane tank were extracted and were amplified. The changes of archaea populations are shown in Figure 4.4

From the result, the profile of archaea community showed a little bit different. Band AR1 always presented in acid tank since week 0 until week 4 and disappeared in week 5. Band AR2 was detected in every week in acid tank. Band AR3 was observed in both of acid and methane tank. Band AR4 was only observed in methane tank in week 4.

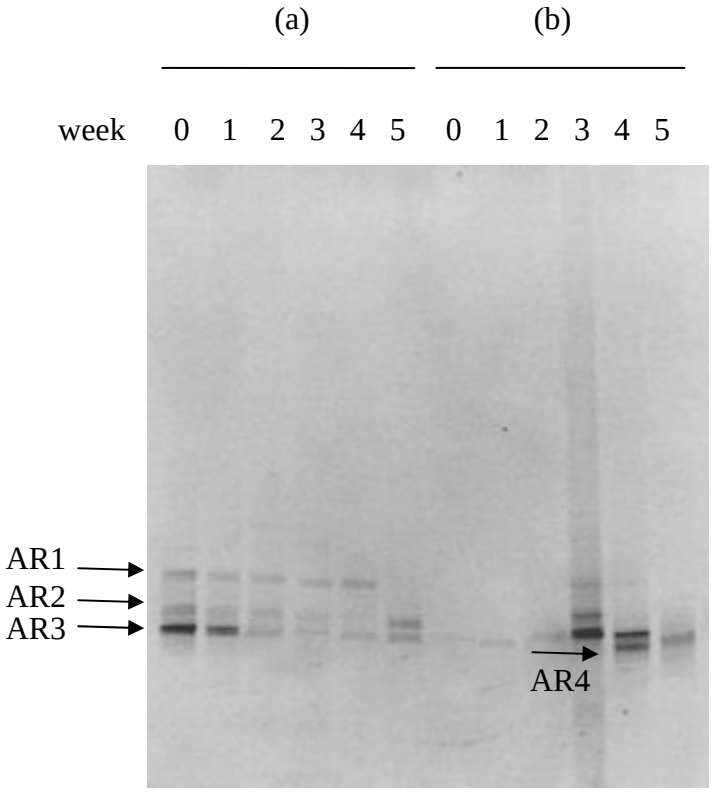

Figure 4.4 DGGE profile of archaea communities from bioreactor using biodiesel wastewater as substrate; condition: 30-70% denaturant (a): biosludges from acid tank weeks 0-5, (b): biosludge from methane tank weeks 0-5

Dominant DNA bands (AR1, AR2, AR3 and AR4) were excise for sequencing. The results of comparison 16S rDNA of archaea communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software are shown in Table 4.5.

Band AR1 showed 100% sequence similarity to uncultured archaeon. Band AR2 showed 96% sequence similarity to uncultured *Methanosaeta* spp. which were found in granular sludge in an upflow anaerobic sludge blanket reactor (Hirasawa, *et al*., 2008). They are acetoclastic methanogens that use acetate as their sole energy source, and it is metabolized into methane and carbon dioxide (Keyser, *et al*., 2006). Band AR3 showed 96% sequence similarity to uncultured Methanosarcinales archaeon that were found in two-phase biogas reactor systems. They synthesize methane as an end product of their energy by utilizing acetate (Klocke, *et al*., 2008). Band AR4 showed 96% sequence similarity to *Methanobacterium beijingense* that had been isolated from anaerobic digester. They are hydrogenotrophic methanogens that used H_2/CO_2 and formate for growth and produced methane (Ma, *et al*., 2005).

Table 4.5 The result of comparison archaea communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software

DNA band	**Clone no.**	**Archaea strains**	**Accession no.**	**Sequence Identity (%)**	**References**
AR1	1	uncultured archaeon	FM242736	182/182 (100%)	Byrne, *et.al.*, 2009
	2	uncultured archaeon	FM242736	156/156 (100%)	Byrne, *et.al.*, 2009
	3	uncultured archaeon	FM242736	182/182 (100%)	Byrne, *et.al.*, 2009
	4	uncultured archaeon	FM242736	182/182 (100%)	Byrne, *et.al.*, 2009

Table 4.5 The result of comparison archaea communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software (continued)

DNA band	**Clone no.**	**Archaea strains**	**Accession no.**	**Sequence Identity (%)**	**References**
	5	uncultured archaeon	FM242736	155/155 (100%)	Byrne, *et.al.,* 2009
AR2	1	uncultured archaeon	FJ853487	145/150 (96%)	Khuchareontaworn, *et.al.* (unpublished)
	2	uncultured *Methanosaeta* sp.	GU475191	145/150 (96%)	Zhang, *et.al.* (unpublished)
	3	uncultured archaeon	FJ853487	145/150 (96%)	Khuchareontaworn, *et.al.* (unpublished)
	4	uncultured *Methanosaeta* sp.	GU475186	145/150 (96%)	Zhang, *et.al.* (unpublished)
	5	uncultured *Methanosaeta* sp.	GU475190	143/148 (96%)	Zhang, *et.al.* (unpublished)
AR3	1	uncultured archaeon	HQ008077	145/150 (96%)	Hughes, *et.al.*, (unpublished)
	2	uncultured Methanosarcinales archaeon	FN646493	145/150 (96%)	Rotaru, *et.al.* (unpublished)
	3	uncultured Methanosarcinales archaeon	FN646491	145/150 (96%)	Rotaru, *et.al.* (unpublished)

Table 4.5 The result of comparison archaea communities in bioreactors fed with biodiesel wastewater as substrate to GenBank database using BLASTn software (continued)

DNA band	Clone no.	Archaea strains	Accession no.	Sequence Identity (%)	References
	4	uncultured Methanosarcinales archaeon	FN646468	139/142 (97%)	Rotaru, *et.al.* (unpublished)
	5	uncultured Methanosarcinales archaeon	FN646493	139/142 (97%)	Rotaru, *et.al.* (unpublished)
AR4	1	uncultured Methanobacteriaceae archaeon	DQ402014	145/151 (96%)	Pei, *et.al.*, (unpublished)
	2	uncultured archaeon	FM242736	158/158 (100%)	Byrne, *et.al.*, 2009
	3	uncultured archaeon	FM242736	159/159 (100%)	Byrne, *et.al.*, 2009
	4	uncultured Methanobacteriaceae archaeon	AB236091	140/146 (95%)	Sakai, *et.al.*, 2009
	5	*Methanobacterium beijingense*	AY552778	131/136 (96%)	Ma, *et.al.*, 2005

4.2.3.2 Cluster analysis of DGGE banding pattern

DGGE analysis were performed to compare the archaea composition of the reactor fed with biodiesel wastewater in both of acid and methane tanks and DGGE banding patterns were used to construct the dendograms as seen in Figure 4.5. The distribution and compositional changes of the archaea reflect the clusters from the

DGGE band patterns. This cluster appeared to be very stable because each sub-clusters were very similar For example, the DGGE band patterns belong to acid tank in weeks 0-4 were defined by 100% similarity. The DGGE band patterns belong to methane tank in weeks 0-2 were defined by 100% similarity.

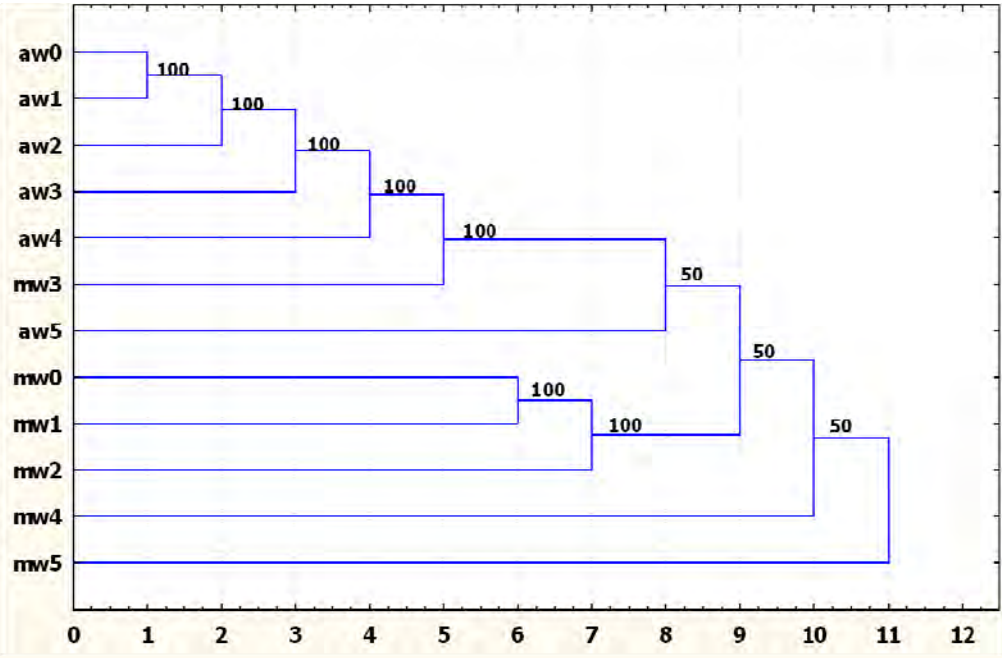

Figure 4.5 Cluster analysis of archaea DGGE banding patterns; aw0-aw5: biosludges from acid tank weeks 0-5, mw0-mw5: biosludges from methane tank weeks 0-5

4.2.4 Analysis of archaea communities in two-stage anaerobic digestion system using two different substrates: organic waste and biodiesel wastewater

The analysis of archaea communities was conducted using DGGE technique. DNA from bioreactors fed with organic waste and biodiesel wastewater in both of acid and methane tank in steady state were extracted and were amplified. The DGGE result is shown in Figure 4.6.

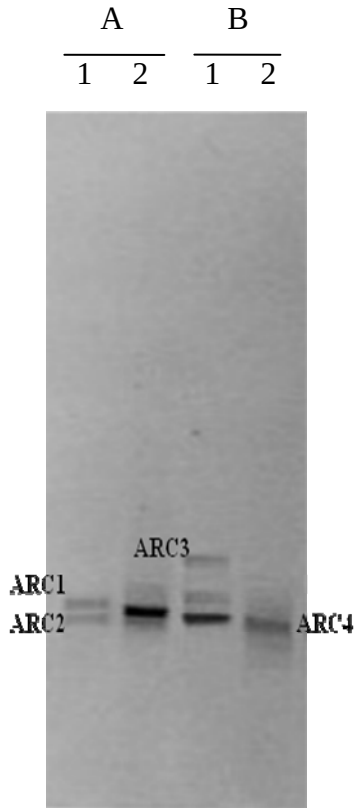

Figure 4.6 DGGE profile of archaea communities in different substrates; condition: 30-70% denaturant gradient (A): biosludges from acid tank and methane tank of bioreactor using organic waste as substrate, (B): biosludges from acid tank and methane tank of bioreactor using biodiesel wastewater as substrate

In comparison between using organic waste and biodiesel wastewater as substrate, the result showed no difference of the DGGE profiles. Dominant DNA bands were excised for sequencing. The results of comparison of archaea communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software are shown in Table 4.6.

Band ARC1 showed 96% sequence similarity to uncultured *Methanosaeta* sp. Band ARC2 showed 96% sequence similarity to uncultured Methanosarcinales archaeon. Band ARC3 showed 100% sequence similarity to uncultured archaeon. Band AR4 showed 96% sequence similarity to uncultured *Methanobacterium beijingense*.

Table 4.6 The result of comparison archaea communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software

DNA band	Clone no.	Archaea strains	Accession no.	Sequence Identity (%)	References
ARC1	1	uncultured archaeon	FJ853487	145/150 (96%)	Khuchareontaworn, *et.al.* (unpublished)
	2	uncultured *Methanosaeta* sp.	GU475191	145/150 (96%)	Zhang, *et.al.* (unpublished)
	3	uncultured archaeon	FJ853487	145/150 (96%)	Khuchareontaworn, *et.al.* (unpublished)
	4	uncultured *Methanosaeta* sp.	GU475186	145/150 (96%)	Zhang, *et.al.* (unpublished)
	5	uncultured *Methanosaeta* sp.	GU475190	143/148 (96%)	Zhang, *et.al.* (unpublished)
ARC2	1	uncultured archaeon	HQ008077	145/150 (96%)	Hughes, *et.al.*, (unpublished)
	2	uncultured Methanosarcinales archaeon	FN646493	145/150 (96%)	Rotaru, *et.al.* (unpublished)
	3	uncultured Methanosarcinales archaeon	FN646491	145/150 (96%)	Rotaru, *et.al.* (unpublished)
	4	uncultured Methanosarcinales archaeon	FN646468	139/142 (97%)	Rotaru, *et.al.* (unpublished)

Table 4.6 The result of comparison archaea communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software (continued)

DNA band	Clone no.	Archaea strains	Accession no.	Sequence Identity (%)	References
	5	uncultured Methanosarcinales archaeon	FN646493	139/142 (97%)	Rotaru, *et.al.* (unpublished)
ARC3	1	uncultured archaeon	FM242736	182/182 (100%)	Byrne, *et.al.*, 2009
	2	uncultured archaeon	FM242736	156/156 (100%)	Byrne, *et.al.*, 2009
	3	uncultured archaeon	FM242736	182/182 (100%)	Byrne, *et.al.*, 2009
	4	uncultured archaeon	FM242736	169/169 (100%)	Byrne, *et.al.*, 2009
	5	uncultured archaeon	FM242736	155/155 (100%)	Byrne, *et.al.*, 2009
ARC4	1	uncultured Methanobacteriaceae archaeon	DQ402014	145/151 (96%)	Pei, *et.al.*, (unpublished)
	2	uncultured archaeon	FM242736	158/158 (100%)	Byrne, *et.al.*, 2009
	3	uncultured archaeon	FM242736	159/159 (100%)	Byrne, *et.al.*, 2009

Table 4.6 The result of comparison archaea communities in bioreactors using two different substrates: organic waste and biodiesel wastewater to GenBank database using BLASTn software (continued)

DNA band	Clone no.	Archaea strains	Accession no.	Sequence Identity (%)	References
	4	uncultured Methanobacteriaceae archaeon	AB236091	140/146 (95%)	Sakai, *et.al.*, 2009
	5	*Methanobacterium beijingense*	AY552778	131/136 (96%)	Ma, *et.al.*, 2005

4.3 Clone libraries of 16S rDNA

DNA from the samples of bioreactor fed with organic waste and biodiesel wastewater as substrate in both of acid and methane tank in the steady state were extracted and were amplified in 16S rDNA by PCR using primer 350F and 1400R. PCR products were ligated to pGEM-T Easy Vecter and transformed into competent *E.coli* JM109. The clone libraries were screened using DGGE analysis

For bioreactor using organic waste as substrate, a total of 32 clones were obtained from acid tank (Figure 4.7). The clone libraries were screened using DGGE analysis and 2 different types of clones were selected for sequencing (Table 4.7). From this analysis, 30 (94%) of total clones were affiliated with *Pseudomonas acephalitica* and 2 clones (6%) were assigned to uncultured Firmicutes bacterium (Figure 4.8).

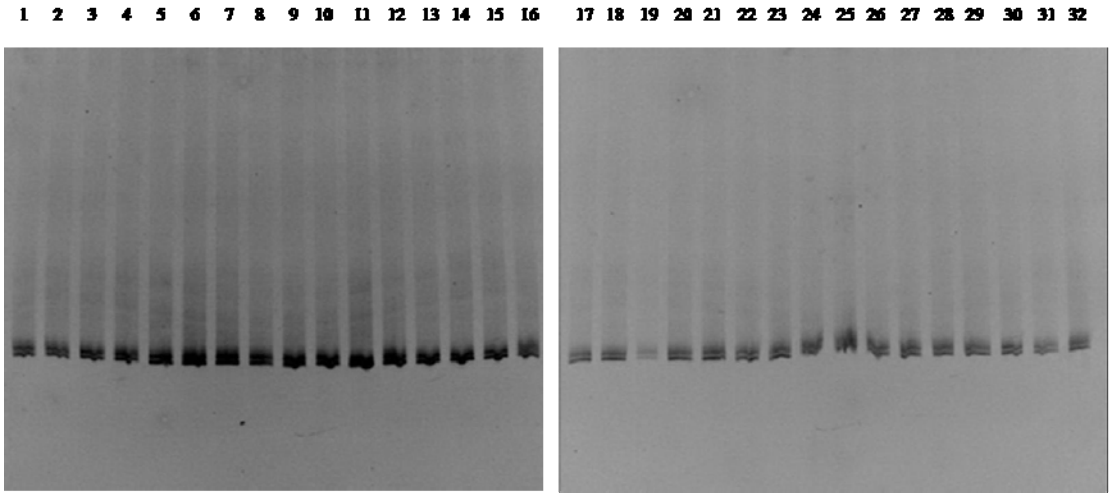

Figure 4.7 DGGE profiles of 16S rDNA clone libraries from acid tank using organic waste as substrate in steady state; condition: 30-70% denaturant gradient

Table 4.7 16S rDNA clone library of the sample from acid tank of bioreactor using organic waste as substrate

DNA band no.	Bacterial strains	Accession no.	Sequence Identity (%)	References
1	*Pseudomonas acephalitica*	AM407893	114/117 (97%)	Pinjari (unpublished)
25	uncultured Firmicutes bacterium	AM706663	109/110 (99%)	Muhling, *et.al.*, 2008

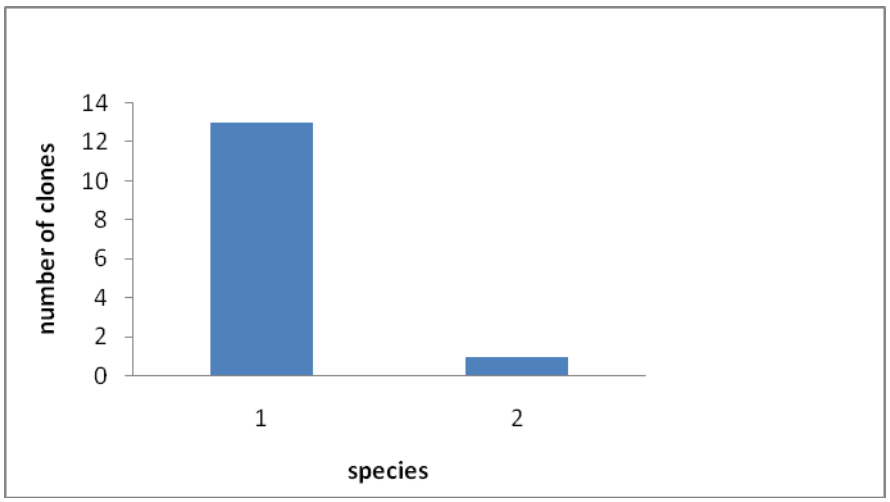

Figure 4.8 Bar diagram showing the distribution of 32 clone sequences among different groups. (1) *Pseudomonas acephalitica* (AM407893). (2) uncultured Firmicutes bacterium (AM706663)

A total of 60 clones were obtained from methane tank (Figure 4.9) and 7 different clones were selected for sequencing (Table 4.8). From this analysis 7 (11%) of total clones were affiliated with *Clostridium* sp. Seventeen clones (28%) were assigned to *Weissella cibaria* that are the lactic acid bacteria (Srionnual, *et al*., 2007). Nine clones (15%) were affiliated with uncultured bacterium. Nine clones (15%) were assigned to *Sedimentibacter* sp. These species is counted to the order of Clostridiales. They are the strictly anaerobic and utilize amino acids and pyruvate as substrates and metabolise acetate and butyrate (Pobeheim, *et al*., 2010). Three clones (5%) were affiliated with *Tissierella praeacuta* that had been found in thermal anaerobic acidogenesis using mesophilic sludge inoculums (Kim, *et al*., 2010). Eleven clones (18%) were assigned to *Clostridium jejuense* and 5 clones (8%) were affiliated with uncultured Firmicutes bacterium (Figure 4.10).

