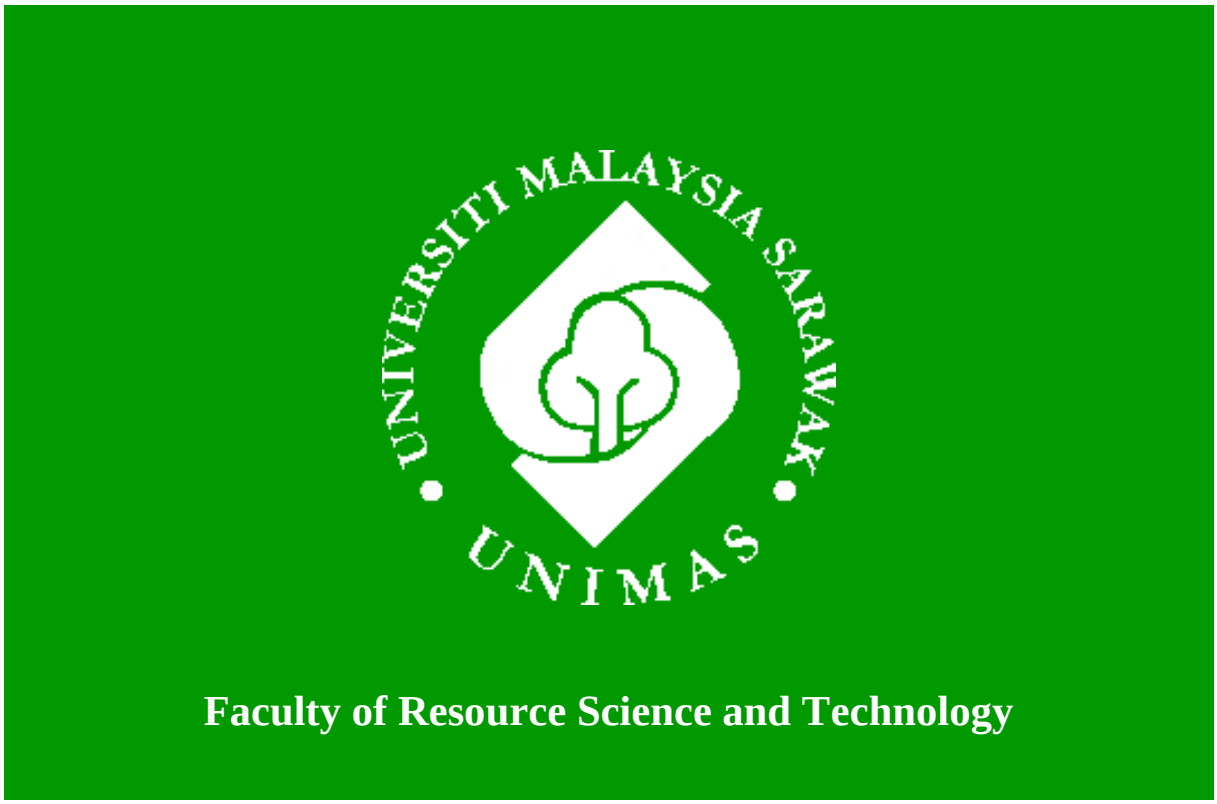

Microbial Characterization and Biogas Production from Palm Oil Mill Wastes

Christy Chan Sien Wei

Master of Science
(Biotechnology)
2016

Microbial Characterization and Biogas Production from Palm Oil Mill Wastes

Christy Chan Sien Wei

A thesis submitted

In fulfillment of the requirements for the degree of master

Faculty Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2016

UNIVERSITI MALAYSIA SARAWAK

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ACKNOWLEDGEMENT

I would like to express my deepest gratitude to Dr Micky Vincent, my main supervisor for his assistance and support throughout my study, for his reviews, advice, patience and encouragement. I am also grateful towards my co-supervisors, Prof Kasing Apun and AP Dr Awang Ahmad Sallehin Awang for their valuable insights, guidance and support throughout my study as well as my examiners for their reviews and advice.

I would also like to thank my laboratory mates and staff for their encouragement and technical advice. Many thanks to the people from Bau Oil Palm Plantation for their hospitality and kindness in sharing their valuable experiences and information.

Last but not least, I would like to express my appreciation to my parents, my sisters and my youngest brother for their unconditional love, support and faith in me.

DECLARATION

I hereby declare that this thesis entitled "Microbial Characterization and Biogas Production from Palm Oil Mill Wastes" is the result of my own research work and effort. It has not been submitted anywhere for any award. Other sources of information that have been used have been acknowledged.

Name of student: Christy Chan Sien Wei

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List of Abbreviations

ANOVA	Analysis of variance
BAPOM	Bau Palm Oil Mill
BOD	Biochemical oxygen demand
BLAST	Basic logical alignment tool
C/N	Carbon to nitrogen
cm^3/d	Centimetre cube per day
CSTR	Continuous stirred tank reactor
COD	Chemical oxygen demand
CPKO	Crude palm kernel oil
CPO	Crude palm oil
DGGE	Denaturing gradient gel electrophoresis
EFB	Empty fruit bunches
FFB	Fresh fruit bunches
FISH	Fluorescent *in situ* hybridization
g	Gram
HRT	Hydraulic retention time
l	Liter
mg	Milligram
µl	Microliter
$mg\ l^{-1}$	Milligram per liter
$µgm\ l^{-1}$	Microgram per mililiter
MPOB	Malaysia Palm Oil Board
CH_4	Methane
O_2	Oxygen

OLR	Organic loading rate
POME	Palm oil mill effluent
POMS	Palm oil mill sludge
ppm	Parts per million
PCR	Polymerase chain reaction
SS	Suspended solid
TS	Total solid
TVS	Total volatile solid
TN	Total nitrogen
T-RFLP	Terminal restriction fragment length polymorphism
UASB	Up-flow anaerobic sludge blanket
UASFF	Up-flow anaerobic sludge fixed-film

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Abstract

Palm Oil Mill Effluent (POME) of palm oil industry is currently being studied extensively due to its high potential in biogas production. Bioconversion of POME to methane via anaerobic digestion involves a consortium of microbes which are responsible in several steps of the biodegradation process. In this study, the microbial community from selected POME was characterized via molecular techniques as well as through culture-based plating in order to determine their composition, and subsequently understand their functions in the anaerobic community. Genomic DNA of the microbial community was extracted using direct extraction technique, followed by PCR targeting the 16S rDNA region. Distinct fragments of approximately 1,100 bp in sizes were successfully amplified using PCR and cloned onto *Escherichia coli* XL-1 Blue. Upon sequencing of the fragments, BLAST queries identified the bacteria as *Thermoanaerobacterium* sp. In addition, two other bacterial species which were successfully isolated from the POME by culturing on DVS agar belonged to the *Bacillus* genus. The ability of the isolates in utilizing different substrates suggested that anaerobic digestion of mixture of POME sludge and solid cud taken from the first compartment of cow's stomach (1:2 and 2:1 ratio) as co-mixture was applicable. Thus, the co-mixture was incubated at 50 °C in a 2 L vessel with initial starter of 400 ml and sampling was conducted every four weeks interval during 12 weeks of incubation. For specific detection of methanogens, 16S rRNA-cloning analysis was carried out. *Methanobrevibacter* sp. and *Methanosaeta* sp. were confirmed to be present within the 2:1 ratio of co-mixture while only *Methanobrevibacter* sp. was found in 1:2 ratio of co-mixture on both Week 0 and Week 4. Duration of anaerobic digestion was reduced to 4 weeks as no methanogens were detected from both co-mixtures on Week 8 and Week 12. By understanding the bacterial community present, improvement of the anaerobic

digestion process to enhance the production of biogas can be carried out. Biogas production from mono- and co-digestion of POME sludge and solid cud from ruminant stomach on Empty Fruit Bunch (EFB) were also investigated at different ratio of POME sludge and solid cud (1:1, 1:2 and 2:1). All digestions were operated at thermophilic condition (50 ˚C) for 4 weeks. Biogas productions from the co-digestion samples were generally higher compared to the mono-digestion experiments. POME sludge to solid cud ratio of 1:2 generated the highest biogas yield with a total of 3,754 cm^3 after 4 weeks of incubation. Biogas production of POME sludge to solid cud ratio was 42.87% and 70.91% higher than single digestion of POME sludge and solid cud, respectively. Although at 1:1 and 2:1 POME sludge to solid cud ratio produced less biogas compare to 1:2 ratio, both co-digestion mixtures generated 33.97% and 41.25% more gas than mono- treatment using only solid cud, which only produced 1,092 cm^3 of biogass after 4 weeks of incubation.

Keywords: Palm Oil Mill Effluent (POME), biogas, solid cud, empty fruit bunch (EFB).

Pengecaman Mikrob dan Penghasilan Biogas daripada Sisa Kilang Minyak Sawit

Abstrak

Efluen Kilang Minyak Sawit (POME) industri minyak sawit dikaji secara meluas kerana potensi yang tinggi dalam pengeluaran biogas. Penukaran POME kepada gas metana melalui pencernaan anaerobik melibatkan beberapa konsortium mikrob. Dalam kajian ini, mikrob daripada POME dipilih menggunakan teknik molekul serta teknik pengkulturan untuk menentukan komposisi dan memahami fungsi mereka dalam proses anaerobik. DNA genomik mikrob diekstrak menggunakan teknik pengekstrakan langsung, diikuti oleh amplifikasi tindakbalas berantai polimerase (PCR) yang mensasarkan gen 16S rRNA. Gen 16S rDNA yang bersaiz kira-kira 1100 bp telah berjaya diamplifikasikan dan diklonkan ke dalam Escherichia coli XL-1 Blue. Hasil penjujukan melalui BLAST telah mengenal pasti bakteria tersebut sebagai Thermoanaerobacterium sp. Di samping itu, dua spesies bakteria lain telah berjaya diasingkan daripada POME melalui pengkulturan pada DVS agar. Hasil penjujukan menunjukkan kedua-dua isolat bakteria adalah daripada genus Bacillus. Keupayaan bakteria tersebut menggunakan substrat yang berbeza menyumbang kepada pencernaan anaerobik yang menggunakan POME dan sisa pepejal yang diambil dari bahagian pertama perut lembu dengan nisbah 1: 2 dan 2: 1 sebagai campuran. Campuran tersebut diperam pada 50 °C dalam bottol 2 L dengan permulaan kulture sebanyak 400 ml dan pensampelan dijalankan setiap 4 minggu semasa 12 minggu pengeraman. Untuk pengesanan spesies methanogens, analisis 16S rRNA-pengklonan telah dijalankan. Methanobrevibacter sp. dan Methanosaeta sp. telah disahkan terdapat dalam campuran nisbah 2:1 manakala hanya Methanobrevibacter sp. didapati dalam campuran 1:2 nisbah pada kedua-dua Minggu 0 dan Minggu 4. Tempoh pencernaan anaerobik telah berjaya dikurangkan kepada 4 minggu kerana tidak ada methanogens

dalam kedua-dua campuran pada Minggu 8 dan Minggu 12. Dengan memahami populasi bakteria yang hadir, penambahbaikan proses pencernaan anaerobik untuk meningkatkan penghasilan biogas telah dijalankan. Penghasilan biogas daripada mono dan campuran POME dengan sisa pepejal daripada bahagian pertama perut lembu dalam nisbah (1:1, 1:2 dan 2:1) pada tempurung kelapa sawit telah disiasat. Semua pencernaan beroperasi dalam keadaan thermophilic (50 °C) selama 4 minggu. Penghasilan biogas daripada campuran sampel pada umumnya lebih tinggi berbanding dengan mono-pencernaan. Pencernaan POME kepada nisbah sisa pepejal 1:2 menjana hasil biogas tertinggi dengan jumlah 3,754 cm^3 selepas 4 minggu pengeraman. Penghasilan biogas daripada campuran nisbah POME kepada sisa pepejal ini adalah 42.87% dan 70.91% lebih tinggi daripada penghadaman tunggal POME dan sisa pepejal. Walaupun nisbah 1:1 dan 2:1 campuran POME kepada sisa pepejal menghasilkan jumlah biogas yang lebih rendah berbanding nisbah 1:2, kedua-dua campuran menghasilkan 33.97% dan 41.25% lebih banyak gas berbanding rawatan tunggan sisa pepejal, yang hanya menghasilkan 1,092 cm^3 biogass selepas 4 minggu pengeraman.

Kata kunci: Efluen Kilang Minyak Sawit, biogas, sisa pepejal, tempurung kelapa sawit

Chapter 1

INTRODUCTION

Oil palm, which is also known as *Elaeis guineensis Jacq.*, was introduced into Malaysia from West Africa by the British colonialists in 1917. It is the most commercially efficient oil producer among the other species in the palmae family. Oil palm produces two types of palm oil; palm kernel oil and crude palm fruit oil that are obtained from the kernel and mesocarp of the fruit, respectively (Corley and Tinker, 2008). Palm kernel contains approximately 80% saturated fatty acid (oleic), and, is mainly used in the manufacturing of soaps, detergents and other toiletries in the oleochemical industries (Basiron, 2007). Crude palm oil which is rich in both saturated (oleic) and unsaturated (palmic) fatty acid is widely used as an ingredient in food, leather, metal and chemical industries. Presently, palm oil is also intensively utilized in the production of biodiesel (palm oil methylester or palm oil diesel) (Reijnders and Huijbregts, 2008), an effect of the Fifth Fuel Policy. This policy was drafted with the aim of leading Malaysia toward utilizing green energy (Lim and Teong, 2010).

Oil palm has the highest yield per hectare compared to other vegetable oils (Corley, 2009). Although oil palm accounts for only 5.5% of global land for cultivation, it produces 32.0% of global oils and fats output in 2012 (Oil World, 2013). Due to increasing demand for palm oil as food source in China, India and South America as well as biodiesel in European Union, global production is increasing at a rate of 9% annually (Tan *et al*., 2009; Koh and Wilcove, 2008).

Global production of palm oil is more than fifty-nine million metrics tonnes, with Malaysia and Indonesia contributing approximately 85% of world production (Fitzherbert *et al*., 2008; Germer and Sauerborn, 2008). Malaysia is currently the second largest palm oil exporter, producing approximately 12.7% (18.91 million tonnes) of total global trade of oils and fats in 2011 (Sime Darby, 2014). As a result, the palm oil industry serves as a vital backbone to the country's economy (Yusoff and Hansen, 2007). In 2011, export revenue of palm oil and its derived products racked in RM80.4 billion, an increase of 34.5% compared to previous year (MPOB, 2011). In addition, palm oil industry provides agriculture employments (Wu *et al*., 2010).