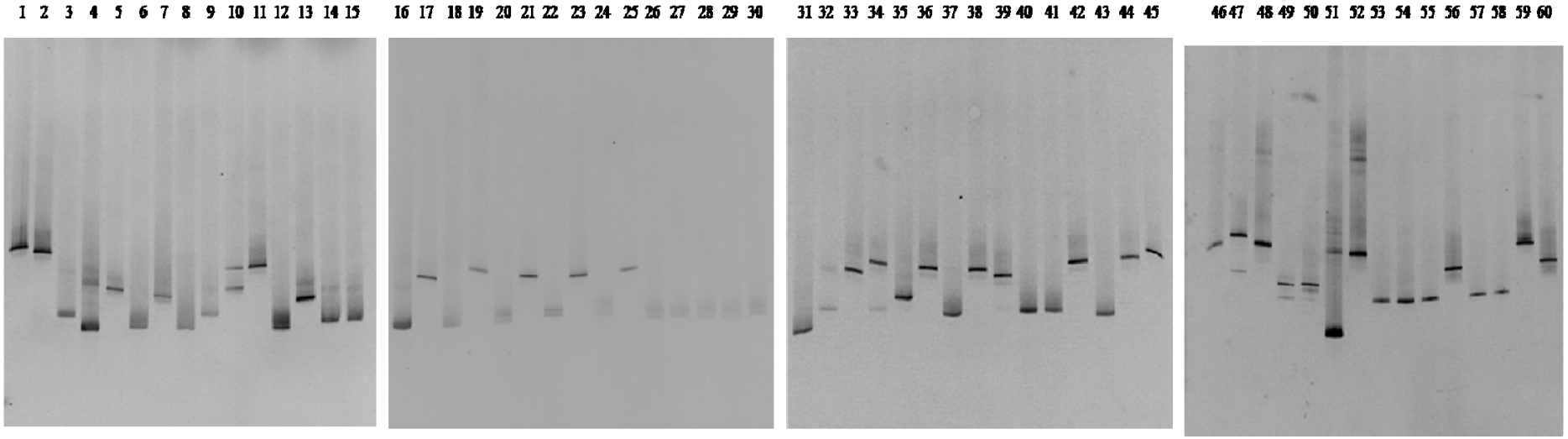

Figure 4.9 DGGE profiles of 16S rDNA clone libraries from methane tank using organic waste as substrate in steady state; condition: 30-70% denaturant gradient

Table 4.8 16S rDNA clone library of the sample from methane tank of bioreactor using organic waste as substrate

DNA band no.	**Bacterial strains**	**Accession no.**	**Sequence Identity (%)**	**References**
1	*Clostridium* sp. Kas107-2	AB114232	838/869 (96%)	Minamisawa, *et.al.*, 2004
3	*Weissella cibaria*	AB494716	788/853 (92%)	Choi, *et.al.* (unpublished)
4	uncultured bacterium	FP083961	849/850 (99%)	Tap, *et.al.*, 2009
5	*Sedimentibacter* sp.	AM933661	830/842 (98%)	Bunge, *et.al.*, 2008
10	*Tissierella praeacuta*	GQ461814	812/846 (95%)	Alauzet, *et.al.* (unpublished)
11	*Clostridium jejuense*	NR_025796	777/847 (91%)	Jeong, *et.al.*, 2004
47	uncultured Firmicutes bacterium	FJ440032	775/847 (91%)	Scupham (unpublished)

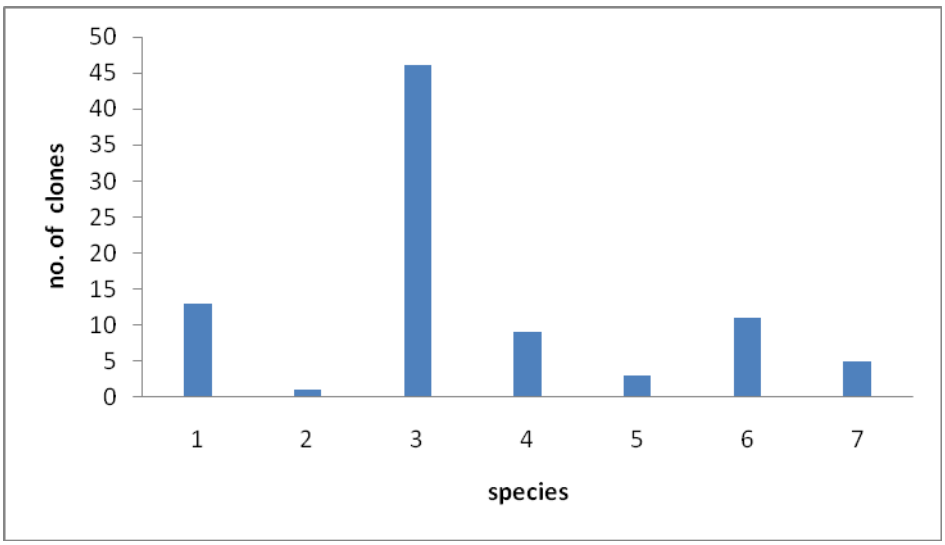

Figure 4.10 Bar diagram showing the distribution of 60 clone sequences among different groups. (1) *Clostridium* sp. (AB114232). (2) *Weissella cibaria*. (AB494716). (3) uncultured bacterium (FP083961) (4) *Sedimentibacter* sp. (AM933661) (5) *Tissierella praeacuta* (GQ461814) (6) *Clostridium jejuense* (NR_025796) (7) uncultured Firmicutes bacterium (FJ440032)

For bioreactor using biodiesel wastewater as substrate, a total of 32 clones were obtained from acid tank (Figure 4.11). The clone libraries were screened using DGGE analysis and 2 different clones were selected for sequencing (Table 4.9). From this analysis, 10 (31%) of total clones were affiliated with *Sphingomonas* sp. that was found in anaerobic digesters (Moletta, *et al*., 2007) and 22 clones (69%) were assigned to *Klebsiella* sp. KUS (Figure 4.12).

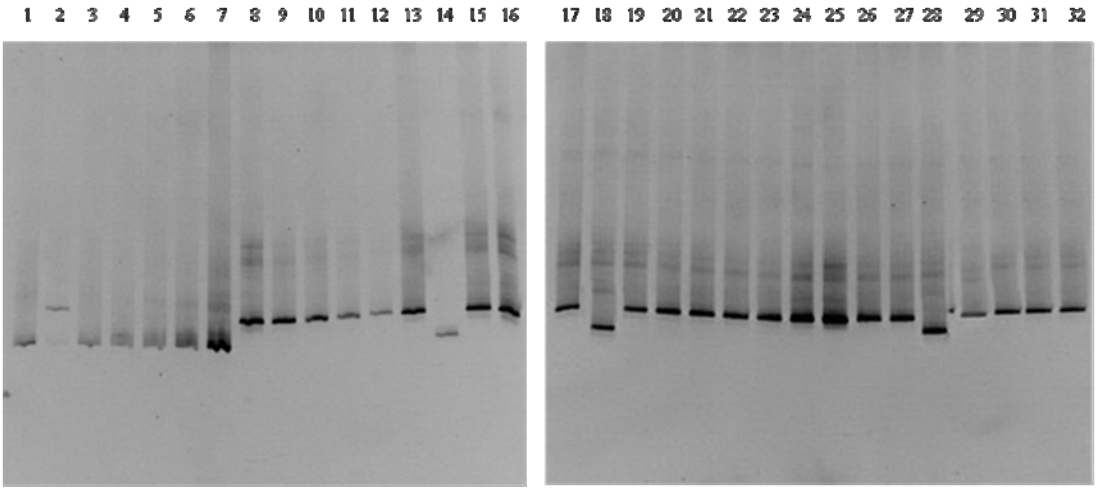

Figure 4.11 DGGE profile of 16S rDNA clone libraries from acid tank using biodiesel wastewater as substrate in steady state; condition: 30-70% denaturant gradient

Table 4.9 16S rDNA clone library of the sample from acid tank of bioreactor using biodiesel wastewater as substrate

DNA band	Bacterial strains	Accession no.	Sequence Identity (%)	References
1	*Sphingomonas* sp. MBHLY-1	HM243762	833/833 (100%)	Cai (unpublished)
8	*Klebsiella* sp. KUS	EF526502	813/813 (100%)	Kumar, *et.al* (unpublished)

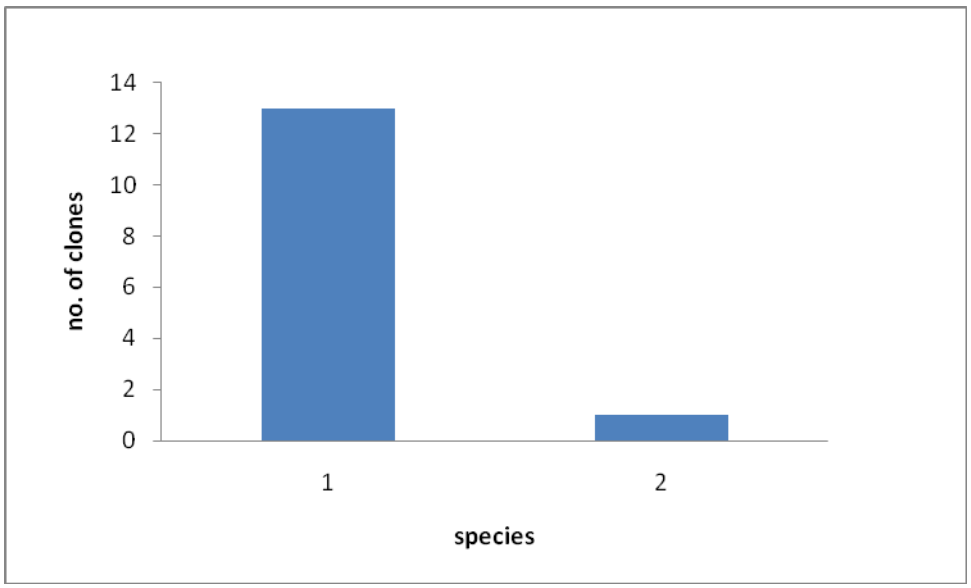

Figure 4.12 Bar diagram showing the distribution of 32 clone sequences among different groups. (1) *Sphingomonas* sp. MBHLY-1 (HM243762). (2) *Klebsiella* sp. KUS (EF526502)

A total of 60 clones were obtained from methane tank (Figure 4.13) and 3different clones were selected for sequencing (Table 4.10). From this analysis 13 (22%) of total clones were affiliated with uncultured bacterium. One clone (2%) was assigned to *Pseudomonas putida* and 46 clones (76%) were affiliated with uncultured Chloroflexi bacterium (Figure 4.14).

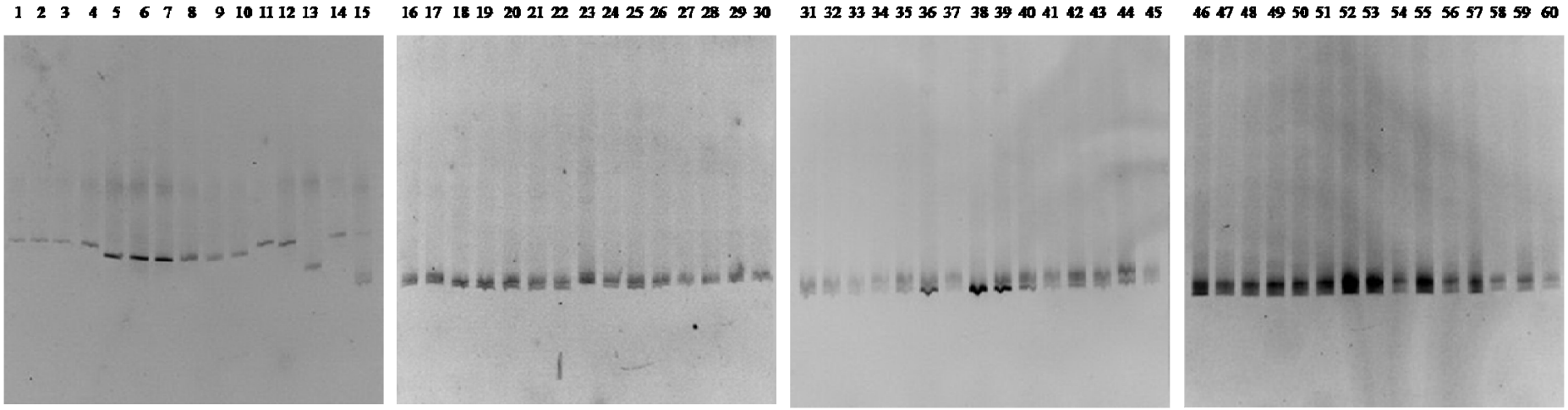

Figure 4.13 DGGE profiles of 16S rDNA clone libraries from methane tank using biodiesel wastewater as substrate in steady state;condition: 30-70% denaturant gradient

Table 4.10 16S rDNA clone library of the sample from methane tank of bioreactor using biodiesel wastewater as substrate

DNA band	Bacterial strains	Accession no.	Sequence Identity (%)	References
2	uncultured bacterium	FM242723	840/888 (94%)	Byrne, *et.al.*, 2009
13	*Pseudomonas putida*	FJ950594	822/825 (99%)	Li, *et.al.*, 2010
16	uncultured Chloroflexi bacterium	GQ143781	754/838 (89%)	Cho, *et.al.* (unpublished)

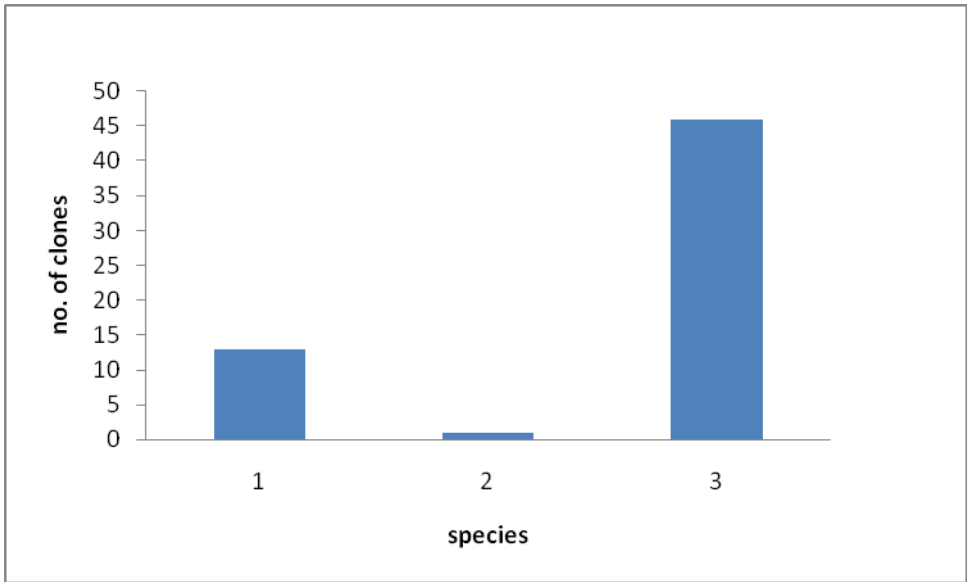

Figure 4.14 Bar diagram showing the distribution of 60 clone sequences among different groups. (1) uncultured bacterium (FM242723) (2) *Pseudomonas putida* (FJ950594) (3) uncultured Chloroflexi bacterium (GQ143781)

In comparison between the result of DGGE analysis and the result of 16S rDNA clone libraries, *Pseudomonas* sp. which found in acid tank of bioreactor using organic waste could be detected in both of DGGE analysis and 16S rDNA clone

libraries. Uncultured Chloroflexi which found in amethane tank of bioreactor using biodiesel wastewater could be detected in both of DGGE analysis and 16S rDNA clone libraries. From the results, it showed that these organisms likely played an important role in this system.

4.4 Detection of genes involved in biogas production by PCR amplification

DNA from the samples of bioreactor fed with organic waste and biodiesel wastewater as substrate in both of acid and methane tanks in the steady state were extracted. Hydrogenase gene (*hydA*) and Methyl-coenzyme M reductase gene (*mcrA*) were used as the targets in this study.

4.4.1 *mcrA* gene

Methyl-coenzyme M reductase (*mcrA*) gene codes for Methyl-coenzyme M reductase which is the key enzyme of methanogenesis. This enzyme catalyses the reduction of methyl-coenzyme M leading to the release of methane (Ellermann, *et al*., 1988). Polymerase chain reaction (PCR) was conducted to detect *mcrA* gene in these biosludge samples which involved in biogas production. The expected product size of *mcrA* is 464-491 bp. From the result, *mcrA* could be detected in every sample because the biogas in the system had methane content in both of acid tank and methane tank and both of substrates; organic waste and biodiesel wastewater (Figure 4.15).

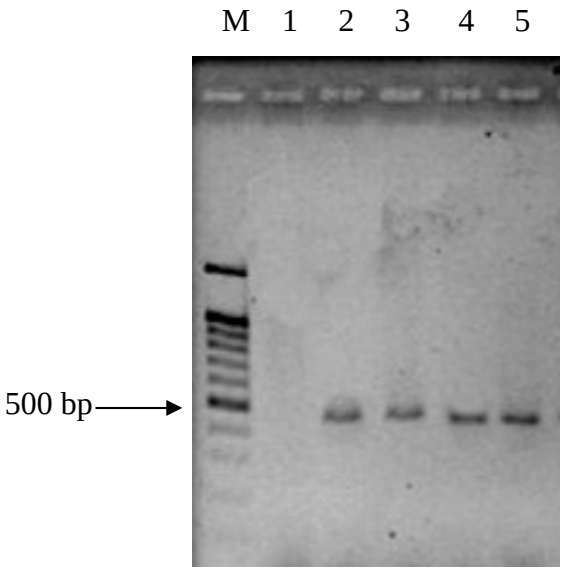

Figure 4.15 Detection of *mcrA* gene in biosludges from bioreactor. Lane M: 100 bp ladder DNA marker, Lane 1: negative control, Lane 2-3: biosludges from acid tank and methane tank of bioreactor using organic waste as substrate, Lane 4-5: biosludges from acid tank and methane tank of bioreactor using biodiesel wastewater as substrate

After that, the bands in lanes 2-5 (Figure 4.15) were extracted and ligated into pGEM-T Easy vector and transformed into *E. coli* JM109. The required colonies were then selected. Ten clones from the band in lanes 2-5 were picked. Extracted plasmids were digested by *Eco*RI in order to check the presence of PCR product.

4.4.1.1 *mcrA*-organic waste-acid tank

The presence of PCR product of the samples from biosludges from acid tank of bioreactor using organic waste as substrate is shown in Figure 4.16.

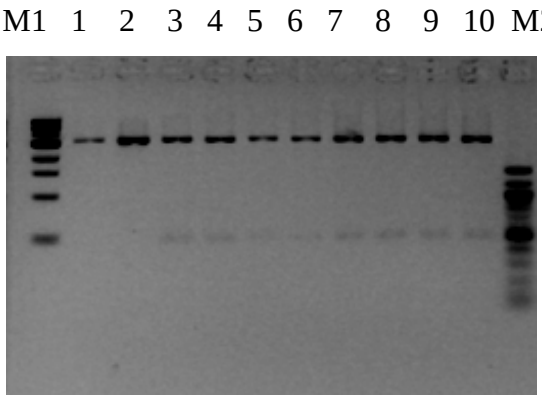

Figure 4.16 Recombinant plasmids after digested by restriction enzyme *Eco*RI
Lane M1: 1 kb marker, Lane 1-10: selected colonies mH1-10, respectively, M2: 100 bp ladder DNA marker

From Figure 4.16, all colonies could be detected the presence of PCR products except for samples mH1 and mH2 in lane 1 and lane 2, respectively. Thus, other samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. Digestion of clones by restriction enzyme *Bsu*RI exhibited the difference between 8 clones and sorted these clones into 2 groups as described below: (Figure 4.17)

Group 1: clone mH3, mH6
Group 2: clone mH4, mH5, mH7, mH8, mH9, mH10

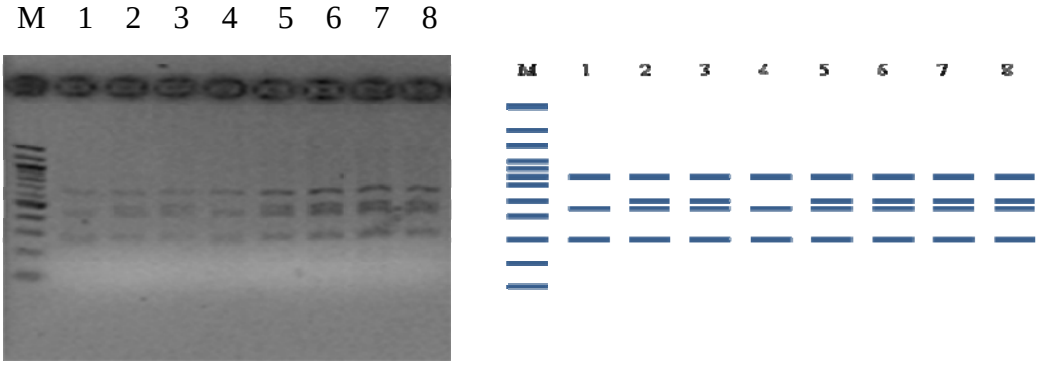

Figure 4.17 Recombinant plasmids after digested by restriction enzyme *Bsu*RI
Lane M: 100 bp ladder DNA marker, Lane 1-8: clones mH3-10, respectively

Therefore, all clones were digested again with restriction enzyme *Hin*fI. Digestion of clones by restriction enzyme *Hin*fI exhibited that all clones shown the same pattern (Figure 4.18).

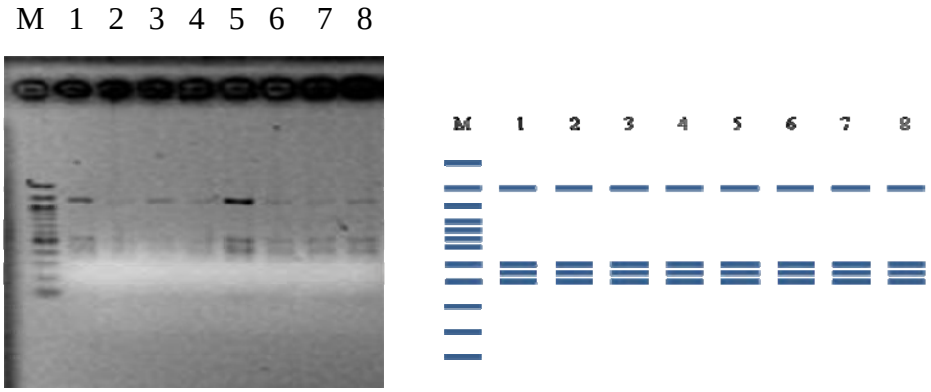

Figure 4.18 Recombinant plasmids after digested by restriction enzyme *Hin*FI
Lane M: 100 bp ladder DNA marker, Lane 1-8: clones mH3-10, respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I. Digestion of clones by restriction enzyme *Rsa*I exhibited that all clones shown the same pattern (Figure 4.19).

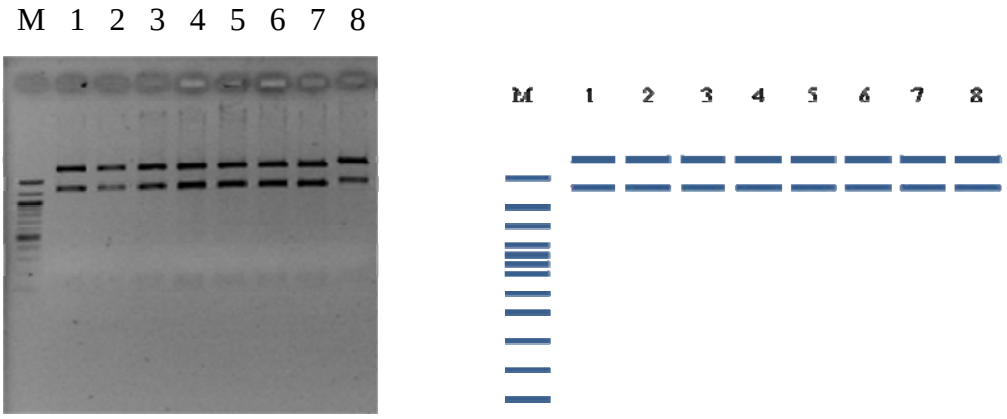

Figure 4.19 Recombinant plasmids after digested by restriction enzyme *Rsa*I
Lane M: 100 bp ladder DNA marker, Lane 1-8: clones mH3-10, respectively

Digestion of clones by all restriction enzyme exhibited the difference between 8 clones and sorted these clones into 2 groups as described below:

Group 1: mH3, mH6
Group 2: mH4, mH5, mH7, mH8, mH9, mH10

4.4.1.2 *mcrA*-organic waste-methane tank

The presence of PCR product of biosludges from methane tank of bioreactor using organic waste as substrate is shown in Figure 4.20.

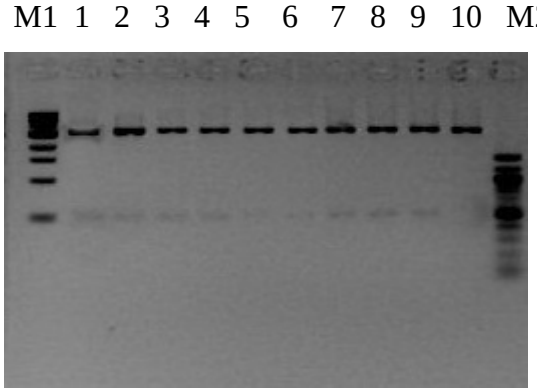

Figure 4.20 Recombinant plasmids after digested by restriction enzyme *Eco*RI
Lane M1: 1 kb marker, Lane 1-10: selected colonies mO1-10, respectively, M2: 100 bp ladder DNA marker

From Figure 4.20, all colonies could be detected the presence of PCR products except sample mO10. Thus, other samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. The results exhibited that all clones shown the same pattern (Figure 4.21).

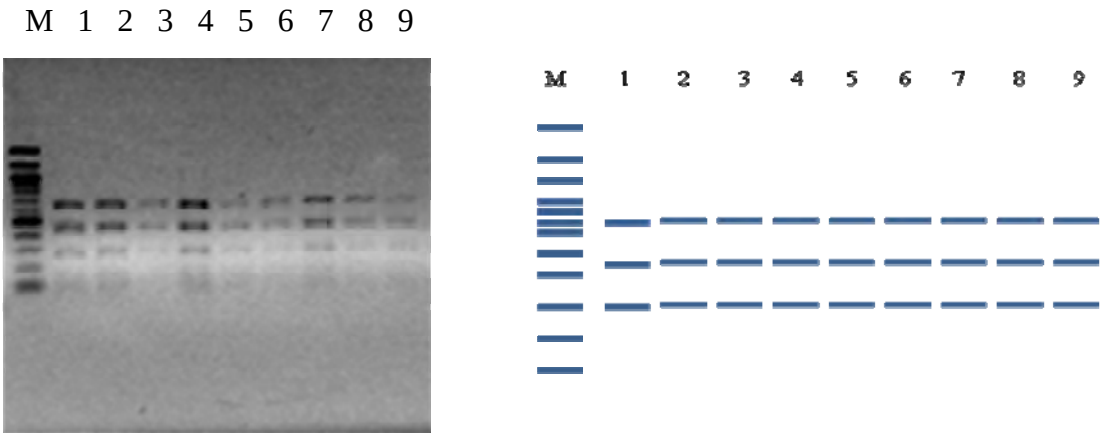

Figure 4.21 Recombinant plasmids after digested by restriction enzyme *Bsu*RI
Lane M: 100 bp ladder DNA marker, Lane 1-9: clones mO1-9, respectively

Therefore, all clones were digested again with restriction enzyme *Hin*fI. Digestion of clones by restriction enzyme *Hin*fI exhibited that all clones shown the same pattern (Figure 4.22).

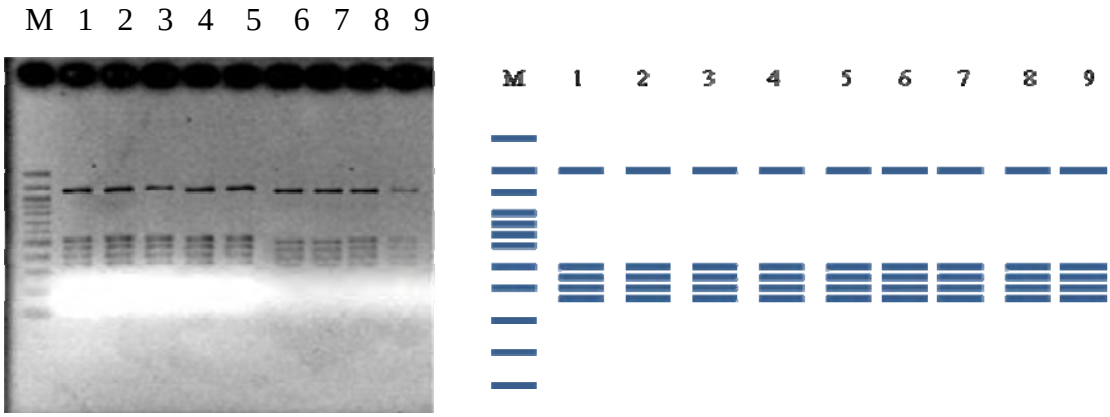

Figure 4.22 Recombinant plasmids after digested by restriction enzyme *Hin*FI
Lane M: 100 bp ladder DNA marker, Lane 1-9: clones mO1-9, respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I. Digestion of clones by restriction enzyme *Rsa*I exhibited that all clones shown the same pattern (Figure 4.23).

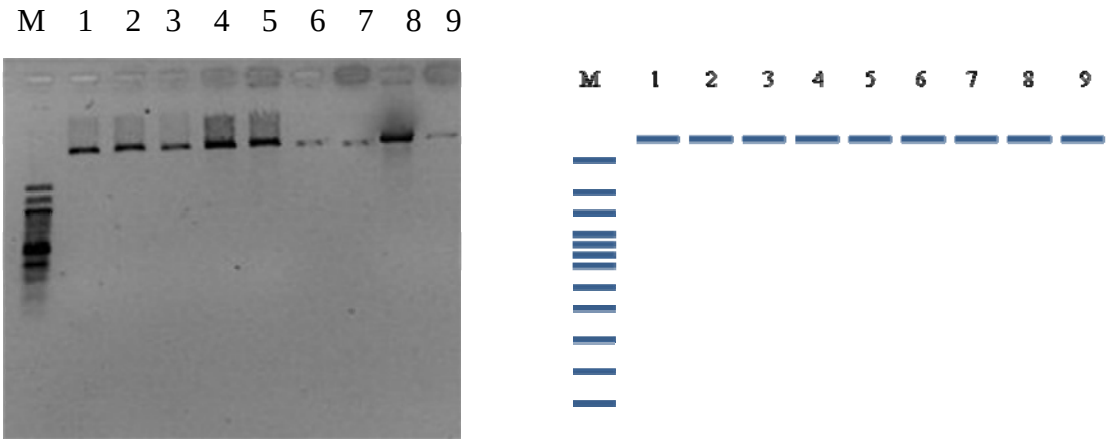

Figure 4.23 Recombinant plasmids after digested by restriction enzyme *Rsa*I
Lane M: 100 bp ladder DNA marker, Lane 1-9: clones mO1-9, respectively

Digestion of clones by all restriction enzyme exhibited that all clones shown the same pattern.

4.4.1.3 *mcrA*-biodiesel wastewater-acid tank

The presence of PCR product of biosludges from acid tank of bioreactor using biodiesel wastewater as substrate is shown in Figure 4.24.

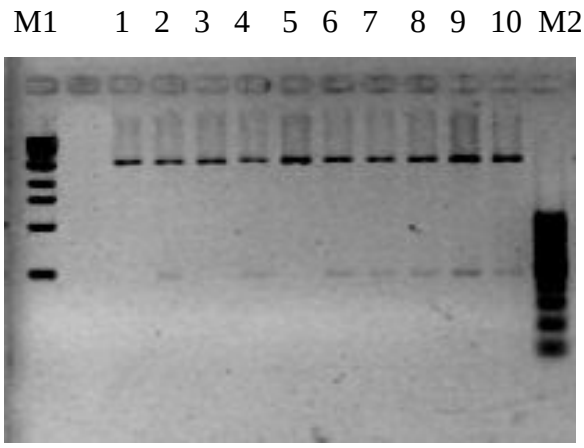

Figure 4.24 Recombinant plasmids after digested by restriction enzyme *Eco*RI
Lane M1: 1 kb marker, Lane 1-10: selected colonies mA1-10, respectively, M2: 100 bp ladder DNA marker

From Figure 4.24, all colonies could be detected the presence of PCR products except sample mA1, mA3 and mA5, respectively. Thus, other samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. The results exhibited that all clones shown the same pattern (Figure 4.25).

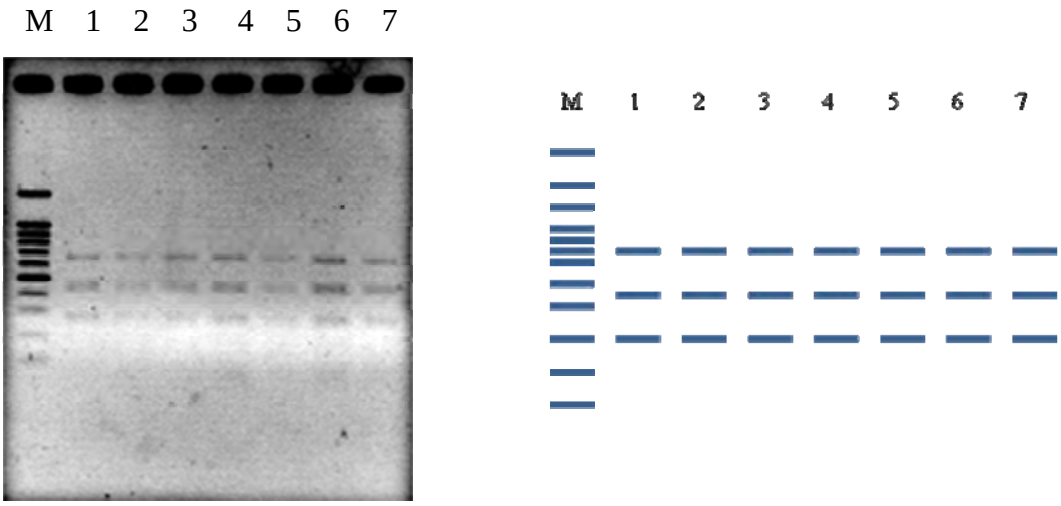

Figure 4.25 Recombinant plasmids after digested by restriction enzyme *Bsu*RI
Lane M: 100 bp ladder DNA marker, Lane 1: clones mA2, Lane 2: mA4, Lane 3-7: mA6-10 respectively

Therefore, all clones were digested again with restriction enzyme *Hin*fI. Digestion of clones by restriction enzyme *Hin*fI exhibited the difference between 7 clones and sorted these clones into 2 groups as described below: (Figure 4.26)

Group 1: mA2, mA4, mA6, mA7, mA8, mA10
Group 2: mA9

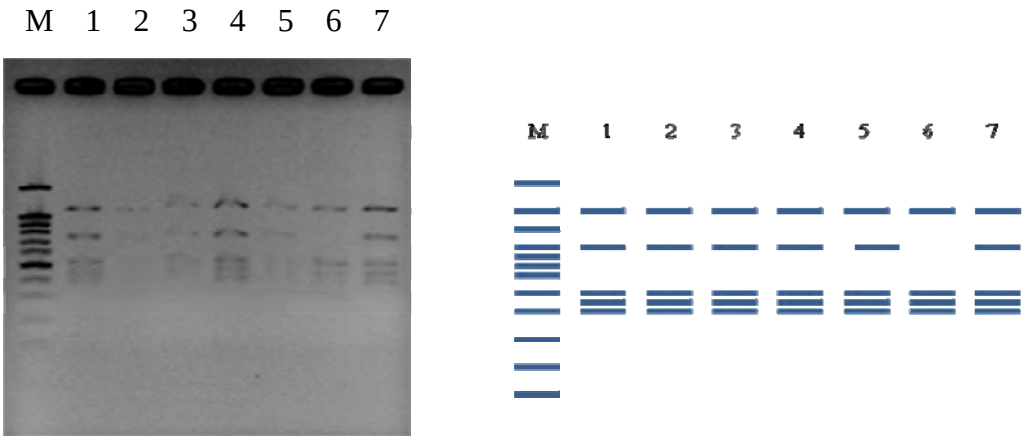

Figure 4.26 Recombinant plasmids after digested by restriction enzyme *Hin*FI Lane M: 100 bp ladder DNA marker, Lane 1: clones mA2, Lane 2: mA4, Lane 3-7: mA6-10 respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I expect sample mA9. Digestion of clones by restriction enzyme *Rsa*I exhibited the difference between 6 clones and sorted these clones into 2 groups as described below: (Figure 4.27).

Group 1: mA2, mA4, mA6, mA7, mA8
Group 2: mA10

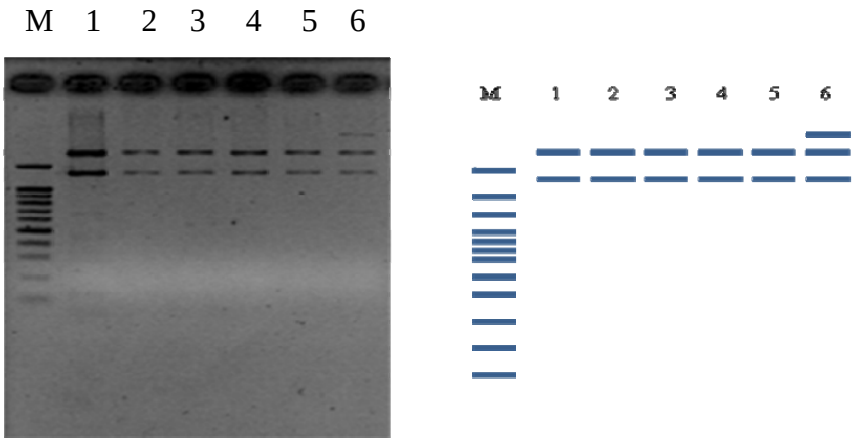

Figure 4.27 Recombinant plasmids after digested by restriction enzyme *Rsa*I Lane M: 100 bp ladder DNA marker, Lane 1: clones mA2, Lane 2: mA4, Lane 3-5: mA6-8, Lane 6: mA10, respectively

Digestion of clones by all restriction enzyme exhibited the difference between 7 clones and sorted these clones into 3 groups as described below:

Group 1: mA2, mA4, mA6, mA7, mA8
Group 2: mA9
Group 3: mA10

4.4.1.4 *mcrA*-biodiesel wastewater-methane tank

The presence of PCR product of biosludges from methane tank of bioreactor using biodiesel wastewater as substrate is shown in Figure 4.28.