Despite the economic benefits, the production of palm oil is facing several challenges from non-governmental organizations (NGOs) regarding effects of the palm oil industries on the environment. The most extensive pollutant from palm oil mill is palm oil mill effluent (POME). It is estimated that 50% of water used in one tonne crude palm oil production is discharged as POME (Ma, 1999). Malaysia alone produces an average of 53 million m^3 POME from 14.8 million tonnes of palm oil produced in 2005 (Madaki and Lau, 2013). POME contains high Biochemical Oxygen Demand (BOD) (25,000 mg l^{-1}) and Chemical Oxygen Demand (COD) (50,000 mg l^{-1}) which is 100 times more compared to domestic sewage (Embrandiri *et al*., 2013). Although it is non-toxic, POME can pollute the ecosystem of rivers due to its acidity and residual oil (Madaki and Lau, 2013).

With more than 430 palm oil mills in Malaysia (Wu *et al*., 2010), the industry is obligated to treat its effluent prior to discharging it to the surrounding streams. Ponding system is the most common treatment method for POME, and, more than 85% of palm oil mills in Malaysia are adopting this method (Ma *et al*., 1993). However, ponding system requires

longer retention time and larger areas as it consists of a de-oiling tank, acidification, anaerobic and facultative ponds (Ma and Ong, 1985). In addition, ponding system releases a large amount of greenhouse gases such as methane and carbon dioxide into the atmosphere.

One way to rectify this problem is to introduce anaerobic digestion treatment of POME as POME contains high concentration of protein, nitrogenous compounds, carbohydrate, lipids and minerals which can be converted into biogas using microbial processes in anaerobic digesters (Abdullah and Sulaiman, 2013). Anaerobic digestion is a series of process in which microorganisms degrade biodegradable materials in the absent of oxygen to produce biogas such as methane gas. Anaerobic digestion in the bioconversion of organic matter into biogas has been applied for different types of organic wastes such as dairy cattle wastes (Wohlt *et al*., 1990), domestic wastes (Gallert *et al*., 2003; Krzystek *et al*., 2001) and fruit and vegetable waste (Bouallagui *et al*., 2005; Parawira *et al*., 2005; Kalia *et al*., 2000).

Over the years, anaerobic digestion of POME has attracted many researchers using different treatment technologies. Studies that have been performed for anaerobic treatments of POME include continuous stir tank reactor (CSTR) (Yacob *et al*., 2006), anaerobic filter bioreactor (AFB) (Wang and Banks, 2007), up-flow anaerobic sludge blanket reactor (UASB) (Borja *et al*., 1996) and fluidized bed reactor (Poh and Chong, 2009). Although anaerobic digestion is suitable in treating high organic content wastes such as POME, unfavourable temperature, pH, carbon/nitrogen ratio and presence of inhibitors would affect the performance of the digestion (Esposito *et al*., 2012).

One method that is recommended to improve biomass anaerobic digestion is the use of mixed microbial cultures. According to Khalid *et al*. (2011), anaerobic digestion using mixed cultures improve the yields of anaerobic digestion of solid or organic wastes as it improve balance of nutrients and better biogas production. This is supported by several other studies that have shown that mixture of agriculture, municipal and industrial wastes produced higher methane gas compared to single digestion (Fezzani and Cheikh, 2010; Agdag and Sponza, 2007).

Currently anaerobic co-digestion of sewage sludge, animal manure and organic fraction of municipal solid wastes using co-substrate such as industrial wastes, agricultural wastes and municipal wastes (Mata-Alvarez *et al*., 2014) are widely used. Therefore, in this study, biogas production of mono-digestion of POME and co-digestion of POME and solid cud from ruminant stomach were carried out as there are limited studies on co-digestion of POME. Molecular techniques such polymerase chain reaction (PCR) and cloning were used to investigate the microbial community in POME as well as co-mixture of POME and solid cud at different mixture ratios.

Objectives

The objectives of this study are:

1. To isolate and characterize the microbial consortium in POME.
2. To investigate the microorganisms presence in the mixture of POME with solid cud from ruminant stomach as microbial inoculum in anaerobic digester.
3. To determine biogas production using co-digestion of POME and solid cud from ruminant stomach as inoculum.

Thesis organization

This thesis is divided into six chapters, each with specific scopes. The scopes are as presented below.

Chapter 1 introduces the history, usage as well as production of palm oil and palm oil mill wastes in Malaysia. Applications of co-digestion of wastes and anaerobic digesters on POME treatment are also examined. The research objectives of the experimental work are also presented in Chapter 1.

Chapter 2 reviews the history, characteristic, production of palm oil, generation of wastes as well as treatment technologies of POME. Using published findings, this chapter provides details on anaerobic digestion, anaerobic co-digestion and anaerobic communities. Information on types of anaerobic digester applied on POME treatment and potential of biogas from POME are also provided in this chapter.

Chapter 3 entitled "**Molecular Identification of Microbial Population in Palm Oil Mill Effluent (POME)"** discusses on the first objective which is the isolation and characterization of microbial consortium of POME. Efficiency of anaerobic digester in POME treatment is determined by the bacterial community involved in anaerobic digestion process. Thus, it is important to understand the microbial population of POME in order to provide optimum condition for microbial population which could contribute to greater methane production. In this chapter, the bacteria community in POME is determined by constructing 16S rDNA clone libraries and, subsequently by sequencing analysis. This chapter has been accepted by **Journal of Palm Oil Research** on November 2014.

This is followed by chapter 4 which is entitled **"Identification of Methane Producing Bacteria from Palm Oil Mill Effluent (POME) with Solid Cud from Ruminant Stomach".** In this chapter, the second objective of this study, to determine the presence of methanogens in different mixtures of POME and solid cud from ruminant stomach was investigated. In order to produce higher biogas yield and reduce digestion time, inoculum source of anaerobic digestion is crucial. Therefore, co-mixture of POME with solid cud from ruminant stomach in different ratio is applied for 12 weeks. Methanogenic bacteria from the different ratio inoculum are sampled every four weeks and identified through 16S rRNA technique. This chapter has been published in **Journal of Biochemistry, Microbiology and Biotechnology, 2 (1): 23-26 (2014)**.

Chapter 5 which is entitled **"Biogas production from Co-mixture of Palm Oil Mill Effluent and Ruminant Solid Cud"** is the last of the main chapters. This chapter aims to address the third objective, to investigate biogas production using co-digestion of POME and solid cud from ruminant stomach as inoculum. Anaerobic co-digestion is a feasible option to overcome the drawbacks of single substrate anaerobic digestion. Despite this fact, it is still important to choose suitable co-substrate and blend ratio to increase biogas yield without affecting the performance of the digester. Therefore in this study, the biogas potentials between mono-digestion of POME and solid cud with co-digestion of POME with solid cud were investigated. Anaerobic digesters of mono-digestion and co-digestion of different mixing ratio are incubated at 50°C and the incubation time was shortened from 12 weeks to 4 weeks. This chapter will be submitted to **Pertanika Journal of Science & Technology** for publication.

The final chapter which is Chapter 6 contains the general discussions and conclusions of these studies. Chapter 6 also suggests the direction of future research of these studies.

Chapter 2

LITERATURE REVIEW

2.1 Historical background

The oil palm, *Elaeis guineensis* originated from West Africa where the main palm belt covered from Sierra Leone, Liberia, Ivory Cost, Ghana and Cameroon to the equatorial regions of the Republics of Congo and Zaire (Hartley, 1988). The development of oil palm as plantation crops started in South East Asia through the introduction of four seedlings from Mauritius and Amsterdam which were planted in the Botanical Garden in Bogor in 1848. The first commercial oil palm plantation was established by M. Adrien Hallet in Sumatera, Indonesia. The development of the industry in Malaysia is contributed by Henri Fauconnier through his association with Hallet. In 1917, Fauconnier established the first commercial oil palm planting at Tennamaram Estate, Kuala Selangor by using seedling obtained from Sumatera, Indonesia (Tate, 1996).