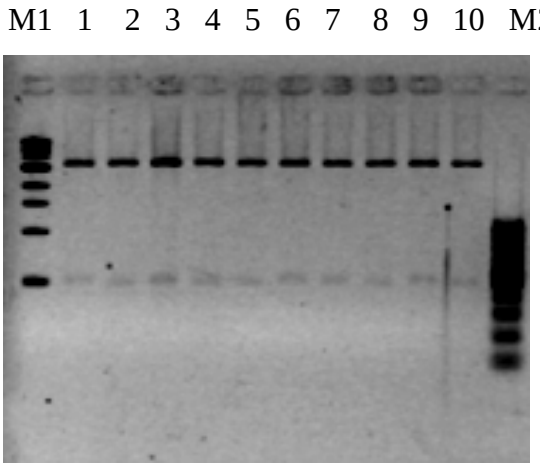

Figure 4.28 Recombinant plasmids after digested by restriction enzyme *Eco*RI
Lane M1: 1 kb marker, Lane 1-10: selected colonies mB1-10, respectively, M2: 100 bp ladder DNA marker

From Figure 4.28, all colonies could be detected the presence of PCR products. Thus, all samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. Digestion of clones by restriction enzyme *Hin*fI exhibited the difference between 10 clones and sorted these clones into 2 groups as described below: (Figure 4.29)
Group 1: mB1, mB3, mB6, mB7, mB9, mB10
Group 2: mB2, mB4, mB5, mB

M 1 2 3 4 5 6 7 8 9 10

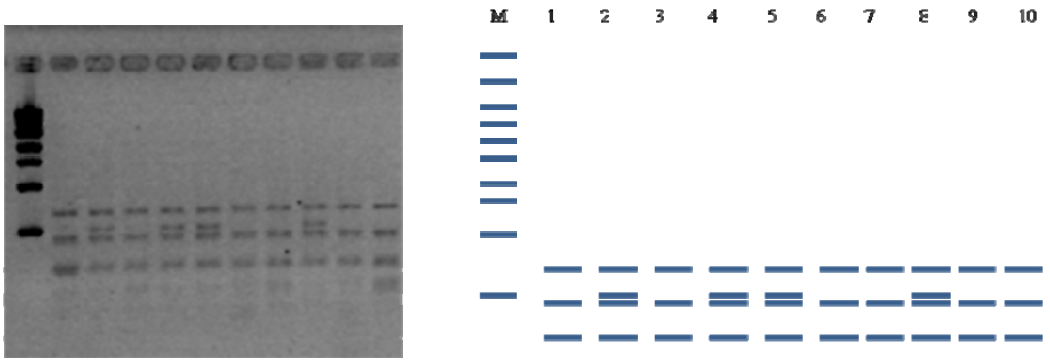

Figure 4.29 Recombinant plasmids after digested by restriction enzyme *Bsu*RI Lane M: 1 kb marker, Lane 1-10: clones mB1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Hin*fI. Digestion of clones by restriction enzyme *Hin*fI exhibited the difference between 10 clones and sorted these clones into 2 groups as described below: (Figure 4.30)

Group 1: mB1, mB2, mB3, mB4, mB5, mB7
Group 2: mB6, mB8, mB9, mB10

M 1 2 3 4 5 6 7 8 9 10

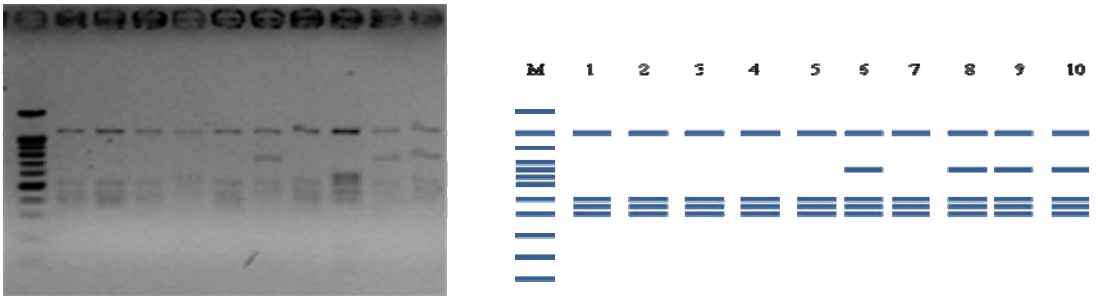

Figure 4.30 Recombinant plasmids after digested by restriction enzyme *Hin*FI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones mB1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I. The results exhibited that all clones shown the same pattern (Figure 4.31).

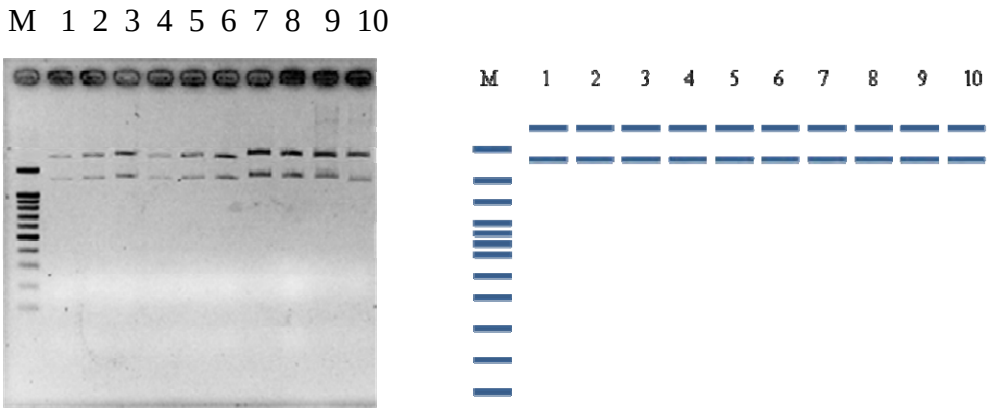

Figure 4.31 Recombinant plasmids after digested by restriction enzyme *Rsa*I Lane M: 100 bp ladder DNA marker, Lane 1-10: clones mB1-10, respectively

Digestion of clones by all restriction enzyme exhibited the difference between 10 clones and sorted these clones into 3 groups as described below:

Group 1: mB1, mB3, mB7
Group 2: mB2, mB4, mB5, mB8
Group 3: mB6, mB9, mB10

After that, clones mH3, mH4, mO1, mA2, mA9, mA10, mB1, mB2 and mB6 which were representative from each group were selected to compare the pattern of digestion of clones by all restriction enzyme. The results exhibited the difference between 10 clones and sorted these clones into 5 groups as described below (Figure 32):

Group 1: mH3 (Lane 1), mB2(Lane 8)
Group 2: mH4 (Lane 2), mA9 (Lane 5), mB1 (Lane 7)
Group 3: mO1 (Lane 3)
Group 4: mA2 (Lane 4), mB6(Lane 9)
Group 5: mA10 (Lane 6)

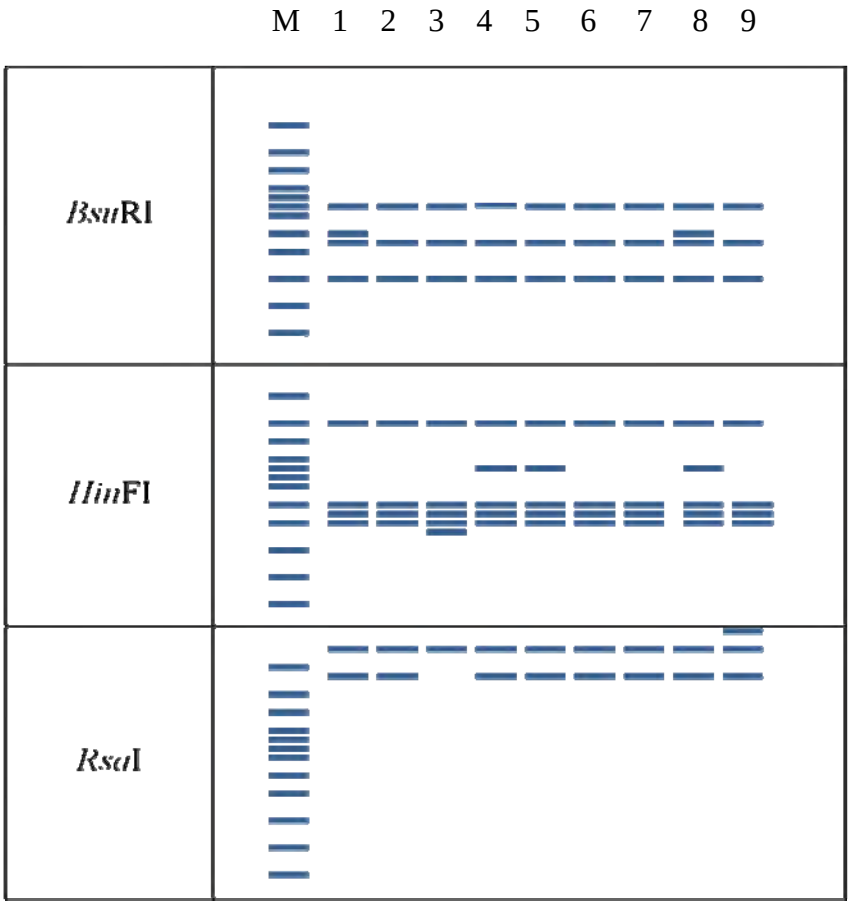

Figure 4.32 Selected recombinant plasmids from acid tank and methane tank of bioreactor using organic waste as substrate after digested with three restriction enzymes. Lane M: 100 bp ladder DNA marker, Lane 1: clones mH3, Lane 2: clones mH4, Lane 3: clones mO1, Lane 4: Cloned mA2, Lane 5: clones mA9, Lane 6: clones mA1, Lane 7: clones mB1, Lane 8: clones mB2, Lane 9: clones mB6

Therefore, clones mH3, mH4, mO1, mA2 and mA10 were selected to analyze the sequence of nucleotide base. After compared sequences to GenBank using software BLASTx (http://www.ncbi.nlm.nih.gov/), all clones were similar to methyl-coenzyme M reductase alpha subunit (Tables 4.11-4.15). Gene product of mH3 showed high sequence similarity to methyl-coenzyme M reductase alpha subunit of uncultured methanogenic archaeon (96%). Gene products of mH4, mO1, mA2 and mA10 were matched closely to methyl-coenzyme M reductase alpha subunit of uncultured Methanomicrobiales archaeon (95%).

Table 4.11 Sequence analysis of gene product of recombinant plasmid mH3

Accession number	Description	Identity (%)	References
AAT45707	methyl-coenzyme M reductase alpha subunit (uncultured Methanomicrobiales archaeon)	138/153 (90%)	Banning, *et al*., 2005
AAX84590	methyl-coenzyme M reductase subunit A (uncultured methanogenic archaeon)	147/152 (96%)	Kovacik, *et al*., 2010
ADD82267	methyl-coenzyme M reductase alpha subunit (uncultured methanogenic archaeon)	140/153 (91%)	Nava, *et al*. (unpublished)
BAF46706	methyl-coenzyme M reductase (*Methanoculleus* sp. HC-1)	133/152 (87%)	Shimizu, *et al*. (unpublished)
CAK95768	methyl-coenzyme M reductase alpha subunit (uncultured *Methanoculleus* sp.)	134/153 (87%)	Hallberg and Johnson (unpublished)

Table 4.12 Sequence analysis of gene product of recombinant plasmid mH4

Accession number	Description	Identity (%)	References
BAF74605	methyl CoM reductase subunit alpha (uncultured Methanomicrobiales archaeon)	143/150 (95%)	Nunoura, *et al*., 2008
ABU90061	methyl-coenzyme M reductase alpha subunit (uncultured methanogenic archaeon)	143/156 (91%)	Ufnar, *et al*. (unpublished)

Table 4.12 Sequence analysis of gene product of recombinant plasmid mH4 (continued)

Accession number	Description	Identity (%)	References
ACD35158	methyl coenzyme M reductase subunit alpha (uncultured archaeon)	145/156 (92%)	Nettmann, *et al*. (unpublished)
NP_613940	methyl coenzyme M reductase, alpha subunit (*Methanopyrus kandleri* AV19)	109/156 (69%)	Slesarev, *et al*. (unpublished)
AAQ56624	methyl coenzyme M reductase alpha subunit (*Methanocaldococcus infernus* ME)	107/156 (68%)	Nercessian *et al*., 2005

Table 4.13 Sequence analysis of gene product of recombinant plasmid mO1

Accession number	Description	Identity (%)	References
BAF74605	methyl CoM reductase subunit alpha (uncultured Methanomicrobiales archaeon)	143/150 (95%)	Nunoura, *et al*., 2008
ABU90061	methyl-coenzyme M reductase alpha subunit (uncultured methanogenic archaeon)	143/156 (91%)	Ufnar, *et al*. (unpublished)
ACD35158	methyl coenzyme M reductase subunit alpha (uncultured archaeon)	145/156 (92%)	Nettmann, *et al*. (unpublished)
NP_613940	methyl coenzyme M reductase, alpha subunit (*Methanopyrus kandleri* AV19)	109/156 (69%)	Slesarev, *et al*. (unpublished)

Table 4.13 Sequence analysis of gene product of recombinant plasmid mO1 (continued)

Accession number	Description	Identity (%)	References
AAQ56624	methyl coenzyme M reductase alpha subunit (*Methanocaldococcus infernus* ME)	107/156 (68%)	Nercessian *et al*., 2005

Table 4.14 Sequence analysis of gene product of recombinant plasmid mA2

Accession number	Description	Identity (%)	References
BAF74605	methyl CoM reductase subunit alpha (uncultured Methanomicrobiales archaeon)	137/144 (95%)	Nunoura, *et al*., 2008
ABU90057	methyl-coenzyme M reductase alpha subunit (uncultured methanogeni archaeon)	137/150 (91%)	Ufnar, *et al*. (unpublished)
ABF19166	methyl-coenzyme M reductase subunit A (uncultured archaeon)	137/145 (94%)	Rastogi, *et al*., 2008
AAX84599	methyl-coenzyme M reductase subunit A (uncultured methanogenic archaeon)	134/148 (90%)	Kovacik, *et al*., 2010
ACL80616	methyl coenzyme M reductase I (uncultured archaeon)	133/150 (88%)	Steinberg and Regan (unpublished)

Table 4.15 Sequence analysis of gene product of recombinant plasmid mA10

Accession number	Description	Identity (%)	References
ABU90061	methyl-coenzyme M reductase alpha subunit (uncultured methanogenic archaeon)	134/147 (91%)	Ufnar, *et al*. (unpublished)
BAF74605	methyl CoM reductase subunit alpha (uncultured Methanomicrobiales archaeon)	135/142 (95%)	Nunoura, *et al*., 2008
ABN54670	methyl-coenzyme M reductase alpha subunit (uncultured archaeon)	132/144 (91%)	Mihajlovski, *et al*., 2008
AAX84599	methyl-coenzyme M reductase subunit A (uncultured methanogenic archaeon)	133/147 (90%)	Kovacik, *et al*., 2010
ACL80616	methyl coenzyme M reductase I (uncultured archaeon)	130/147 (88%)	Steinberg and Regan (unpublished)

The previous studies reported that the presence of *mcrA* gene could represent the availability of methanogens which are the species play a pivotal role in the production of biogas. Radl *et al.* (2007) detected the *mcrA* genes to observe for the methanogens in soils.

4.4.2 *hydA* gene

Hydrogenase (*hydA*) gene codes hydrogenases enzyme which plays a central role in hydrogen metabolism in anaerobic microorganisms. Hydrogenases are most often involved in the oxidation of hydrogen and catalyze the reduction of protons. Moreover, hydrogenases have been found in some methanogens and they catalyze an

intermediary step in CO_2 reduction with H_2 to methane. Polymerase chain reaction (PCR) was conducted to detect *hydA* gene in these biosludge samples which involved in biogas production. The expected product size of *hydA* is 300 bp. From the result, *hydA* could be detected in every sample because the biogas in the system had hydrogen content in both of acid tank and methane tank and both of substrates; organic waste and biodiesel wastewater (Figure 4.33).

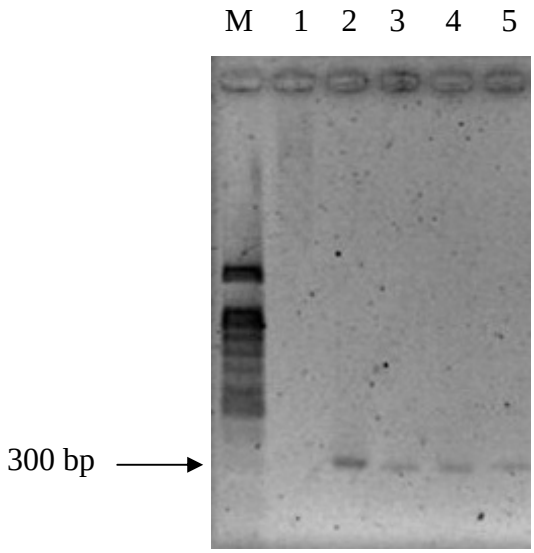

Figure 4.33 Detection of *hydA* gene in biosludges from bioreactor. Lane M: 100 bp ladder DNA marker, Lane 1: negative, Lane 2-3: biosludges from acid tank and methane tank of bioreactor using organic waste as substrate, Lane 4-5: biosludges from acid tank and methane tank of bioreactor using biodiesel wastewater as substrate

After that, the bands in lanes 2-5 (Figure 4.33) was extracted and ligated into pGEM-T Easy vector, transformed into *E. coli* JM109. The required colonies were then selected. Ten clones from the bands in lanes 2-5 were picked. Extracted plasmids were digested by *Eco*RI in order to check the presence of PCR product.

4.4.2.1 *hydA*-organic waste-acid tank

The presence of PCR product of biosludges from acid tank of bioreactor using organic waste as substrate is shown in Figure 4.34.

M 1 2 3 4 5 6 7 8 9 10

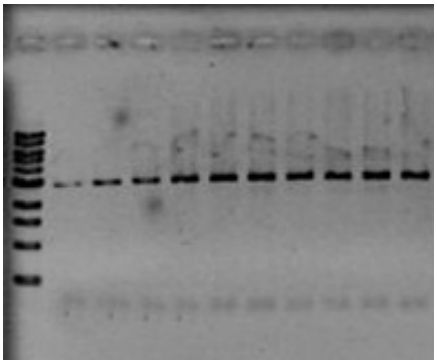

Figure 4.34 Recombinant plasmids after digested by restriction enzyme *Eco*RI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hH1-10, respectively

From Figure 4.34, all colonies could be detected the presence of PCR products. Thus, all samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. Digestion of clones by restriction enzyme *Bsu*RI exhibited the difference between 10 clones and sorted these clones into 2 groups as described below: (Figure 4.35)

Group 1: hH1, hH2, hH3, hH4, hH5, hH6, hH7, Hh8, hH9

Group 2: hH10

M 1 2 3 4 5 6 7 8 9 10

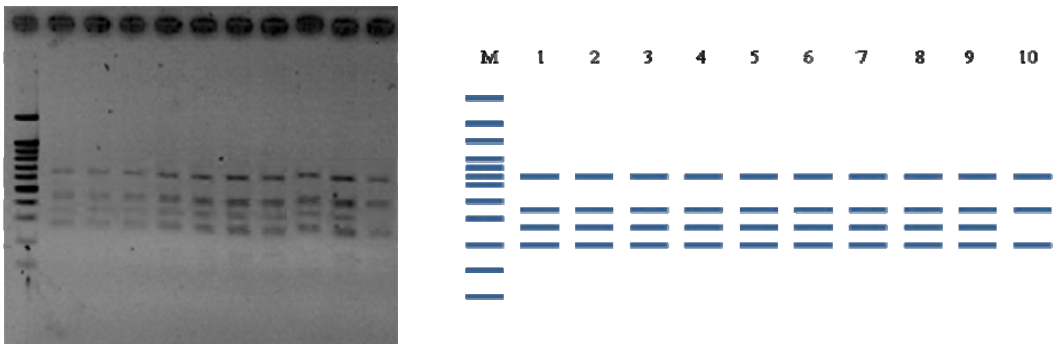

Figure 4.35 Recombinant plasmids after digested by restriction enzyme *Bsu*RI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hH1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Hin*FI. The results exhibited that all clones shown the same pattern (Figure 4.36).

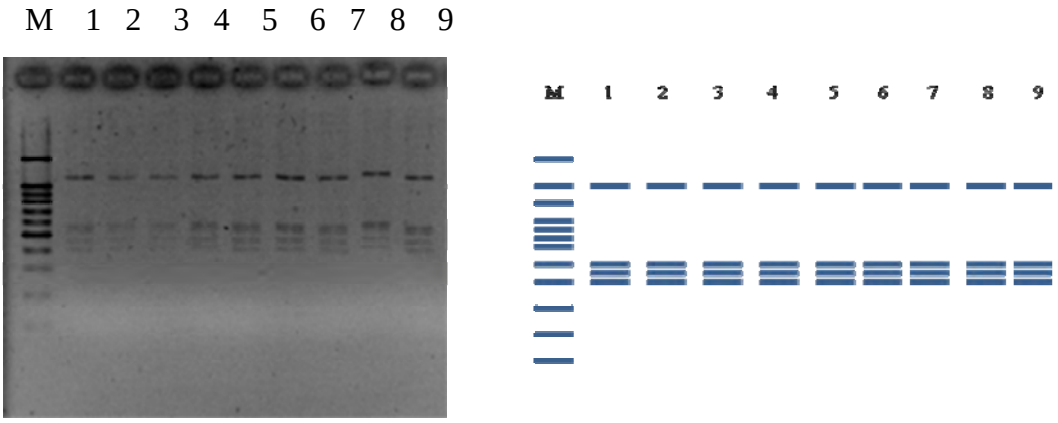

Figure 4.36 Recombinant plasmids after digested by restriction enzyme *Hin*FI Lane M: 100 bp ladder DNA marker, Lane 1-9: clones hH1-9, respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I. The results exhibited that all clones shown the same pattern (Figure 4.37).

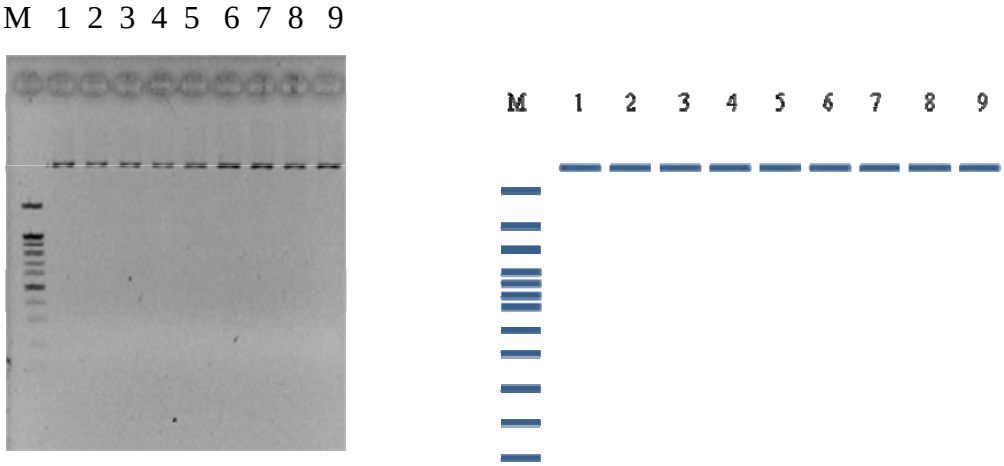

Figure 4.37 Recombinant plasmids after digested by restriction enzyme *Rsa*I Lane M: 100 bp ladder DNA marker, Lane 1-9: clones hH1-9, respectively

Digestion of clones by all restriction enzyme exhibited the difference between 10 clones and sorted these clones into 2 groups as described below:
Group 1: hH1, hH2, hH3, hH4, hH5, hH6, hH7, hH8, hH9
Group 2: hH10

4.4.2.2 *hydA*-organic waste-methane tank

The presence of PCR product of biosludges from methane tank of bioreactor using organic waste as substrate is shown in Figure 4.38.

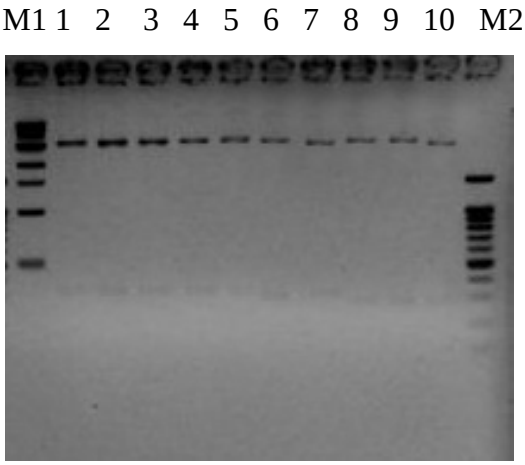

Figure 4.38 Recombinant plasmids after digested by restriction enzyme *Eco*RI Lane M1: 1 kb marker, Lane 1-10: selected colonies hO1-10, respectively, M2: 100 bp ladder DNA marker

From Figure 4.38, all colonies could be detected the presence of PCR products. Thus, all samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. The results exhibited that all clones shown the same pattern (Figure 4.39).

M 1 2 3 4 5 6 7 8 9 10

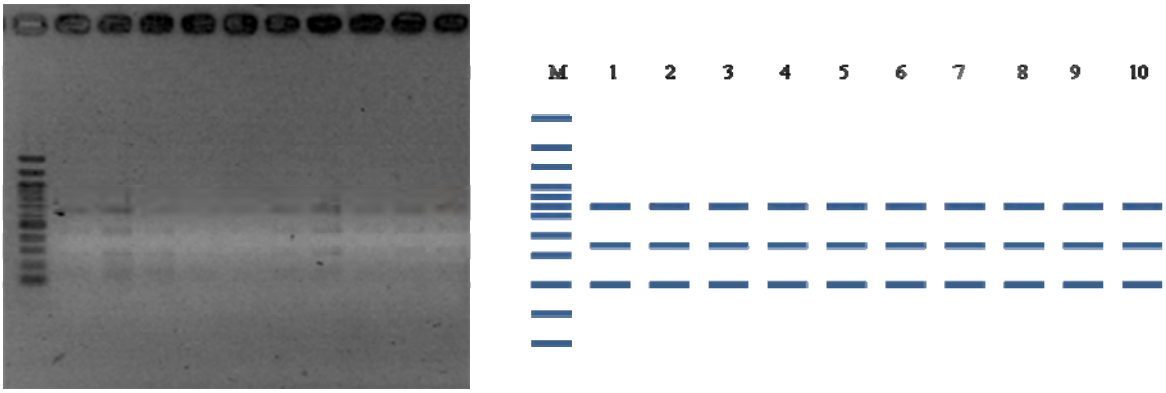

Figure 4.39 Recombinant plasmids after digested by restriction enzyme *Bsu*RI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hO1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Hin*fI. Digestion of clones by restriction enzyme *Hin*fI exhibited the difference between 10 clones and sorted these clones into 2 groups as described below: (Figure 4.40)

Group 1: hO1, hO2, hO3, hO4, hO5
Group 2: hO6, hO7, hO8, hO9, hO10

M 1 2 3 4 5 6 7 8 9 10

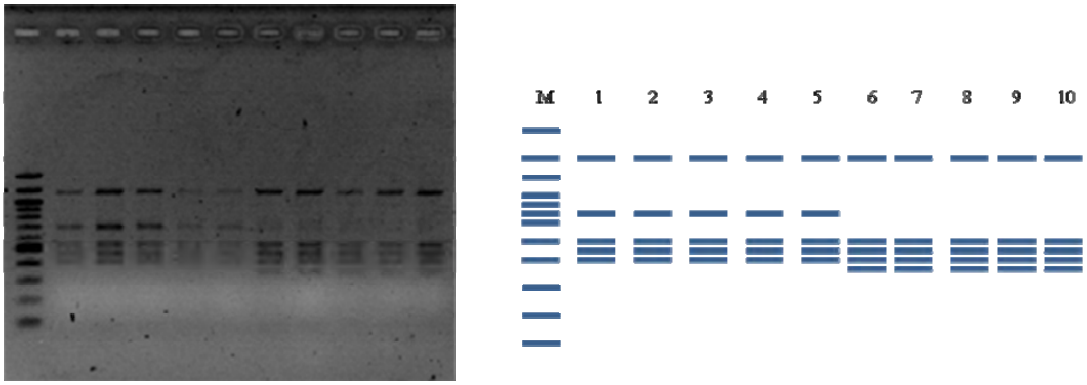

Figure 4.40 Recombinant plasmids after digested by restriction enzyme *Hin*FI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hO1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I. Digestion of clones by restriction enzyme *Rsa*I exhibited the difference between 10 clones and sorted these clones into 2 groups as described below: (Figure 4.41)

Group 1: hO1, hO2, hO3, hO4, hO5

Group 2: hO6, hO7, hO8, hO9, hO10

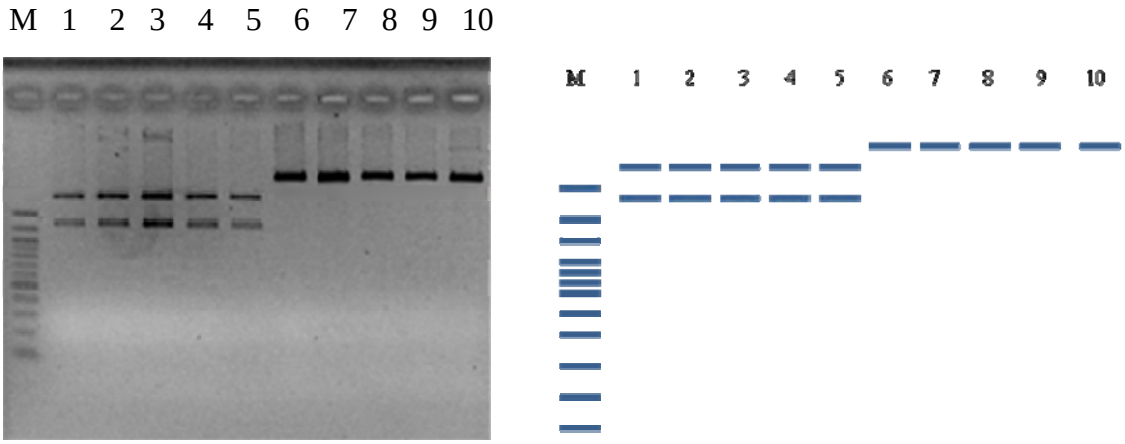

Figure 4.41 Recombinant plasmids after digested by restriction enzyme *Rsa*I Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hO1-10, respectively

Digestion of clones by all restriction enzyme exhibited the difference between 10 clones and sorted these clones into 2 groups as described below:

Group 1: hO1, hO2, hO3, hO4, hO5

Group 2: hO6, hO7, hO8, hO9, hO10

4.4.2.3 *hydA*-biodiesel wastewater-acid tank

The presence of PCR product of biosludges from acid tank of bioreactor using biodiesel wastewater as substrate is shown in Figure 4.42.

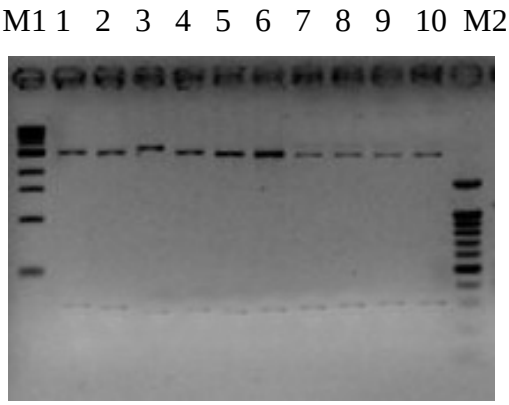

Figure 4.42 Recombinant plasmids after digested by restriction enzyme *Eco*RI Lane M1: 1 kb marker, Lane 1-10: selected colonies hA1-10, respectively, M2: 100 bp ladder DNA marker

From Figure 4.42, all colonies could be detected the presence of PCR products. Thus, all samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. The results exhibited that all clones shown the same pattern (Figure 4.43).

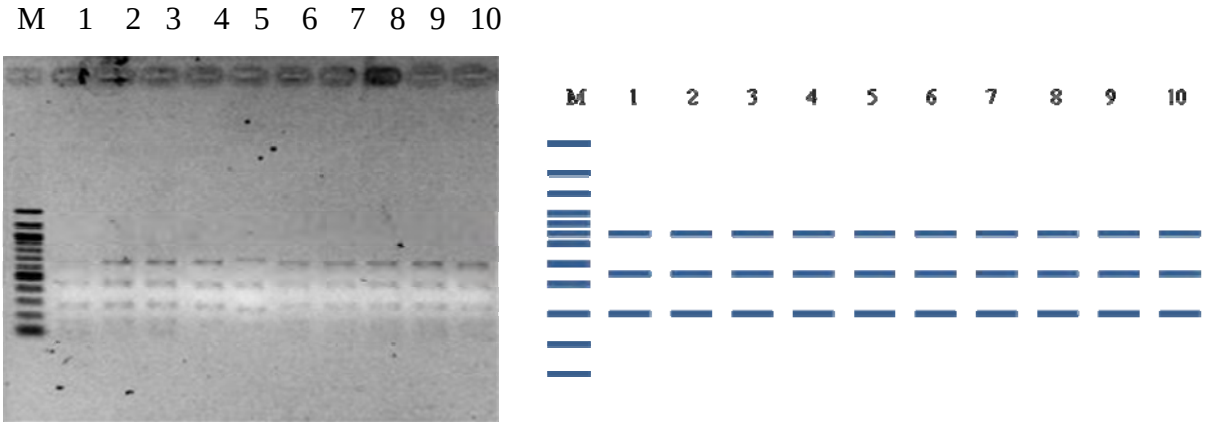

Figure 4.43 Recombinant plasmids after digested by restriction enzyme *Bsu*RI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hA1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Hin*fI. The results exhibited that all clones shown the same pattern (Figure 4.44)

M 1 2 3 4 5 6 7 8 9 10

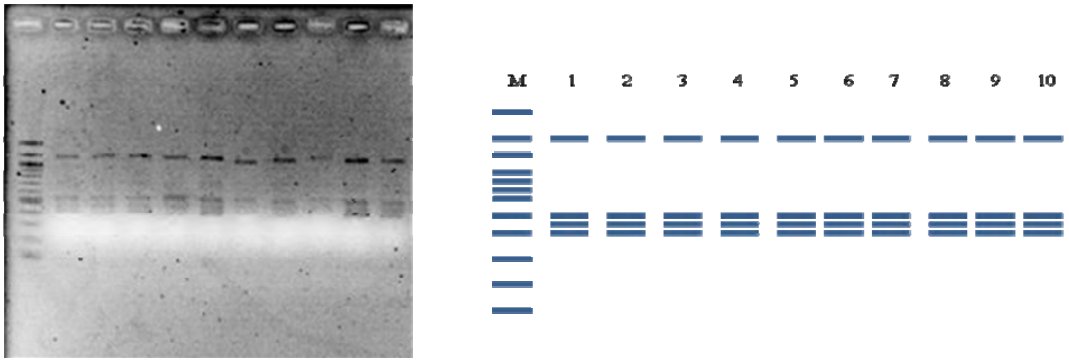

Figure 4.44 Recombinant plasmids after digested by restriction enzyme *Hin*FI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hA1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I. The results exhibited that all clones shown the same pattern (Figure 4.45).

M 1 2 3 4 5 6 7 8 9 10

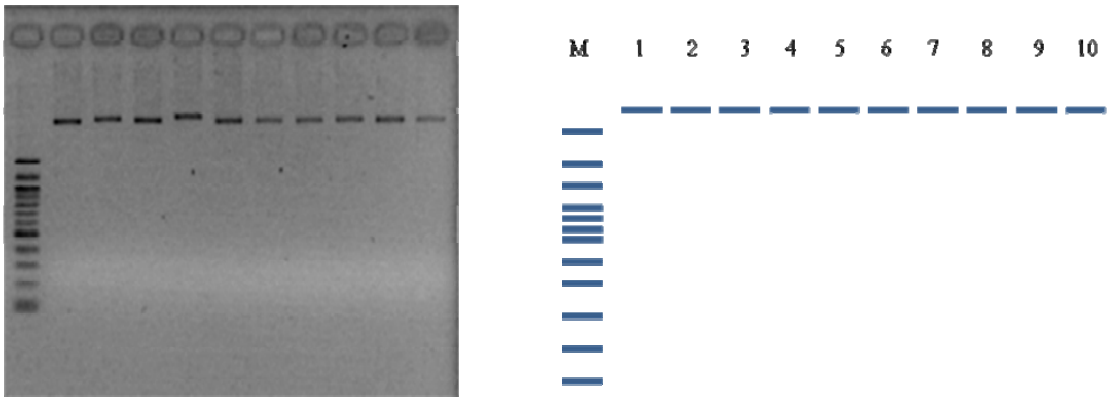

Figure 4.45 Recombinant plasmids after digested by restriction enzyme *Rsa*I Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hA1-10, respectively

Therefore, all clones of biosludges from methane tank of bioreactor using organic waste as substrate were completely digested with three restriction enzymes. The results exhibited that all clones shown the same pattern.

4.4.2.4 *hydA*-biodiesel wastewater-methane tank

The presence of PCR product of biosludges from methane tank of bioreactor using biodiesel wastewater as substrate is shown in Figure 4.46.

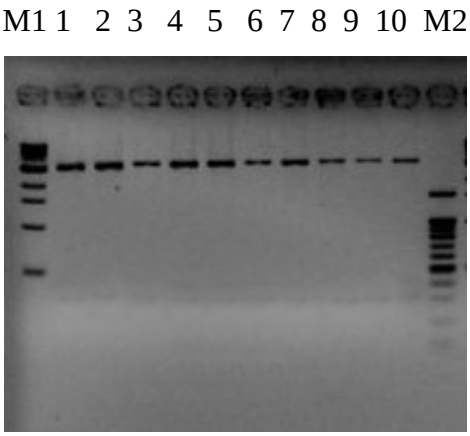

Figure 4.46 Recombinant plasmids after digested by restriction enzyme *Eco*RI Lane M1: 1 kb marker, Lane 1-10: selected colonies hB1-10, respectively, M2: 100 bp ladder DNA marker

From Figure 4.46, all colonies could be detected the presence of PCR products. Thus, all samples were digested by restriction enzyme *Bsu*RI in order to group the same pattern of PCR products. The results exhibited that all clones shown the same pattern (Figure 4.47).

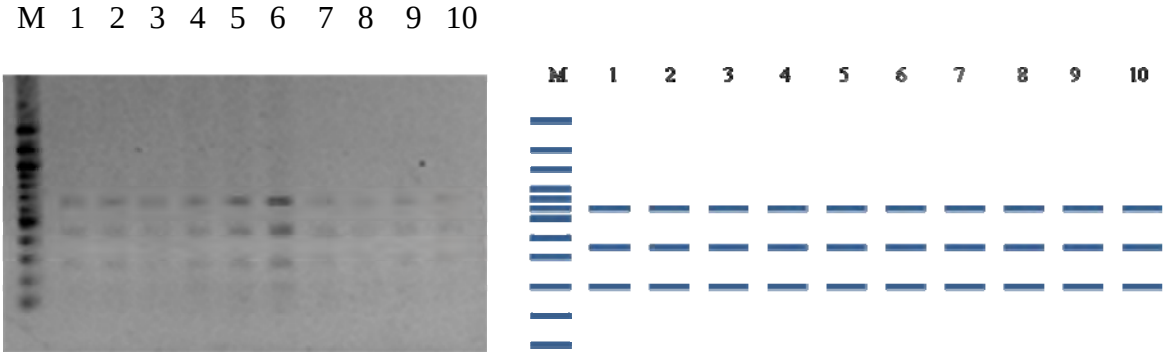

Figure 4.47 Recombinant plasmids after digested by restriction enzyme *Bsu*RI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hB1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Hin*fI. The results exhibited that all clones shown the same pattern (Figure 4.48)

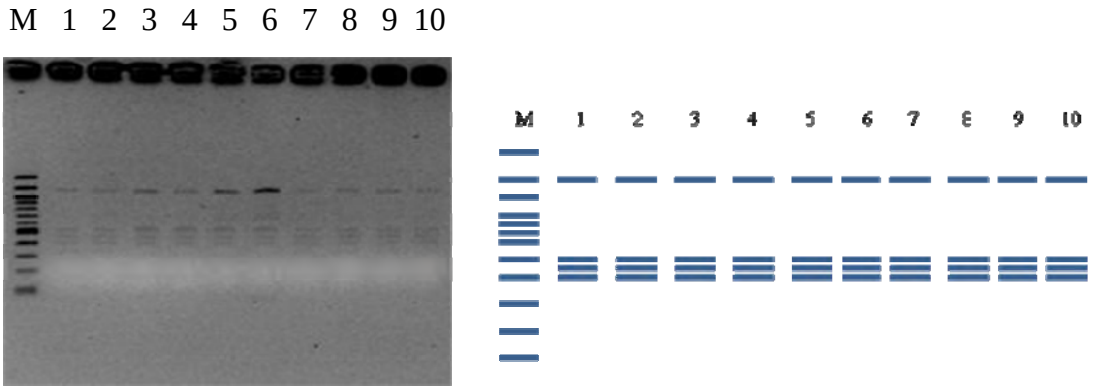

Figure 4.48 Recombinant plasmids after digested by restriction enzyme *Hin*FI Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hB1-10, respectively

Therefore, all clones were digested again with restriction enzyme *Rsa*I. The results exhibited that all clones shown the same pattern (Figure 4.49).