According to Singh (1976), the advancement of the industry can be classified into three distinct phases, starting with the Research and Development (R&D) phase from the late 1800s to 1916, while the development phase started in 1917 until 1960. With declining prices of two major national commodities, tin and natural rubber as well as competition with synthetic rubber, the expansion phase of oil palm industry began in 1960 in response to the government Diversification Policies. Federal Land Development Autority (FELDA) which was established in 1956 with the socio-economic responsibility of eradicating poverty played a major part in the expansion phase by developing plantation land for the rural and poor citizens.

The palm oil industry underwent two further phases with commencement of large scale planting in Sabah and Sarawak in 1970, and, extension of further upstream operation to Indonesia due to adequate supply of workers and vast area for plantation development (Hai, 2002).

2.2 The Oil Palm

E. guineensis Jacq., or commonly known as oil palm belongs to the family *Palmaceae* in the genus *Elaeis*. The genus contains two main species*: E. guineensis* and *E. melanococca*. Oil palm is a monoecious crop (Adam *et al*., 2005) as it bears both male and female flowers on the same tree which only differentiated after approximately two years. Each tree produces compact bunches weighing between 10 to 25 kilograms with 1000 to 3000 fruitlets per bunches (Ohimain *et al*., 2012). Each fruitlet consists of a fibrous mesocarp layer and the endocarp which contains the kernel as shown in Figure 2.1.

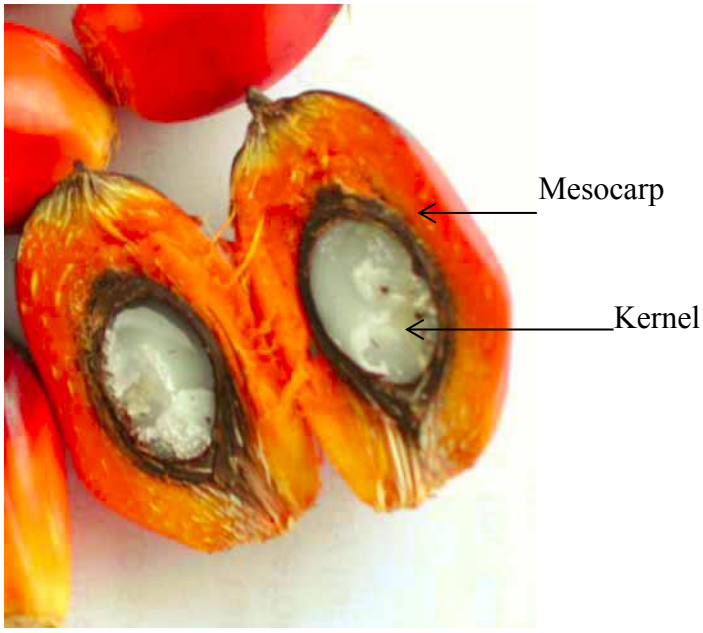

Figure 2.1: Cross section of a fruitlet (Hai, 2002).

The most common cultivars of *E. guineensis*; the *Dura*, *Tenera* and *Pisifera* are classified according to endocarp thickness and mesocarp content. According to Latiff (2000), *Dura* palms have 2-8 mm thickness of endocarp with medium mesocarp content (35%-55% of fruit weight) while the *Tenera* race has 0.5-3.0 mm thickness of endocarp with high mesocarp content (60%-90% of fruit weight). Although *Pisifera* palm does not have endocarp, it has high content of mesocarp which is 95% of the fruit weight. In Malaysia, the cultivar planted is the *Tenera* hybrid, a cross between *Dura* and *Pisifera*, which yields about 4 to 5 tonnes of crude palm oil (CPO) per hectare per year and about 1 tonne of palm kernels (MPOC, 2012).

According to a report by Malaysia Palm Oil Council (MPOC) (2012), oil palm produces two types of oil, CPO from the mesocarp and crude palm kernel oil (CPKO) from palm kernel which has wide application in oleochemical industries. CPO is processed by physical refining process to produce refined oil for further end use application. Palm oil has a balance ratio of saturated and unsaturated fatty acid which contain 40% oleic acid (monounsaturated fatty acid), 10% linoleic acid (polyunsaturated fatty acid), 45% palmitic acid (saturated fatty acid) and 5% stearic acid (saturated fatty acid) (MPOC, 2012). Palm oil is relatively stable to oxidation process due to low level of linoleic acid and linolenic acid in the oil. This composition results in palm oil as an edible oil which is suitable in variety of food application.

The composition and properties of palm kernel oil differ significantly from palm oil. Palm kernel oil consists of mainly saturated fatty acid which is similar to the composition of coconut oil. Table 2.1 shows the fatty acid composition of palm kernel oil, its similarities to coconut oil and differences from palm oil (Krishna *et. al*., 2010). High content of lauric

acid in palm kernel oil produces sharp melting point that enables palm kernel oil to be used in enrobing and dipping products.

Table 2.1: Fatty acid composition in coconut, palm kernel and palm oil

Fatty acid	Palm oil	Palm kernel oil	Coconut oil
C8:0	-	-	7.0
C10:0	-	1.2	5.4
C12:0	0.2	51.6	48.9
C14:0	1.1	22.9	20.2
C16:0	42.6	12.2	8.4
C18:0	3.8	1.3	2.5
C18:1	41.9	10.8	6.2
C18:2	10.4	-	1.4

Source: Krishna et al., (2010).

Palm oil is used in a wide variety of applications compared to other vegetable oils due to its unique chemical composition. Approximately 75% of global palm oil usage is for food products such as cooking oil, shortening and margarine, 22% is for industries purposes which include soap, detergents, toiletries, cosmetics and candles while the remaining is used for biofuel production (WWF, 2013) (Figure 2.2).

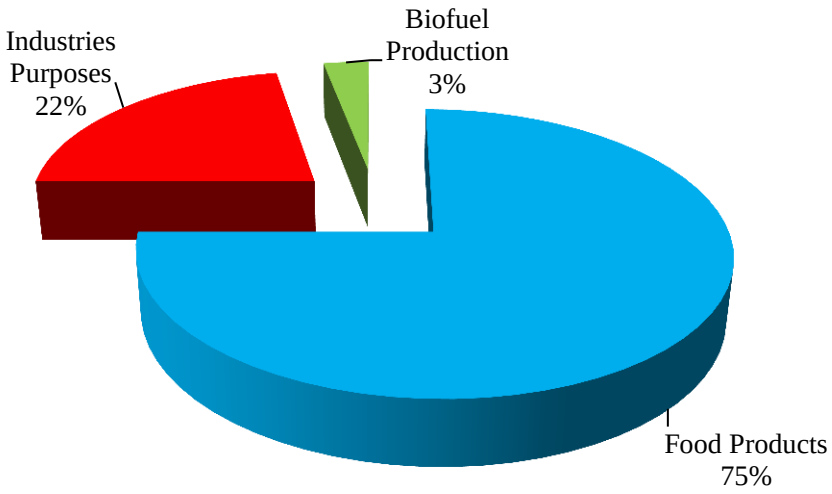

Figure 2.2: Application of palm oil in different sectors (WWF, 2013).

2.3 Palm oil in Malaysia

Among the 10 major oilseeds, oil palm produced 32.0% of global oil and fats output in 2012 (Oil World, 2013) (Figure 2.3). From an average of 1.26 million tonnes during 1958 to 1962, its production surged to about 17.93 million tonnes during 1996 to 2000 and further increased to 45.10 million tonnes in 2009 as shown in Table 2.2. It also surpassed soya bean oil production since 2005.

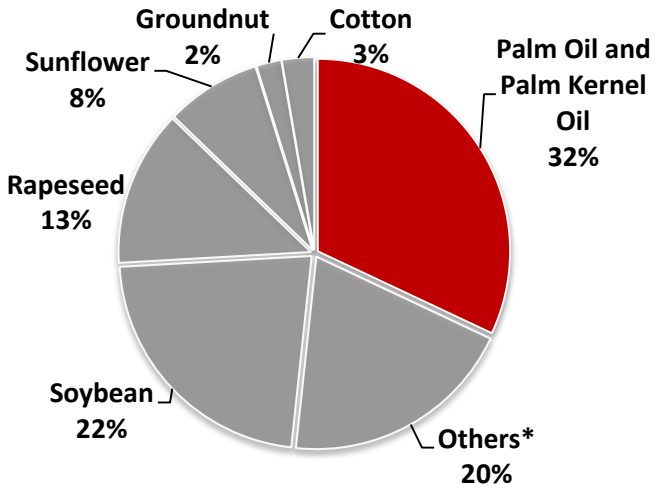

Figure 2.3: Global Production of 17 Major Oil and Fats in 2012 (Total= 186.4 mil tonnes) (Oil World, 2013).

Table 2.2: Average annual production of major oils and fats

Years/ Oil	1958-1962	1996-2000	2009	2010
Butter	4.21	5.75	7.12	7.16
Tallow	3.39	7.65	8.43	8.37
Soyabeans	3.20	22.84	36.10	40.06
Lard	3.19	6.21	7.77	8.03
Groundnut	2.65	4.62	4.12	4.10
Cottonseed	2.26	4.00	4.69	4.60
Sunflower	1.90	9.14	12.97	12.28
Coconut	1.85	3.10	3.22	3.67
Olive	1.30	2.42	2.92	3.26
Palm	**1.26**	**17.93**	**45.10**	**45.59**
Rapeseed	1.13	12.56	21.34	23.51
Linseed	0.92	0.73	0.58	0.65

Source: Chin *et al*., (2013).

Malaysia and Indonesia produce approximately 89% of world production of palm oil in 2012 (WWF, 2013). Production from Malaysia and Indonesia have increased over time

from 12.38 million tonnes (2004) to 22.30 million tonnes (2010) and 13.98 million tonnes (2004) to 16.99 million tonnes (2010) respectively (Abdullah, 2011). Due to vast land availabilities and cheap labour, Indonesia has surpassed Malaysia's production since 2006 to become the world's largest palm oil producer.

Palm oil plantation in Malaysia expanded rapidly due to the government's agricultural diversification programme which was established to overcome the country's reliance on rubber and tin. This practice has successfully converted many rubber plantations into oil palm estates. The planted areas have been expanding rapidly from 1.5 million hectares in 1985 to 4.92 million hectares in 2011 (Figure 2.4). The largest expansion is in Sarawak with an increase of 102,169.00 hectares. According to the Malaysia Palm Oil Board (MPOB) (2011), Sabah remains as the largest palm oil plantation area which accounted 1.43 million hectares of total palm oil planted area (4.92 million hectares), followed by Sarawak with 1.02 million hectares.

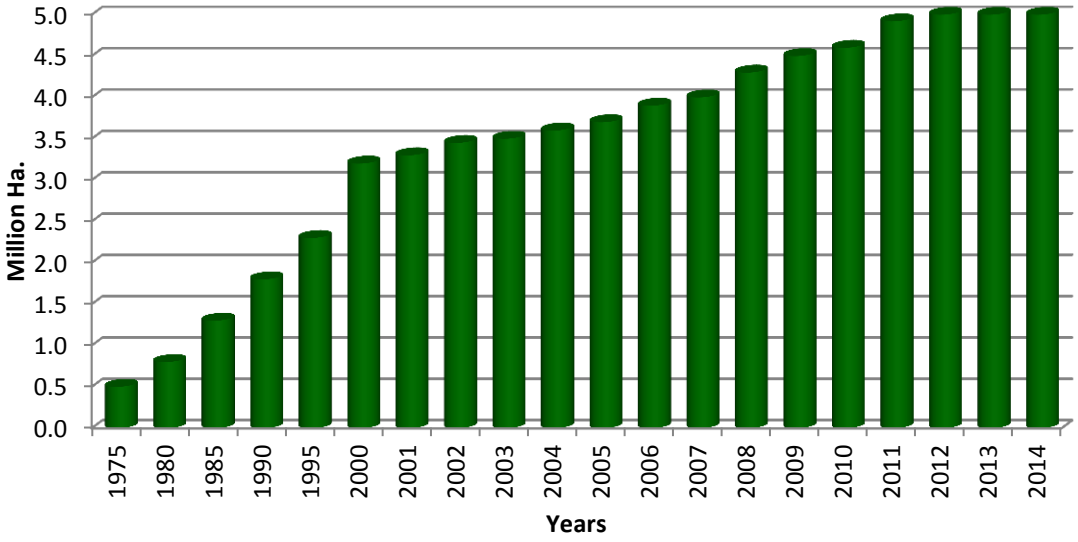

Figure 2.4: Planted area of palm oil in Malaysia (MPOB, 2015).

CPO production in Malaysia has been increasing continuously over the years, from 4.1 million tonnes in 1985 to 6.1 million tonnes in 1990. The production further increased to 16.9 million tonnes in 2010 and reached 18.9 million tonnes in 2011 (Figure 2.5).

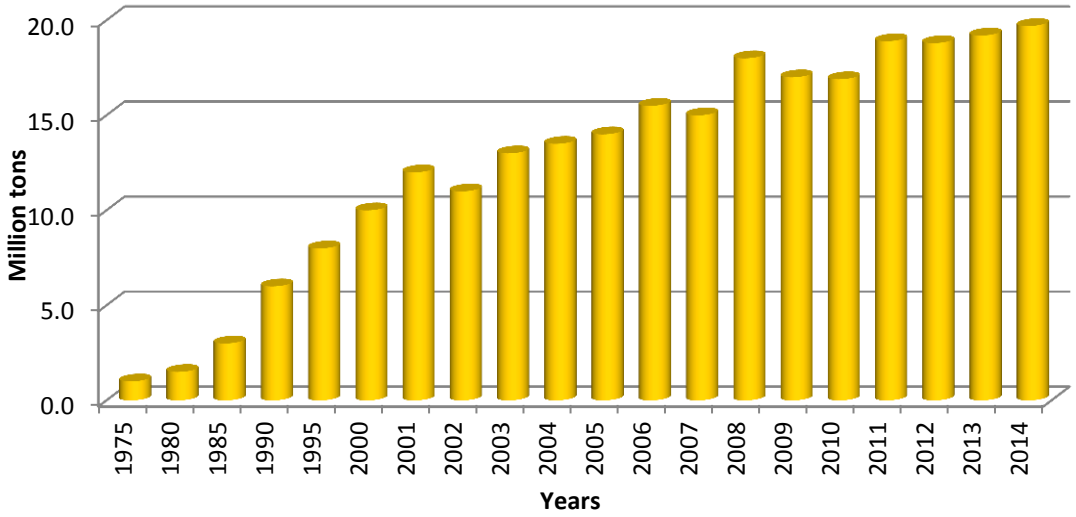

Figure 2.5: Production of crude palm oil (MPOB, 2015).