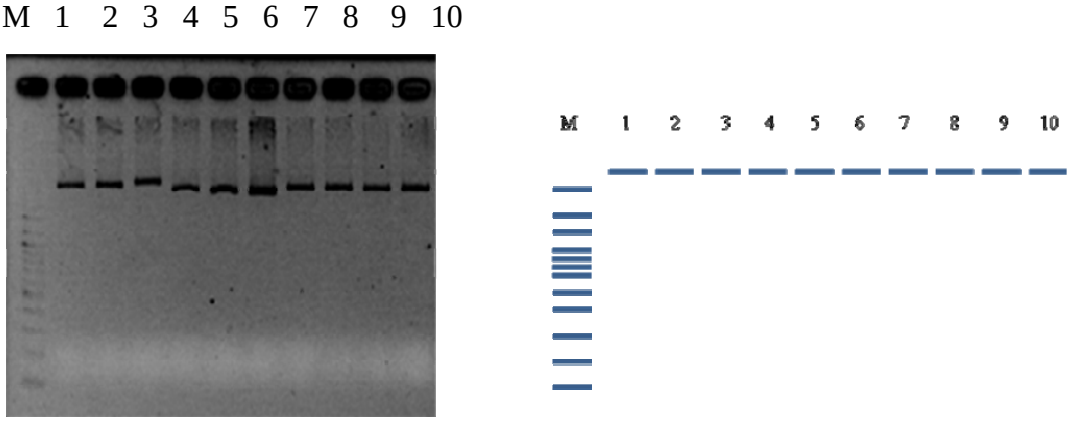

Figure 4.49 Recombinant plasmids after digested by restriction enzyme *Rsa*I Lane M: 100 bp ladder DNA marker, Lane 1-10: clones hB1-10, respectively

Therefore, all clones of biosludges from methane tank of bioreactor using biodiesel wastewater as substrate were digested with three restriction enzymes. The results exhibited that all clones shown the same pattern.

After that, clones hH1, hH10, hO1, hO6, hA1 and hB1 which were representative from each group were selected to compare the pattern of digestion of clones by all restriction enzyme. The results exhibited the difference between 7 clones and sorted these clones into 4 groups as described below (Figure 4.50):

Group 1: hH1 (Lane 1)
Group 2: hH10 (Lane 2), hA1 (Lane5), hB1 (Lane 6)
Group 3: hO1 (Lane 3)
Group 4: hO6 (Lane 4)

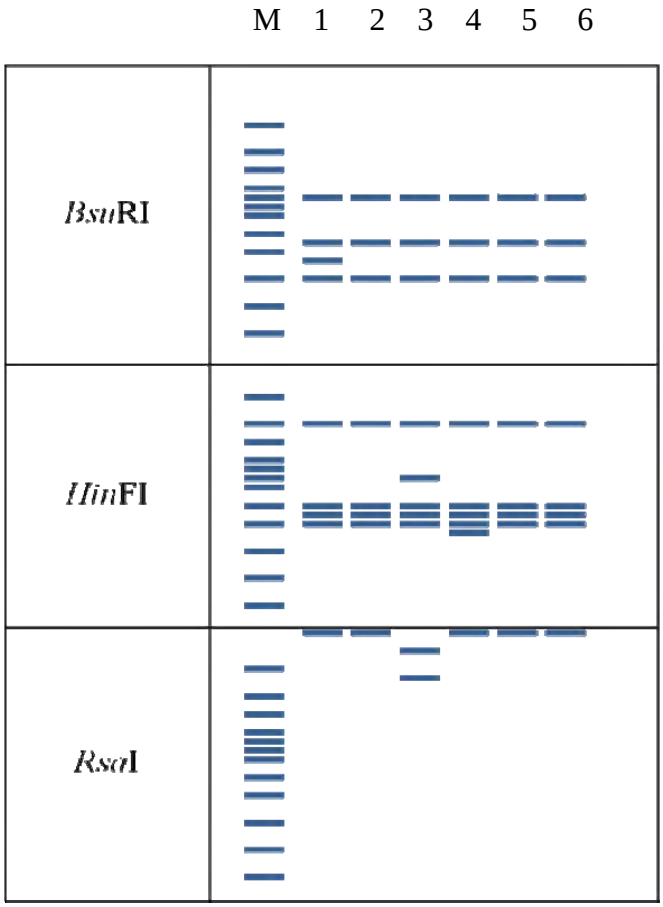

Figure 4.50 Selected recombinant plasmids from acid tank and methane tank of bioreactor using organic waste as substrate after digested with three restriction enzymes, Lane M: 100 bp ladder DNA marker, Lane 1: clones hH1, Lane 2: clones hH10, Lane 3: clones hO1, Lane 4: clones hO6, Lane 5: clones hA1, Lane 6: clones hB1

Therefore, clones hH1, hH10, hO1 and hO6 were selected to analyze the sequence of nucleotide base. After compared sequences to GenBank using software BLASTx (http://www.ncbi.nlm.nih.gov/), all clones were similar to [FeFe]-hydrogenase and [Fe]-hydrogenase (Tables 4.16-4.19). Gene product of hH1 and hH10 showed high sequence similarity to [FeFe]- hydrogenase of uncultured bacterium (85%). Gene products of hO1 and hO6 were matched closely to [Fe]-hydrogenase of *Ruminococcus flavefaciens* (82%).

Table 4.16 Sequence analysis of gene product of recombinant plasmid hH1

Accession number	**Description**	**Identity (%)**	**References**
ACQ94917	iron-iron hydrogenase (uncultured bacterium)	85/100 (85%)	Sahl, *et al*., (unpublished)
ADC53680	iron-iron hydrogenase (uncultured bacterium)	77/96 (80%)	Boyd, *et al*. (unpublished)
YP_002892704	hydrogenase, Fe-only (*Tolumonas auensis* DSM 9187)	71/100 (71%)	Lucas, *et al*., (unpublished)
CAY56130	[Fe-Fe] hydrogenase large subunit (uncultured bacterium)	68/100 (68%)	Schmidt (unpublished)
YP_077035	iron hydrogenase (*Symbiobacterium thermophilum* IAM 14863)	75/83 (90%)	Ueda, *et al*., 2004

Table 4.17 Sequence analysis of gene product of recombinant plasmid hH10

Accession number	**Description**	**Identity (%)**	**References**
ACQ94917	iron-iron hydrogenase (uncultured bacterium)	83/97 (85%)	Sahl, *et al*., (unpublished)

Table 4.17 Sequence analysis of gene product of recombinant plasmid hH10 (continued).

Accession number	Description	Identity (%)	References
ADC53613	iron-iron hydrogenase (uncultured bacterium)	78/97 (80%)	Boyd, *et al*. (unpublished)
YP_002892704	hydrogenase, Fe-only (*Tolumonas auensis* DSM 9187)	67/97 (69%)	Lucas, *et al*., (unpublished)
CAY56130	[Fe-Fe] hydrogenase large subunit (uncultured bacterium)	64/97 (65%)	Schmidt (unpublished)
YP_430562	Iron hydrogenase, small subunit (*Moorella thermoacetica* ATCC 39073)	59/97 (60%)	Pierce, *et al*., 2008

Table 4.18 Sequence analysis of gene product of recombinant plasmid hO1

Accession number	Description	Identity (%)	References
ZP_06141654	hydrogenase, Fe-only [Ruminococcus flavefaciens FD1]	79/96 (82%)	Berg Miller, *et al*., 2009
CBL17696	hydrogenases, Fe-only [Ruminococcus sp. 18P13]	76/96 (79%)	Pajon, *et al*. (unpublished)
CBK79892	hydrogenases, Fe-only [Coprococcus catus GD/7]	78/96 (81%)	Pajon, *et al*. (unpublished)
YP_003823544	hydrogenase, Fe-only [Clostridium saccharolyticumWM1]	75/96 (78%)	Lucas, *et al*. (unpublished)
ZP_04670977	hydrogenase [Clostridiales bacterium1_7_47_FAA]	73/96 (76%)	Allen-Vercoe, *et al*., 2004

Table 4.19 Sequence analysis of gene product of recombinant plasmid hO6

Accession number	Description	Identity (%)	References
ZP_06141654	hydrogenase, Fe-only [Ruminococcus flavefaciens FD-1]	79/96 (82%)	Berg Miller, *et al*., 2009
CBL17696	hydrogenases, Fe-only [Ruminococcus sp. 18P13]	76/96 (79%)	Pajon, *et al*. (unpublished)
CBK79892	hydrogenases, Fe-only [Coprococcus catus GD/7].	78/96 (81%)	Pajon, *et al*. (unpublished)
YP_003823544	hydrogenase, Fe-only [Clostridium saccharolyticum WM1].	75/96 (78%)	Lucas, *et al*. (unpublished)
ZP_04670977	hydrogenase [Clostridiales bacterium 1_7_47_FAA].	73/96 (76%)	Allen-Vercoe, *et al*., 2004

The previous studies reported that the hydrogenase play a central role in hydrogen methabolism in many microorganisms such as sulfate-reducing, photosynthetic, methanogenic, nitrogen-fixing and acetogenic prokaryotes (Vignais and Billoud, 2007).

4.5 Real-time PCR for quantification of *mcrA* gene

A real-time PCR was used to quantify *mcrA* gene target from samples of bioreactor fed with biodiesel wastewater as substrate. In the biogas production, methanogens play an important role and convert H_2/CO_2, acetate, formate or methanol to mehane (Ferry, 1993). Methanogens can be studied specifically using a characteristic functional marker gene *mcrA* coding α-subunit of methyl-coenzyme M reductase, the key enzyme of methanogenesis (Rastogi *et al*., 2008). The presence of

the *mcrA* gene is restricted to methanogenic archaea (Radl, *et al*., 2007), hence its quantity serves an estimate for understanding performance of the system.

This study used primer set *mcrA* F and *mcrA* R which was specific to methyl-coenzyme M reductase enzyme to detect and quantify amount of *mcrA* genes using standard curve as shown in Figure 4.51. The DNA samples extracted from biosludge from acid tank and methane tank of bioreactor using biodiesel wastewater as substrate were detected with SYBR green dyes which were described in Figure 4.52. In acid tank, the amount of *mcrA* genes in week 0 and week 1 was similar which were 4.95×10^3 and 4.79×10^3 *mcrA* gene copies number/g sludge. For week 2, the amount of *mcrA* genes in sludge decrease to 6.15×10^2 *mcrA* gene copies number/g sludge because the amount of volatile fatty acid (VFA) was high (Figure 4.54). The volatile fatty acid (VFA) produced during anaerobic digestion tend to reduce the pH (Figure 4.55) which can inhibit the activity of the metanogens (Appels, *et al*., 2008). On the contrary, the amount of *mcrA* gene in week 3 to the last week tended to increase from 9.30×10^2 to 4.40×10^4 *mcrA* gene copies number/g sludge because the VFA was decrease and pH was higher. For methane tank, the amount of *mcrA* gene tended to increase from 8.41×10^2 to 4.28×10^4 *mcrA* gene copies number/g sludge. On the contrary, the amount of *mcrA* gene was decreased to 8.87×10^2 *mcrA* gene copies number/g sludge in the last week. For the quantification of *mcrA* genes in methane tank revealed a similar pattern as accumulated biogas production (Figure 4.53). The accumulation of biogas in methane tank tended to increase in every week since week0 until week 4 and dropped in the last week.

Biogas that occurred in this system was a total amount of biogas which consisted of methane, hydrogen and carbon dioxide. However, the ratio of methane in biogas was range from 63.32-68.58%. Since this system could not decisively separate the activity of two main groups of microorganisms; acid and methane forming microorganisms, methane could occur in both of acid tank and methane tank. From this reason, *mcrA* gene could be detected in acid tank and methane tank.

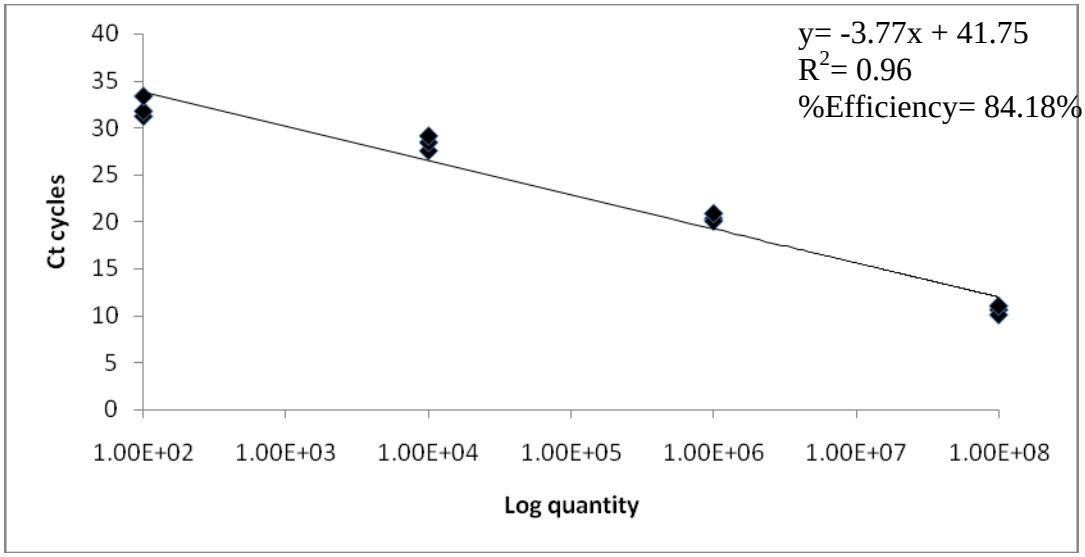

Figure 4.51 Standard curve of the *mcrA* gene copy numbers from real-time PCR amplification assays obtains by plotting the logarithm of the gene copy numper (equivalent to the plasmid copy number) *vs*. the ct value

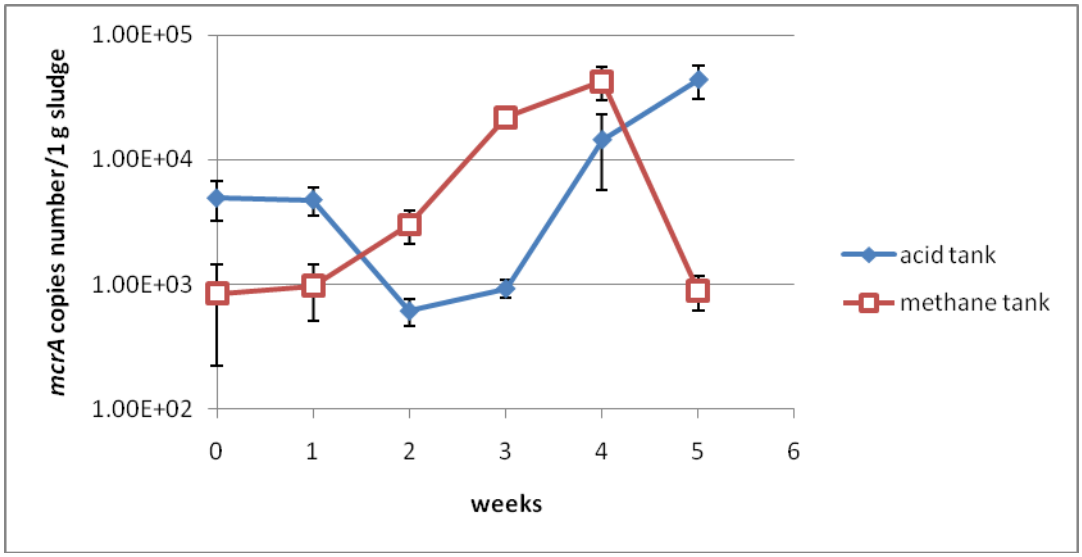

Figure 4.52 *mcrA* gene copy numbers by Real-time PCR using biosludge samples from bioreactor using biodiesel wastewater as substrate; *mcrA* gene copies number/ 1 g sludge

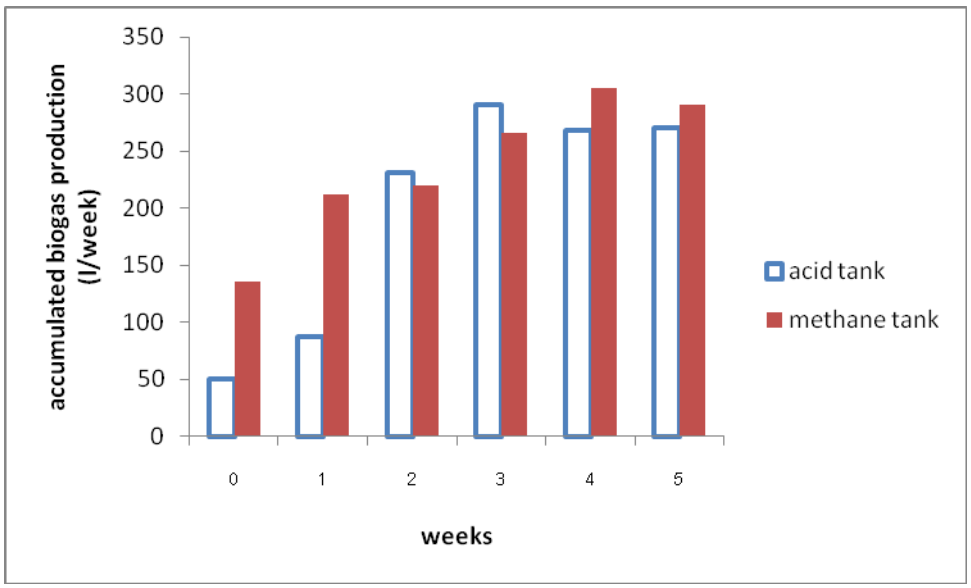

Figure 4.53 Accumulated biogas productions in bioreactor using biodiesel wastewater as substrate (Panadda, 2009)

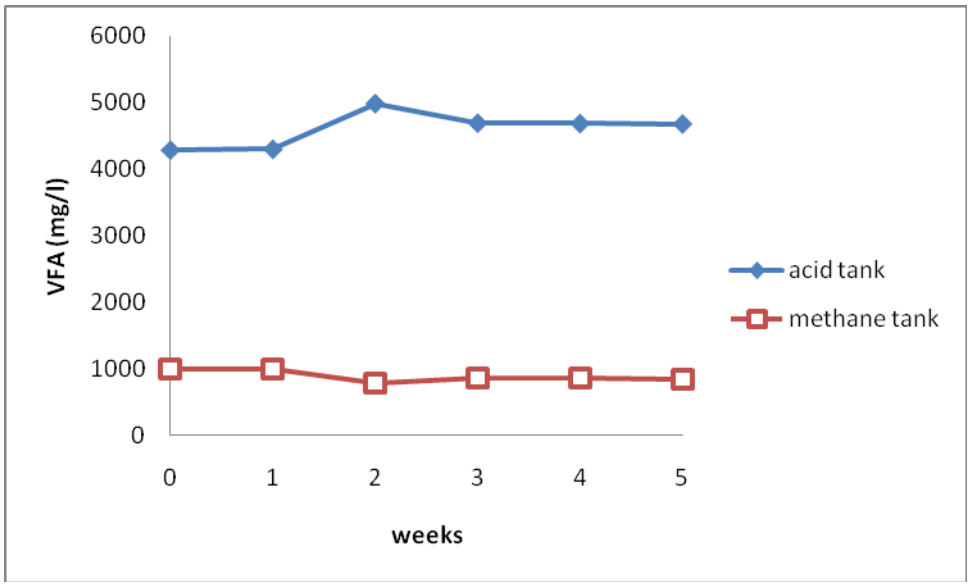

Figure 4.54 Amount of volatile fatty acid (VFA) in bioreactor using biodiesel wastewater as substrate (Panadda, 2009)

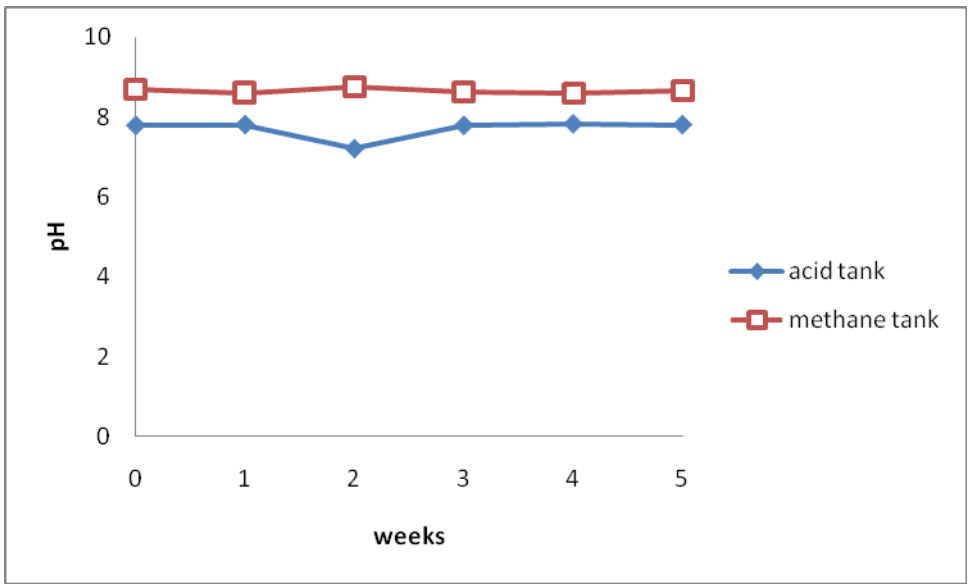

Figure 4.55 pH in bioreactor using biodiesel wastewater as substrate (Panadda, 2009)

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Nowadays, the energy demands have increase continuously while fossil fuel resources are limited and the price of them has become very high. Moreover, fossil fuels are the major cause of global warming. As a sustainable energy source, biogas is one of the alternatives to replace fossil fuel because it is clean and environmental friendly. Biogas is produced by anaerobic digestion by specific microbial communities. Therefore, it is better to understand the functions of the microbial community in the process.