However, in 2009 the production of CPO decreased which was highly due to the age of palm oil tree. Palm oil has a potential to produce volume of fresh fruit brunches (FFB) over a lifespan of 25 years. It was stated in an MPOB report (2011), the optimum yields for palm oil are from the age of 9-18 years, and gradually decline thereafter.

2.4 Palm Oil Production Process

Ripe FFB are transported to palm mill and processed as soon as possible to prevent an increase in free fatty acid content which would result in quality decline in CPO. FFB loaded onto sterilization cage are subjected to steam-heat treatment at a pressure of 3 kg/cm^2 and 140 °C for 75 to 90 minutes. A three-peak sterilization pattern is conducted to prevent further formation of free fatty acid, facilitate stripping of fruits and prepare fruits for subsequence sub-process (Vijaya *et al.*, 2010). Fruitlets from the sterilized bunches are stripped off in a rotary drum-stripper (Wu *et al.*, 2010). The detached fruits are conveyed

into the digester while empty fruit bunches (EFB) are transported to plantation for mulching.

Palm fruits are mashed by rotating arms under steam heated condition in the digester in order to break the oil-bearing cells of the mesocarp (Rupani *et al*., 2010). The digested mash is then pressed using screw presses to extract the oil. CPO from the presses contains a mixture of palm oil (35%-45%), water (45%-55%) and fibrous materials (Rupani *et al*, 2010). The CPO is pumped into a vertical clarification tank for oil separation purpose by settling and centrifugation. Addition of hot water causes the insoluble solid to settle to the bottom of clarifier while the oil droplets flow through the watery mixture on the top (Lam and Lee, 2011). The bottom phase is then drained off as sludge or Palm Oil Mill Effluent (POME).

2.5 Generation of waste from palm oil mills

Huge quantities of wastes are generated in palm oil mill industry. More than 70% (by weight) of the processed FFB is left as wastes (Zafar, 2015). The process of oil extraction from FFB produces liquid waste commonly known as POME. POME also contains solid both total dissolved solids and suspended solid which are known as palm oil mill sludge (POMS) (Rupani *et al.*, 2010). Other solid wastes and by-products that are produced in the extraction process are EFB, potash ash, palm kernel, fibre and shell (Singh *et al*., 2010).

2.6 Palm Oil Mill Effluent (POME)

Extraction of CPO from FFB requires large amount of water. Sterilization of FFB, clarification of extracted CPO and hydrocyclone separation of kernel and shell contributed 36%, 60% and 4% of POME respectively in the mills (Rupani *et al*., 2010). According to

Madaki and Lau (2013), a well managed palm oil mill will generate about 2.5 cm^3 of POME per tonne of CPO produced. However, the generation of POME will continue to increase with the increase of palm oil mill from 334 mills in 1999 to 426 mills in 2011 (MPOB, 2014). Raw POME is a thick brownish liquid which is discharged at a temperature of 80 to 90 °C. It is acidic with a pH ranging about 4-5 (Md Din *et al*., 2006) due to the organic acid produced in the fermentation process. The characteristics of raw POME are presented in Table 2.3.

Table 2.3: Characteristics of raw POME

	Parameter*			
General Parameters	Mean	Range	Metals & Other Constituents	
pH	4.2	3.4-5.2	Phosphorus	180
Oil & Grease(O&G)	6,000	150-18,000	Potassium	2,270
Biochemical Oxygen Demand (BOD; 3days, 30°C)	25,000	10,000-44,000	Magnesium	615
Chemical Oxygen Demand	50,000	16,000-100,000	Calcium	440
Total Solid (TS)	40,500	11,500-79,000	Boron	7.6
Suspended Solid (SS)	18,000	5,000-54,000	Iron	47
Total Volatile Solid (TVS)	34,000	9,000-72,000	Manganese	2.0
Ammoniacal Nitrogen (AN)	35	4-80	Copper	0.9
Total Nitrogen (TN)	750	80-1,400	Zinc	2.3

*All parameter's units in mg/l except pH.
Source: MPOB, (2014).

The raw or partially treated POME has high degradable organic content. POME is non-toxic and contains substantial amounts of N, P, K, Mg and Ca which are the vital nutrients elements for plant growth (Embrandiri *et al*., 2013). The characteristics and nutrient composition of POME is given in Table 2.4. However due to high content of organic matter, oil and grease, COD and BOD, POME is identified as a major source of aquatic pollution by depleting dissolved oxygen when discharged untreated into water body.

Table 2.4: Nutrient Composition of Raw and Treated POME

Type of POME	BOD (mg/l)	N (mg/l)	P (mg/l)	K (mg/l)	Mg (mg/l)
Raw POME	25,000	945	154	1,958	345
Anaerobically Digested POME:					
Stirred Tank	1,300	900	120	1,800	300
Supernatant	450	450	70	1,200	280
Slurry	190	320	40	1,495	260
Bottom Slurry	1,000-3,000	3,552	1,180	2,387	1,509
Aerobically Digested POME:					
Supernatant	100	50	12	2,300	539
Bottom Slurry	150-300	1,495	461	2,378	1,004

Source: MPOB, (2014).

2.7 Regulatory Control of Effluent Discharge

In order to control industrial pollution, regulatory control over discharge from palm oil mill under the Environmental Quality Act, 1974 is being carried out by the Department of Environmental (DOE) (Chin *et al.*, 2013). Mill owners are obliged to follow the regulations before discharging the mill effluent into river and land. DOE imposes a more stringent limit of BOD (20 parts per million (ppm) on industrial effluent which will be discharged into rivers at environmentally sensitive areas of Sabah and Sarawak (Madaki and Lau, 2013). Therefore a more effective treatment process has to be applied to meet the stringent standard requirement on effluent discharge. The effluent discharge standards that are applicable to palm oil mills are presented in Table 2.5.

Table 2.5: Effluent discharge standards from palm oil mill (MPOB, 2014)

Parameter	Unit	Parameter Limits	Remarks
Biochemical Oxygen Demand (BOD; 3-Days, 30 °C)	mg/l	100	
Chemical Oxygen Demand (COD)	mg/l	-	
Total Solids	mg/l	-	
Suspended Solids	mg/l	400	
Oil and Grease	mg/l	50	
Ammoniacal Nitrogen	mg/l	100	Value of filtered sample
Total Nitrogen	mg/l	-	Value of filtered sample
pH	-	5-9	
Temperature	°C	45	

Source: MPOB, (2014).

2.8 Treatment Technologies for POME

In order for the discharge of POME into the river to be safe for human usage and the ecology, various treatment technologies are employed jointly or independently in the treatment of POME. The physical treatment for POME includes pre-treatment steps such as screening, sedimentation and oil removal in oil traps before secondary treatment in biological treatment systems (Industrial Processes and the Environment, 1999). Biological treatments rely on a consortium of active microorganisms to breakdown the organic matters into end-products such as methane (CH_4), carbon dioxide (CO_2), hydrogen sulphide and water. Thus, a large number of biological treatments have been employed in order to create the most cost-effective treatment system.

The most commonly practiced treatment system in palm oil mills is the ponding systems in which more than 85% of the palm oil mills in Malaysia are currently employing this method (Ma *et al.*, 1993). The ponding system is a multistage process consisting of de-oiling tank, acidification tank, anaerobic ponds and facultative or aerobic ponds (Alade *et al.*, 2011). Although ponding systems are more economically applicable and have the

capacity to tolerate a wider range of organic loading rate (OLR) (Poh and Chong, 2009), the major concern about ponding system is the emission of large amount of CH_4 gas into the atmosphere. Ponding treatments generate higher CH_4 gas with an average of 54.4% as compared to open digestion tank (Yacob *et al.,* 2006). Shirai *et al.* (2003) estimated that 0.36 million tons of CH_4 gas will be emitted from ponding systems in 2020.

Anaerobic digestion is the most suitable method in treating effluent with high concentration of organic content (Perez *et al.,* 2001) such as POME. Anaerobic digestion is a process of decomposition of complex organic substrates into simple compounds such as CH_4 and CO_2 in anaerobic condition. Anaerobic digestion has several advantages such as producing CH_4 gas as valuable end product and minimizing the amount of final sludge disposal (De Baere, 2000).

2.9 Chemical Basis of Anaerobic Digestion

Anaerobic digestion occurs in four stages namely, hydrolysis, acidogenesis, acetogenesis, and methanogenesis which produces biogas as end product (Insam *et al.*, 2010; Weiland, 2010) as shown in Figure 2.6. For a stable degradation process, the former and latter stages must be in equilibrium. According to Weiland (2010), hydrolysis is the limiting factor when the rate of conversion in acetogenesis and methanogenesis stages are faster. Other parameters affecting the process include ammonia concentration, trace elements, temperature and retention time in bioreactor (Braun *et al.,* 2010).

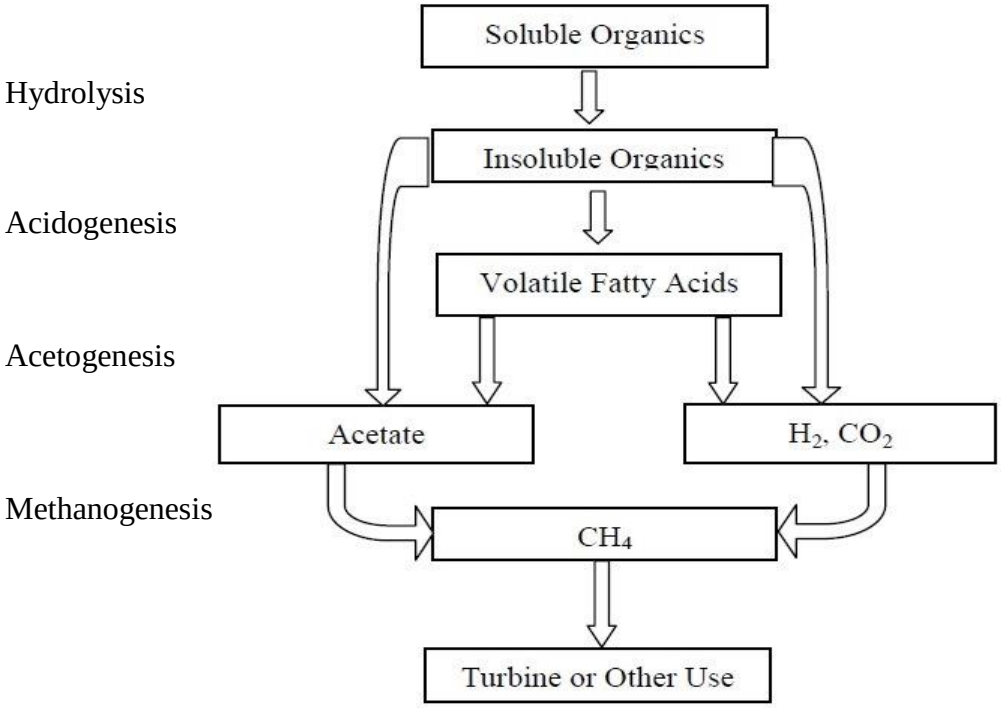

Figure 2.6: Process of anaerobic digestion (Ostrem and Themelis, 2004).

2.9.1 Hydrolysis

During hydrolysis, complex organic materials are broken down into soluble monomers. However, the hydrolytic stage is relatively limited especially for raw cellulolytic materials (Zieminski and Frac, 2012). Only 50% of the organic compound undergo degradation due to lack of enzymes involve in degradation process (Parawira *et al.*, 2008). On the other hand, carbohydrates are converted more rapidly to simple sugars which are subsequently fermented to volatile acid (Saady, 2011).

2.9.2 Acidogenesis

During the acidogenesis process, acidogenic bacteria convert soluble monomers into simple organic compounds such as propionic, formic, lactic and butyric, ketones and alcohols that are required for the acetogenesis stage (Ray *et al.*, 2013). However, decrease in pH due to the products excreting out from the cell is one of the most common factors of

reactor failure. Therefore, the presence of acidogenic and acid scavenging microbes are critical in ensuring the stability of digestion process (Insam *et al*., 2010).

Reaction in acid-forming stages are shown below (Ostrem and Themelis, 2004):

$$C_6H_{12}O_6 \longleftrightarrow 2CH_3CH_2OH + 2CO_2$$

(Glucose) (Ethanol)

$$C_6H_{12}O_6 + 2H_2 \longleftrightarrow 2CH_3CH_2COOH + 2H_2O$$

(Glucose) (Propionate)

2.9.3 Acetogenesis

Fermentation of acidogenesis products produces acetate, CO_2 and hydrogen (H_2) which are needed in the methanogenesis process. H_2 which is one of the end products of acetogenesis is important in converting compounds such as propionic and butyric acid into acetate. However, the reaction can only proceed if the partial pressure of H_2 is low (Ralph and Dong, 2010).

Important reactions in acetogenesis stage are as follow (Ostrem and Themelis, 2004):

$$C_6H_{12}O_6 + 2H_2O \longleftrightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

(Glucose) (Acetate)

$$CH_3CH_2OH + 2H_2O \longleftrightarrow CH_3COO^- + 2H_2 + H^+$$

(Ethanol) (Acetate)

$$2HCO_3^- + 4H_2 + H^+ \longleftrightarrow CH_3COO^- + 4H_2O$$

(Bicarbonate) (Acetate)

2.9.4 Methanogenesis

Methanogenic bacteria are involved in the methanogenesis stage by either converting methyl compound, acetate or reduce H_2 into CH_4. Methanogens work well in neutral environment (Zieminski and Frac, 2012). Thus, a balance between acidogenesis and methanogenesis microbial is important for the stability of anaerobic digestion (Braun *et al.*, 2010).

Methanogenesis reactions are as follows (Ostrem and Themelis, 2004):

Acetate conversion

$$2CH_3CH_2OH + CO_2 \longleftrightarrow 2CH_3COOH + CH_4$$

(Ethanol) (Acetate)

Followed by

$$CH_3COOH \longleftrightarrow CH_4 + CO_2$$

(Acetate) (Methane)

Methanol conversion

$$CH_3OH + H_2 \longleftrightarrow CH_4 + H_2O$$

(Methanol) (Methane)

Carbon dioxide reduction by hydrogen

$$CO_2 + 4H_2 \longleftrightarrow CH_4 + H_2O$$

(Carbon dioxide) (Methane)

2.10 Microorganisms in anaerobic digestion

Anaerobic digestion involves four different groups of microorganisms which convert complex macromolecules into CH_4. These microorganisms are known as primary fermentation bacteria, secondary fermentation bacteria, acetogenic bacteria and

methanogens (Zieminski and Frac, 2012). Cooperation of these microorganisms enable production of sugars and simple organic compounds which are utilised by other group of bacteria.