Hence, the aim of this study is to analyze the microbial diversity in biogas production within continuous stirred tank reactor (CSTR) fed with organic waste and biodiesel wastewater as substrate. Microbial community structure was analyzed by PCR-DGGE and 16S rDNA clone libraries. In addition, this study assessed the genes involved in biogas production: hydrogenase genes and methyl-coenzyme M reductase genes by PCR amplification and real-time PCR.

In this study, the samples were taken from the lab bench scale two-stage anaerobic digestion in two different substrates: organic waste and biodiesel wastewater. For bioreactor which using biodiesel wastewater as substrate, sample were collected during operation in every week for 6 weeks since the start-up state until steady state. For bioreactor which using organic waste as substrate, sample were collected at steady state.

Based on bacterial community analyses for biodiesel wastewater-feeding reactor, DGGE bands in acid tank and methane tank were different but DGGE bands in the same tank were similar. For archaea community, the DGGE bands in acid tank and methane had a little different and less diverse (Table 5.1). It is known that the conditions can affect the species of microbial community.

Table 5.1 Bacteria and archaea found in bioreactor using biodiesel wastewater as substrate.

	Acid tank	**Methane tank**
Bacteria	*Megasphaera sueciensis* *Pectinatus* sp. uncultured *Pseudomonas* sp. *Clostridium* sp. uncultured Bacteroidetes	*Clostridium kluyveri* *Propionibacterium* sp. *Pseudomonas* sp. uncultured Bacteroidetes
Archaea	uncultured archaeon *Methanosaeta* sp. Methanosarcinales archaeon	Methanosarcinales archaeon *Methanobacterium beijingense*

Comparison of bacterial community between using organic waste and biodiesel wastewater as substrate, the DGGE bands were different. It is known that type of substrate can affect the species of microbial community. For archaea community, the result showed a little bit the DGGE profiles (Table 5.2).

Table 5.2 Bacteria and archaea were found in bioreactor using two different substrates: organic waste and biodiesel wastewater

Substrate	**Bacteria**	**Archaea**
Organic waste	Bacteroidetes bacterium *Pseudomonas* sp. *Syntrophomonas* uncultured *Dialister* sp.	*Methanosaeta* sp. uncultured Methanosarcinales archaeon
Biodiesel wastewate	*Megasphaera sueciensis* *Pectinatus* sp. *Clostridium acetobutylicum* *Klebsiella pneumonia* uncultured *Chloroflexus* sp.	*Methanosaeta* sp. uncultured Methanosarcinales archaeon uncultured archaeon *Methanobacterium beijingense*

The result of 16S rDNA clone libraries, for bioreactor using organic waste as substrate, a total of 32 clones were obtained from acid tank. The clone libraries were screened using DGGE analysis and 2 different clones were selected for sequencing. In methane tank, a total of 60 clones were obtained and 7 different clones were selected for sequencing. For bioreactor using biodiesel wastewater as substrate, a total of 32 clones were obtained from acid tank and 2 different clones were selected for sequencing. In methane tank, a total of 60 clones were obtained and 3different clones were selected for sequencing (Table 5.3).

Table 5.3 16S rDNA clone libraries of bioreactor using two different substrates: organic waste and biodiesel wastewater

Substate	**Tank**	**No of clones**	**Microorganisms**
Organic waste	acid	32	*Pseudomonas acephalitica* (94%) uncultured Firmicutes bacterium (6%)
	methane	60	*Weissella cibaria* (28%) *Clostridium jejuense* (18%) uncultured bacterium (15%) *Sedimentibacter* sp., (15%) *Clostridium* sp. (11%) uncultured Firmicutes bacterium (8%) *Tissierella praeacuta* (5%)
Biodiesel wastewater	acid	32	*Klebsiella* sp. (69%) *Sphingomonas* sp. (31%)
	methane	60	uncultured Chloroflexi bacterium (76%) uncultured bacterium (22%) *Pseudomonas putida* (2%).

Hydrogenase genes (*hydA*) and methyl-coenzyme M reductase genes (*mcrA*) are the genes which involved in biogas production system. Hydrogenases play a central role in hydrogen metabolism and methyl-coenzyme M reductase is the key

enzyme of methanogenesis. Both of enzymes were detected in this study and they could be detected in acid tank and methane tank of two reactors.

For the real-time PCR, it was used to quantify *mcrA* gene target from samples of bioreactor fed with biodiesel wastewater as substrate. For the result, the amount of *mcrA* genes of acid tank in week 0 and week 1 was similar. For week 2, the amount of *mcrA* genes in sludge was decrease because the amount of volatile fatty acid (VFA) was high. The volatile fatty acid (VFA) produced during anaerobic digestion tend to reduce the pH which can inhibit the activity of the metanogens. On the contrary, the amount of *mcrA* gene in week 3 to last week trended to increase because the VFA was decrease and pH was higher. For methane tank, the amount of *mcrA* gene tended to increase. On the contrary, the amount of *mcrA* gene was decrease in the last week. For the quantification of mcrA genes in methane tank revealed a similar pattern as biogas yield production (production of biogas in methane tank trended to increase in every week and dropped in the last week.

Based on these data, a greater understanding on types of microorganisms in biogas production was obtained. The ability to monitor microorganisms and understand their ecology is essential to effectively control the start-up and operation of anaerobic bioreactors. This knowledge can be used to design effective biogas production by providing the preferred conditions for microorganisms in the two-stage anaerobic digestion system. For example, controlling of pH and temperature in the system had suitable for activity of microorganisms. Quantitative data are required for an empiric model which could facilitate the development of a better process performance monitoring. In addition, molecular techniques such as PCR-DGGE, 16S rDNA clone libraries and real-time PCR have been successfully applied to monitor and identify microorganisms in biogas production, so these techniques could be applied to analyzed microbial diversity in other reactors or other substrates.

5.2 Recommendations

1. For DNA extraction from sludge or soil should be increased time of mixing between sample and DNA extraction buffer because it can increase of DNA yield. For DNA purification should be purified at least 2 times for purified DNA.
2. Based on archaea community analysis was showed a less diverse structure. The other primers might be used for study archaea community.
3. Screening of clone library by using DGGE analysis could be changed % denaturant gradient gel which give the better results
4. For further studies could be analyzed microbial diversity in other type of biogas reactor or other type of substrate.
5. For further investigation could be analyzed the group-specific microorganisms in biogas production by real-time PCR assays.
6. For further investigation could be analyzed the active group- microorganisms in biogas production by RNA extraction from the samples.

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APPENDICES

APPENDIX A

Media Preparation

Luria Bertani (LB) broth

Tryptone	10	g
Yeast extracts	5	g
NaCl	5	g
Deionized water	to 1,000	ml

Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

LB agar

Add 15 g of agar to LB broth 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

SOC medium

Solution A

Yeast extracts	5	g
Tryptone	20	g
NaCl	0.58	g
$MgCl_2$	2	g
$MgSO_4.7H_2O$	2.46	g
KCl	0.18	g

Make final volume to 980 ml with deionized water. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

Solution B

Glucose	3.6	g
Deionized water	20	ml

Sterilize by filter through filter paper pour size 0.22 µm. Mix solutions A and B and store at -20°C until being used.

Ψb broth

Yeast extracts	5	g
Tryptone	20	g
$MgSO_4.7H_2O$	5	g

Mix them in deionized water. Adjust pH to 7 with 1 N NaOH (Appendix II). Make volume with deionized water to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

In order to make agar medium, add 15 g of agar to 1,000 ml of Ψb broth before autoclaving.

APPENDIX B

Chemicals

70% Ethanol

99% Ethanol	700	ml
Sterilized deionized water	300	ml

20% sodium dodecyl sulfate, SDS

SDS	20	g

Dissolve slowly in 80 ml 0f 60°C-sterilized deionized water. When it completely dissolved, add sterilized deionized water to make final volume of 100 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

20% Proteinase K

Proteinase K	20	mg
sterilized deionized water	1	ml

10 mM Tris-HCl solution, pH 8

Trizma base ($C_4H_{11}NO_3$)	1.2	g

Dissolve in 800 ml of deionized water, and then adjust pH to 8 with HCl. Add deionized water to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

0.5 M EDTA solution

EDTA ($C_{10}H_{14}O_8Na_2.2H_2O$)	186.1	g
NaOH	20	g

Dissolve EDTA in 800 ml deionized water. Add NaOH, mix and wait until the solution cool down to room temperature. Adjust pH to 8 and make volume to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

TE buffer

10 mM Tris-HCl, pH 8	10	ml
0.5 M EDTA solution	0.2	ml

Make volume to 1,000 ml using deionized water. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

Phenol/chloroform solution

Mix phenol which has been saturated with Tris-HCl and chloroform in ratio of 1:1 (v/v) by stirring for 15 min. Store at 4°C until being used.

Chloroform/isoamylalcohol solution

Mix chloroform qith isoamylalcohol in ratio of 24:1 (v/v). Store at 4°C until being used.

DNA extraction buffer

10 mM Tris-HCl solution, pH 8	50	ml
0.5 M EDTA	10	ml
10% SDS	30	ml
Deionized water	10	ml

Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

50X TAE buffer

Tris-HCl	242	g
0.5 M EDTA, pH 8	100	ml
Glacial acetic acid	57.1	ml

Dissolve all chemicals in 800 ml deionized water. After complete dissolve, add deionized water to 1,000 ml. Sterilize by autoclaving with pressure 15 lb/inch^2 at 121°C for 15 min.

0.9% agarose gel

Agarose gel	0.9	g
1X TAE buffer	100	ml

Melt using microwave oven.

2% agarose gel

Agarose gel	2	g
1X TAE buffer	100	ml

Melt using microwave oven.

10 mg/ml ethidium bromide

Ethidium bromide	0.1	mg
Deionized water	10	mg

Mix well and store in the dark place. When prepare, wearing glove is require since ethidium bromide is proved carcinogen.

Amplicilin, Ap

Amplicilin	100	mg
Deioniized water	1	ml

Sterilize by filter through filter paper pour size 0.22 µm.

2% 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)

X-gal	20	mg
Dimethylformamide (DMF)	1	ml

Sterilize by filter through PTFE filter pour size 0.22 µm.

1 M isopropyl-β-D-thiogalactopyranoside (IPTG)

IPTG	238	mg
Deionized water	1	ml

Sterilize by filter through filter paper pour size 0.22 µm.

TfbI solution

Potassium acetate (CH_3COOK)	0.295	g
Rubidium chloride (RbCl)	1.21	g
Calcium chloride ($CaCl_2.2H_2O$)	0.148	g
Manganeses chloride ($MnCl_2$)	0.99	g
Glycerol	15	ml

Dissolve in 70 ml of deionized water. Adjust pH to 5.8 using 0.2 M acetic acid. Add deionized to make volume to 100 ml. Sterilize by filter through filter paper pour size 0.22 µm.

TfbII solution

2-[*N*-morpholino]ethanesulfonic acid (MES)	0.29	g
Rubidium chloride (RbCl)	0.121	g
Calcium chloride ($CaCl_2.2H_2O$)	1.103	g
Glycerol	15	ml

Add deionized water to 100 ml. Sterilize by filter through filter paper pour size 0.22 µm.

Chemicals used in DGGE

10% ammonium persulfate

Ammonium persulfate	0.1	g
Deionized water	1	ml

0.5 mg/ml ethidium bromide solution

10 mg/ml ethdium bromide solution	10	µl
Deionized water	200	ml

0% denaturing solution in 8% acrylamide gel

40% acrylamide/bis	20	ml
50X TAE buffer	2	ml
Deionized water	78	ml

100% denaturing solution in 8% acrylamide gel

40% acrylamide/bis	20	ml
50X TAE buffer	2	ml
Formamide	40	ml
Urea	42	g
Add deionized water	to 100	ml

APPENDIX C

Sequence results

The sequence results of 16S rDNA of bacterial communities in bioreactors fed with biodiesel wastewater as substrate

A1_1

GAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAG
ACGGCCTTCGGGTCGTAAAGCTCTGTTATATGGGACGAACGTATCTATGGCCAATACCCATAGATAG
TGACGGTACCGTAAGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATCGAATTC

A1_2

TGATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTCTTACGGTACCGTCACTATCTATGGG
TATTGGCCATAGATACGTTCGTCCCATATAACAGAGCTTTACGATCCGAAGACCTTCCTCGTTCACGC
GGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTG

A1_3

TTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTCTTACGGTACCGTCACTATCTATGGGTAT
TGGCCATAGATACGTTCGTCCCATATAACAGAGCTTTACGACCCGAAGGCCGTCTTCACTCACGCGG
CGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCCTCCCG

A1_4

TGCTGGCACGTAGTTAGCCGTGGCTTTCTCTTACGGTACCGTCACTATCTATGGGTATTGGCCATAGA
TACGTTCGTCCCATATAACAGAGCTTTACGACCCGAAGGCCGTCTTCACTCACGCGGCGTTGCTCCGT
CAGGCTTTCGCCCATTGCGGAAGATTCCCCACTG

A1_5

CTGGCACGTAGTTAGCCGTGGCTTTCTCTTACGGTACCGTCACTATCTATGGGTATTGGCCATAGATA
CGTTCGTCCCATATAACAGAGCTTTACGACCCGAAGGCCGTCTTCACTCACGCGGCGTTGCTCCGTCA
GGCTTTCGCCCATTGCGGAAGATTCCCCAC

A2_1

TTACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGA
ACGAGGAAGGTCTTCGGATCGTAAAGTTCTGTTGCAGGGGACGAACGGCACTATAGCCAATAAGTAT
AGTGAATGACGGTACCCTGTTAGAAAGCCACGGCTAACTACGTGCC

A2_2

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ATACTTATTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTT
CCTCGTTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCT
GCCTCCCGT

A2_3

CGTATTACCGCGGCTGCTGCCCTGGCACGTAGTTAGCCGTGGCTTTCTAACAGGGTACCGTCATTCAC
TATACTTATTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGT
TCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCTG

A2_4

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ATTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGTTCACGCG
GCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCA

A2_5

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ATTCACTATACTTATTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTT
CCTCGTTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCAC

A3_1

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CCCATATGGTCGACCTGCAGGCGGCCGCGAAT

A3_2

TCGCCAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCC
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CCGGGA

A3_3

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CCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATCGA

A3_4

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AATTCTCCACTGCT

A3_5

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GCCTCCCGTA

A4_1

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GCCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCATGGCTGGATCAGGGTTGCCCC
CATTGTCCAAAATTCCCCACTGCTG

A4_2

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TAGCCGTTGCCATTTCTTCCCCGCCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCA
TGGCTGGATCAGGGTTGCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAAA

A4_3

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GAAGCACCGGCTAACTACGTGCCAGCA

A4_4

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A4_5

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GCCTCCCGT

A5_1

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ATTGTGCAATATTCCCCACTGCTGCCTCCCGTAA

A5_2

GATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTGTTTTCAGGGTCCGTCATTTGTTTCGTCCC
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A5_3

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CATTGCCCAATATTCCCCACTGCTGCCTCCCG

A5_4

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A5_5

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A6_1

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GCATGGCTGGATCAGATTTCCATCCATTGTCCAATATCCCTCACTG

A6_2

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A6_3

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A6_4

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TCGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCC

A6_5

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A7_1

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A7_2

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A7_3

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A7_4

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A7_5

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C1_1

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CCC

C1_2

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C1_3

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C1_4

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ATTACCG

C1_5

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C2_1

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C2_2

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C2_3

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C2_4

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C2_5

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AGCACCGGCTAACTACG

C3_1

GCGGTAATACAGCAGCCAGCAGCCGCGTACGGGAGGCAGCAGTTAAGAATTTTGCGCAATGGGCGC
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ACGAATAAGCAG

C3_2

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C3_3

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C3_4

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GACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCA

C3_5

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C4_1

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C4_2

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C4_3

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C4_4

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C4_5

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C5_1

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C5_2

CGACATATCCTGATCGCCAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCCAACGCGTT
GGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTTACCGCGGCTGCTG
CCTCCCGTATTACCGCGGCTGCTGCCTCCCGTAAATCGAATTCC

C5_3

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AGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGAC

C5_4

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C5_5

CTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGT
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CCCGTAAATCGAATTCCCGCGGCCGCCATGGCGGCCG

C6_1

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GGCCGGGAGCATGCGACGTCGGGCC

C6_2

ATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGA
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CGTAAATCGAATTCCC

C6_3

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GAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCAC

C6_4

CGCGAATTCACTAGTGATTTACGGGAGGCAGCAGCCGCGGTAATACGGCAGCCAGAAGCCGCCTAC
GGGAGGCAACAGCCGCGGTAATACGGGAGGCACCAACTCCCGTAGTCGAATTGCCGCGGCCGACCT
GGCGACCGGGAGCCTGCTACGTCCGACCCAGATCCCCATATAGTGAGTC

C6_5

TTACGGGAGGCAGCAGCCGCGGTAATACGGGAGGCAGCAGCCGCGGTAATACAGTAGCCAGCAGCC
GCGGTAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCT
ATAGTGAGT

The sequence results of 16S rDNA of bacterial communities in bioreactors using two different substrates: organic waste and biodiesel wastewater

B1_1

TTACGGGAGGCAGCAGTGAGGGATATTGGACAATGGATGGAAATCTGATCCAGCCATGCCGCGTGC
AGGAAGACGGCCCTATGGGTTGTAAACTGCTTTTATACGGGAGCAATAAGGTCTACGCGTAGACTGA
TGAGAGTACCGTAAGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCG

B1_2

GCAGCAGTGAGGGATATTGGACAATGGATGGAAATCTGATCCAGCCATGCCGCGTGCAGGAAGACG
GCCCTATGGGTTGTAAACTGCTTTTATACGGGAGCAATAAGGTCTACGCGTAGACTGATGAGAGTAC
CGTAAGAATAAGCATCGGCTAACTCCGTG

B1_3

ACGGGAGGCAGCAGTGAGGGATATTGGACAATGGATGGAAATCTGATCCAGCCATGCCGCGTGCAG
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AGAGTACCGTAAGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCG

B1_4

GGATATTGGACAATGGATGGAAATCTGATCCAGCCATGCCGCGTGCAGGAAGACGGCCCTATGGGTT
GTAAACTGCTTTTATACGGGAGCAATAAGGTCTACGCGTAGACTGATGAGAGTGCCGTAAGAATAAG
CATCGGCTAACTCCGTGCCAGCAGCCG

B1_5

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CTAACTCCGTGCCAGCAGCCG

B2_1

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B2_2

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B2_3

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B2_4

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B2_5

AATTCGATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAG
TCAAATATTAGTTAACTGCTCTTCCTCCCAACTTAAAGTGCTTTACAATCCTAAGACCTTCTTCACAC
ACGCGGCATGGCTGGATCAG

B3_1

CTAGTGATTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTTATTCTTACGGTACTCTCATCAGTCT
ACGCGTAGACCTTATTGCTCCCGTATAAAAGCAGTTTACAACCCATAGGGCCGTCTTCCTGCACGCG
GCATGGCTGGATCAGATTTCCATCCATTGTCCAATATCCCTCACTG

B3_2

TTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTCTTAAGGTACCGTCATTCCCCAGTCAGTT
ACTACTGAGGATATTCGTCCCTTAAAACAGAGCTTTACGACCCTAAGGCCTTCTTCGCTCACGCGGCG
TCGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCC

B3_3

CTTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTCTTAAGGTACCGTCATTCCCCAGTCAGT
TACTACTGAGGATATTCGTCCCTTAAAACAGAGCTTTACGACCCTAAGGCCTTCTTCGCTCACGCGGC
GTCGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCCA

B3_4

TTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTCTTAAGGTACCGTCACCTTTACTGGATTT
TTCCCAGTTAAGTCTTCGTCCCTTAAACAGAGCTTTACGACCCTAAGGCCTTCTTCGCTCACGCGGCG
TCGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCC

B3_5

CGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCGACGCCGCGTGAGCG
AAGAAGGCCTTAGGGTCGTAAAGCTCTGTTTAAGGGACGAAGACTTAACTGGGAAAAATCCAGTAA
AGGTGACGGTACCTTAAGAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGT

B4_1

TGGCACGTAGTTAGCCGTGGCTTCCTCGTCAGGTACCGTCATTGGAAATGGCTATTTGCCATCTCCAC
ATTCGTCCCCGACAACAGAGCTTTACGAGTCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCGTCAG
GCTTGCGCCCATTGCGGAAGATTCCCCACTGCTGCCT

B4_2

ATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTA
AAGCTCTGTGATCGGGGACGAATGGCTGGTATGCTAATACCATATCAGAGTGACGGTACCCGAATAG
CAAGCCACGGCTAACTACGTGCCAGCA

B4_3

CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTAAAGC
TCTGTGATCGGGGACGAATGGCTGGTATGCTAATACCATATCAGAGTGACGGTACCCGAATAGCAAG
CCACGGCTAACTACGTG

B4_4

TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTAAAGCTCTGTGA
TCGGGGACGAATGGCTGGTATGCTAATACCATATCAGAGTGACGGTACCCGAATAGCAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAA

B4_5

ACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGACGGCCTTCGGGTTGTAAAGCTCTGTGATCGGGGACGAATGGCTGGTATGCTAATACCATATC
AGAGTGACGGTACCCGAATAGCAAGCCACGGCTAACTACGTGCCAGCA

B5_1

GAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAG
ACGGCCTTCGGGTCGTAAAGCTCTGTTATATGGGACGAACGTATCTATGGCCAATACCCATAGATAG
TGACGGTACCGTAAGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATCGAATTC

B5_2

GAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAG
ACGGCCTTCGGGTCGTAAAGCTCTGTTATATGGGACGAACGTATCTATGGCCAATACCCATAGATAG
TGACGGTACCGTAAGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATCGAATTC

B5_3

TTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTCTTACGGTACCGTCACTATCTATGGGTAT
TGGCCATAGATACGTTCGTCCCATATAACAGAGCTTTACGACCCGAAGGCCGTCTTCACTCACGCGG
CGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCCTCCCG

B5_4

TTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGATGAGGTTAT
TAACCTCACCGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCA
TGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTA

B5_5

CGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGATGAGGTTATTAACCT
CACCGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTG
CATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGC

B6_1

TGATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAACAGGGTACCGTCATTCACTATACT
TATTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGTTCACGC
GGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACT

B6_2

ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAACAGGGTACCGTCATTCACTATACTTA
TTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGTTCACGCGG
CGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCCTCCCGT

B6_3

TGCTGCCTCCCGTATTACCGCGGCTGCTGCCCTGGCACGTAGTTAGCCGTGGCTTTCTAACAGGGTAC
CGTCATTCACTATACTTATTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGA
CCTTCCTCGTTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCC
TCCCGTAA

B6_4

GATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAACAGGGTACCGTCATTCACTATACTT
ATTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGTTCACGCG
GCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACT

B6_5

ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAACAGGGTACCGTCATTCACTATACTTA
TTGGCTATAGTGCCGTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGTTCACGCGG
CGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACT

B7_1

TGATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTCCTTGTACGGTACCGTCATTATCGTCCCG
TACAACAGAGCTTTACGATCCGAAGACCTTCTTCACTCACGCGGCGTTGCTGCATCAGGGTTTCCCCC
ATTGTGCAATATTCCCCACTGCTGCCTCCCGTAA

B7_2

GATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTGTTTTCAGGGTCCGTCATTTGTTTCGTCCC
CTGTCAAAGAAGTTTACAACCCGAAAGCCTTCTTCCTTCACGCGGCGTTGCTGGGTCAGGCTTGCGCC
CATTGCCCAATATTCCCCACTGCTGCC

B7_3

GATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTGTTTTCAGGGTACCGTATTTGTTTCGTCCC
CTGTCAAAGAAGTTTACAACCCGAAAGCCTTCTTCCTTCACGCGGCGTTGCTGGGTCAGGCTTGCGCC
CATTGCCCAATATTCCCCACTGCTGCCTCCCG

B7_4

ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTGTTTTCAGGGTACCGTCATTTGTTTCGTCCC
CTGTCAAAGAAGTTTACAACCCGAAAGCCTTCTTCCTTCACGCGGCGTTGCTGGGTCAGGCTTGCGCC
CATTGCCCAATATTCCCCACTGCTGCCTC

B7_5

ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGT
GAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTACGGGACGATAATGACGGTACCGTACAAGGAAG
CCACGGCTAACTA

B8_1

TTTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAACCTC
ATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG

B8_2

TTTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAACCTC
ATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG

B8_3

TGATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGATGAGG
TTATTAACCTCACCGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGC
GGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGC

B8_4

AGTGATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGATGA
GGTTATTAATCTCACCGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACAC
GCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCT

B8_5

GATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGATGAGGT
TATTAATCTCACCGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCG
GCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACT

B9_1

TTTACGGGAGGCAGCAGCAGGGAATTTTGCGCAATGGGCGAAAGCCTGACACAGCGACGCCGCGTG
GGCGATGAAGGCCTTCGGGTTGTAAAGCCCTTTTCTGGGGGAAGAGAGAGGACGGTACCTCAGGAA
TAAGCGTCGGCTAACTACGTGCCAGCAGCCGCGGTAATC

B9_2

TACGGGAGGCAGCAGCAGGGAATTTTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGGG
CGATGAAGGCCTTCGGGTTGTAAAGCCCTTTTCTGGGGGAAGAGAGAGGACGGTACCTCAGGAATA
AGCGTCGGCTAACTACGTGCCAGCAGCCGCGGTAATCG

B9_3

GTGATTACCGCGGCTGCTGGCACGTAGTTAGCCGACGCTTATTCCTGAGGTACCGTCCTCTCTCTTCC
CCCAGAAAAGGGCTTTACAACCCGAAGGCCTTCATCGCCCACGCGGCGTCGCTGCGTCAGGCTTTCG
CCCATTGCGCAAAATTCCCTGCTGCTGCCTCC

B9_4

TGATTACCGCGGCTGCTGGCACGTAGTTTGCCGGGGCTTCCTCGTATGGTACCGTCTTCCGCTCTTCC
CATACAACAGGGCTTTACATCCCGAAGGATTTCTTCACCCACGCGGCGTCGCTGGGTCAGGGTTCCC
CCCATTGCCCAATATTCCCCACTGCTGCCTCCCGTAA

B9_5

CGGGAGGCAGCAGTGAAGAATATTGCGCAATGGACGAAAGTCTGACGCAGCCACGCCGCGTGAGTG
AAGAAGGCCTTCGGGTCGTAAAGCTCTTTGAGCAGGGAAGAGAGGCCTCGTTGCTAATATCAACGGG
GCGAGACGGTACCTGCAGAACAAGCATCGGCTAACTCCGTGCCAG

The sequence results of archaeal communities

AR1_1, ARC3_1

TCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGA
GTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAAC
TTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGC

AR1_2, ARC3_2

CGGTAAAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCC
TATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGT
TACCCAACTTAATCGCCTTGCAGCACA

AR1_3, ARC3_3

TCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGA
GTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAAC
TTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGC

AR1_4, ARC3_4

TCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTA
TTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATC
GCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCG

AR1_5, ARC3_5

CGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACA
ATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTT
GCAGCACATCCCCCTTTCGC

AR2_1, ARC1_1

CGGCCGCGAATTCACTAGTGATTTACGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACA
GCGATAAGGGAACCTCGAGTGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATA
GCAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAAAATCGAATTCCCGCGGCCGCCATGGCG

AR2_2, ARC1_2

ATTTACGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGAG
TGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGACC
GGTGCCAGCCGCCGCGGTAAAATCGAATTCCCGCGGCCGCCATGGCG

AR2_3, ARC1_3

ATTTACGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGAG
TGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGACC
GGTGCCAGCCGCCGCGGTAAAATCGAATTCCCGCGGC

AR2_4, ARC1_4

ATTTACGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGAG
TGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGACC
GGTGCCAGCCGCCGCGGTAAAATCGAA

AR2_5, ARC1_5

CGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGAGTGCCA
GGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGACCGGTGC
CAGCCGCCGCGGTAAAA

AR3_1, ARC2_1

ATTCACTAGTGATTTACGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGG
GAACCTCGAGTGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCC
GGGCAAGACCGGTGCCAGCCGCCGCGGTAAAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGA

AR3_2, ARC2_2

GATTTACGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGA
GTGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGAC
CGGTGCCAGCCGCCGCGGTAAAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGA

AR3_3, ARC2_3

GATTTACGGGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGA
GTGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGAC
CGGTGCCAGCCGCCGCGGTAAAATCGAATTCCCGCGGCCGCCATG

AR3_4, ARC2_4

GGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGAGTGCCAGG
TTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGACCGGTGCCA
GCCGCCGCGGTAAAATCGAATTCCCGCGGCCGCCATG

AR3_5, ARC2_5

GGAGGCAGCAGCCGCGGTAAACTTTACAATGCTGGCAACAGCGATAAGGGAACCTCGAGTGCCAGG
TTACAAATCTGGCTGTCGAGATGCCTAAAAAGCATTTCATAGCAAGGGCCGGGCAAGACCGGTGCCA
GCCGCCGCGGTAAAATCGAATT

AR4_1, ARC4_1

TTACGGGAGGCAGCAGCCGCGGTAAACCTCCGCAATGTGAGAAATCGCGACGGGGGGACCCCAAGT
GCCACTCTTAACGGGGTGGCTTTTCTTAAGTGTAAAAAGCTTTTGGAATAAGGGCTGGGCAAGACCG
GTGCCAGCCGCCGCGGTAAAATCGAATT

AR4_2, ARC4_2

CGTAAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTA
TAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTA
CCCAACTTAATCGCCTTGCAGCACATC

AR4_3, ARC4_3

TAAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATA
GTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC
CAACTTAATCGCCTTGCAGCACATCC

AR4_4, ARC4_4

ACGGGAGGCAGCAGCCGCGGTAAACCTCCGCAATGTGAGAAATCGCGACGGGGGGACCCCAAGTGC
CACTCTTAACGGGGTGGCTTTTCTTAAGTGTAAAAAGCTTTTGGAATAAGGGCTGGGCAAGACCGGT
GCCAGCCGCCGCG

AR4_5, ARC4_5

GGAGGCAGCAGCCGCGGTAAACCTCCGCAATGTGAGAAATCGCGACGGGGGGACCCCAAGTGCCAC
TCTTAACGGGGTGGCTTTTCTTAAGTGTAAAAAGCTTTTGGAATAAGGGCTGGGCAAGACCGGTGCC
AGCCGCC

The sequence results of *mcrA* genes

mH3

GTTGGGTCCACGGAGTTCTCCGACTGCACCCTCGTCGGGACGAATCGCGAGCGAGTTTGCGGAACCG
CACTGGTCCTGCAGGTCGTAGCCGAAGAACCCGAGGCGTGACCAGCCTTCCTTGTGCATGAGCATGC
ACAGGTACCAGGCGTTCAGCCCGGCGTTGGAGTTCCCGGTCGCAATCGCGGTGGACAGACCGCAGGC
AGCGGCGAGCACACCGGCACGCTGGGAACCGCCGAAGTGGTCCTCCATCATGGTCGGGAACTGCTC
GTACTGCTCCATGCCGTTCAAGCACACTTCGGTCGCAATGTCATTGACGATCTCCTGGGTGGGCTTGA
CCTTGTCCTTGTCGCTTGGGTTCTGCCAGTCGACTTTGTACTTCTGCTTGATGTAGTCCATCCCGTAGT
AGGTGAACTCGTCAAGGATGTTGTCGGTGTAGGCAGCTGTAGCATATTGTGTGAAT

mH4

TGATTGGTGGTGTAGGATTCACACAATATGCTACAGCGGCCTACACCGATGACATCCTGGAGGACTA
CACCTACTGGGCCATCGACCTGGTCAAGAACAAGTACGGCGGGCTGTGCAAGAGCAAGCCCTCCATG
GACCTGATGGAGAAGCTCGGTACCGAGGTCAACTCCTACGCTCTCGAGATGTACGAGAGGTACCCCG
CTGCTATGGAAGCCCACTTCGGTGGGTCCCAGCGTGCCACCGTCGCCGCTGCTGCCACTGGTATCGCT
TGCGCGATGGCCACCGGCAACGCCGACTTCGGTGTCAACGGCTGGTACCTGTCCATGCTCCAGCACA
AGGAGAGGCACGGCCGCCTTGGGTTCTACGGGTACGACCTGCAGGACCAGTGCGGTTCCGCCAACTC
CCTGTCCTACAGGAGCGACGAGGGCCTGCCCTTCGAGCTGAGGGGTCCGAACTACCCAAACTATGCA
ATGAA

mO1

TGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGG
CCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACAT
CCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCA
GCCTGAATGGCGAATGGACGCGCCCTGT

mA2

AGTTCGGACCCCTCAGCTCGAAGGGCAGGCCCTCGTCGCTCCTGTAGGACAGGGAGTTGGCGGAACC
GCACTGGTCCTGCAGGTCGTACCCGTAGAACCCAAGGCGGCCGTGCCTCTCCTTGTGCTGGAGCATG
GACAGGTACCAGCCGTTGACACCGAAGTCGGCGTTGCCGGTGGCCATCGCGCAAGCGATACCAGTG
GCAGCAGCGGCGACGGTGGCACGCTGGGACCCACCGAAGTGGGCTTCCATAGCAGCGGGGTACCTC
TCGTACATCTCGAGAGCGTAGGAGTTGACCTCGGTACCGAGCTTCTCCATCAGGTCCATGGAGGGCT
TGCTCTTGCACAGCCCGCCGTACTTGTTCTTGACCAGGTCGATGGCCCAGTAGGTGTAGTCCTCCAGG
ATGTCATCGGTGTAGGCCGCTGTAGCATATTGTGTGAATCCTACACCACCAAT

mA10

GGACCCCTCAGCTCGAAGGGCAGGCCCTCGTCGCTCCTGTAGGACAGGGAGTTGGCGGAACCGCACT
GGTCCTGCAGGTCGTACCCGTAGAACCCAAGGCGGCCGTGCCTCTCCTTGTGCTGGAGCATGGACAG
GTACCAGCCGTTGACACCGAAGTCGGCGTTGCCGGTGGCCATCGCGCAAGCGATACCAGTGGCAGCA
GCGGCGACGGTGGCACGCTGGGACCCACCGAAGTGGGCTTCCATAGCAGCGGGGTACCTCTCGTACA
TCTCGAGAGCGTAGGAGTTGACCTCGGTACCGAGCTTCTCCATCAGGTCCATGGAGGGCTTGCTCTTG
CACAGCCCGCCGTACTTGTTCTTGACCAGGTCGATGGCCCAGTAGGTGTAGTCCTCCAGGATGTCATC
GGTGTAGGCCGCTGTAGCATATTGTGTGAATCCTACACCAC

The sequence results of *hydA* genes

hH1

AACTCCTCCTGTTGCACCAAATATCAAACCTGCACCTGATGCATCTCCAAAAGGACTATCAAAATGT
GATTTTGGCATTTCAGGTAAATAAATTCCTGCTTCTTTTATCATCTTTGCTAATTCTCTCGTAGTTAAT
CCATAATCTACGTCTTTGTATCCTGATGAATTCATCTCTGGTCTATTGCATTCGAATTTCTTTGCCGAA
CAGGGCATTACCGCTACCGAAACTATATCTTTTGGGTCGATTCCTTTCTGTTGTGCATAAAAAGTCTT
TAATAATGCACCAAATATTTGTTGTGG

hH10

TTGCACCAAATATCAAACCTGCACCTGATGCATCTCCAAAAGGACTATCAAAATGTGATTTTGGCATT
TCAGGTAAATAAATTCCTGCTTCTTTTATCATCTTTGCTAATTCTCTCGTAGTTAATCCATAATCTACG
TCTTTGTATCCTGATGAATTCATCTCTGGTCTATTGCATTCGAATTTCTTTGCCGAACGGGGCATTACC
GCTACCGAAACTATATCTTTTGGGTCGATTCCTTTCTGTTGTGCATAAAAAGTCTTTAATAATGCACC
AAATATTTGTTGTGGTGAAAT

hO1

TTGCACCAAAAATTACACCTGCCCCAGATGACATTCCTAATGGCATATCAAATTCTTCATCAGGCAG
AGATGTGAAATTAATGCCTGCACGTTCAATCATAGTTGCCAGTTCCCTAGTTGTAATAGCATAATCTA
CATCAGGTACGCCTGCTGCATCCTCATCATCACGGCCGATTTCAAATTTCTTAGCAGTACATGGCATA
ACACTAACCATTACTATATCCTTAGGGTTAAGACCCATTTTTTCAGCATAATATGTTTTAGCAATCGC
ACCAAATATTTGTTGTGGT

hO6

CACCAAAAATTACACCTGCCCCAGATGACATTCCTAATGGCATATCAAATTCTTCATCAGGCAGAGA
TGTGAAATTAATGCCTGCACGTTCAATCATAGTTGCCAGTTCCCTAGTTGTAATAGCATAATCTACAT
CAGGTACGCCTGCTGCATCCTCATCATCACGGCCGATTTCAAATTTCTTAGCAGTACATGGCATAACA
CTAACCATTACTATATCCTTAGGGTTAAGACCCATTTTTTCAGCATAATATGTTTTAGCAATC

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