Wastes which contain protein, carbohydrate and lipid are hydrolysed into amino acid, sugar and fatty acid by the hydrolytic enzyme secreted by hydrolytic bacteria (Weiland, 2010). The metabolites that are produced are subsequently converted into volatile fatty acid, alcohols, CO_2 and H_2. Lee *et al.* (2009) reported the genus *Clostridium* as the most common fermentation bacteria in anaerobic condition. However, other microorganisms such as *Acetivibro*, *Bacteroides*, *Selenomonas*, *Rumiococcus*, *Lactobacillus*, *Selemonas* and *Enterobacter* (Insam *et al*., 2010) and *Streptococci* (Weiland, 2010) are also reported to be involved in the degradation of wastes into volatile fatty acid.

Volatile fatty acids produced are then converted to acetates and H_2 by acetate bacteria of the genera *Syntrophomonas* and *Syntrphobacter* (Staszewska, 2011). *Syntrophomonas* sp. oxidize butyric acid, pentatonic and enanthic acid to acetic acid, CO_2 and H_2 (Ali Shah *et al.*, 2014). However, syntrophic or acetogenic bacteria can only convert fatty acid to acetate in low H_2 concentration (Demirel and Scherer, 2008). Thus, these bacteria co-exist with H_2 utilising bacteria such as methanogens and sulphur reducers. Zieminski and Frac (2012) reported that *Desulfovibrio* may cooperate with *Methanobacterium* genus in producing acetic acid and H_2 during anaerobic digestion. CO_2, H_2, acetate and methyl-group compound produced are utilised by methanogenic bacteria to synthesis CH_4 gas (Shima *et al*., 2002).

Methanogenes are divided into two groups that are known as hydrogenotrophic and acetotrophic (Demirel and Scherer, 2008). Demirel and Scherer (2008) reported that hydrogenotrophic methanogens use H_2 or CO_2 to produce CH_4 while acetotrophic methanogens convert acetate to CH_4 (Table 2.6).

Table 2.6: Characteristics of selected methanogenic bacteria

Species	Morphology	Substrate
Methanobacterium brytanii	Long rods	H_2/CO_2
Methanobacterium formicicum	Long rods	H_2/CO_2, formate
Methanobacterium thermoalcaliphilum	Rods	H_2/CO_2
Methanothermobacter thermoautotrophicum	Long rods	H_2/CO_2
Methanothermobacter wolfii	Rods	H_2/CO_2
Methanobrevibacter smithii	Short rods and chain	H_2/CO_2, formate
Methanobrevibacter ruminatium	Short rods and chain	H_2/CO_2, formate
Methanotherums fervidus	Short rods	H_2/CO_2, formate
Methanothermococcus thermolithotrophicus	Cocci	H_2/CO_2, formate
Methanococcus voltaei	Cocci	H_2/CO_2, formate
Methanococcus vannielii	Cocci	H_2/CO_2, formate
Methanomicrobium mobile	Short rods	H_2/CO_2, formate
Methanolacinia paynteri	Short rods	H_2/CO_2
Methanospirillum hungatei	Short rods	H_2/CO_2, formate
Methanosarcinaacetivorans	Irregular cocci	Methanol, acetate
Methanosarcina barkeri	Irregular cocci, irregular packets	H_2/CO_2, methanol, acetate
Methanosarcina mazeii	Irregular cocci, irregular packets	Methanol, actate
Methanosarcina thermophila	Irregular cocci	H_2/CO_2, methanol, acetate
Methanococcoides methylutens	Irregular cocci	Methanol
Methanosaeta consilii	Rods	Acetate
Methanosaeta thermophila	Rods	Acetate

Source: Demirel and Scherer, (2008).

In the studies of anaerobic digestion, different types of methanogenic bacteria have been successfully identified. *Methanosphaerastadtmanii* and *Methanobrevibacter wolinii* were identified as the dominant methanogen species in anaerobic processing of fruit and

vegetable wastes (Bouallagui *et al*., 2004). *Methanosarcina thermophila*, *Methanoculleus thermophiles* and *Methanobacterium formicicum* were also reported to be present during anaerobic digestion (Charles *et al*., 2009). McMahon *et al*. (2004) stated that *Methanosaeta concilii* was the main species among the acetotrophic methanogens in anaerobic digestion of municipal wastes and sewage sludge.

2.11 Methanogens in POME

Very limited studies on microbial aspect of POME anaerobic treatment have been carried out (Khemkhao *et al*., 2012; Sulaiman *et al*., 2009; Tabatabaei *et al*., 2009; Zellner *et al*., 1998). A comprehensive study on methanogenic community of POME anaerobic treatment conducted by Tabatabaei *et al*. (2009) by using fluorescent *in situ* hybridization (FISH) has identified that the majority of methanogens were from the genus *Methanosaeta*. However, further analysis of POME using 16S rRNA cloning with denaturing gradient gel electrophoresis (DGGE) only managed to identify *M. concilli* as the sole species present in POME. The same study also reported that *Methanosarcina* accounted less than one percent of the total methanogens population in POME (Tabatabaei *et al*., 2009). This was due to the high concentration of acetate which favours *M. concilli*.

2.12 Anaerobic Digestion of POME

Anaerobic digestion on POME offer more advantages compared to other alternative treatments as anaerobic digestion does not require energy for aeration. Besides, anaerobic treatment of POME generates CH_4 gas which can be used in the mill to generate more revenue in terms of certified emission reduction (CER) (Poh and Chong, 2009). However, long retention time and low removal efficiencies are some of the challenges in anaerobic process (Park *et al*., 2005). Nonetheless, usage of high-rate anaerobic bioreactor as shown

in Table 2.7 managed to shorten the retention time as well as capture methane gas for utilization.

Table 2.7: Different anaerobic digestion performed for POME treatment

Anaerobic digestion treatment	Comments	References
Anaerobic suspended growth processes		
Continuous stirred tank reactor	COD removal efficiency as high as 83% and 62.5% of biogas production was reported.	Abdurahman *et al*. (2013)
Attached growth anaerobic processes		
Immobilized cell bioreactor	Digester successfully cope with high volumetric loads and an average of 95.7% of substrate was successfully degraded	Borja and Banks (1994b)
Anaerobic filter tank	90% of total COD removal efficiency with an average of 60% of methane gas production from anaerobic POME treatment was recorded	Borja and Banks (1995)
Thermophilic anaerobic filter tank	The start-up period of POME anaerobic digestion was successfully shorten by increasing the temperature at 0.5-1.0°C per day	Mustapha *et al.* (2003)
Anaerobic fluidized bed reactor	Higher methane yield was achieved with shorter Hydraulic Retention Time (HRT) (6h) compared to anaerobic filter (1.5-4.5 days of HRT) in POME treatment.	Poh and Chong (2009)
Anaerobic sludge blanket processes		
Up-flow anaerobic sludge blanket	Treatment of POME recorded high COD removal efficiency (≈98.4%) with the highest operating OLR of 10.63kg COD/m^3 day	Borja and Banks (1994a)
Two-stage up-flow anaerobic sludge blanket reactor	A maximum load of 30g COD/l per day of POME showed a significant decrease in COD as well as efficient conversion of acid to methane.	Borja *et al.* (1996)
Anaerobic baffled reactor	A low level concentration of fatty acid especially for longer HRT (10 days) was maintained and this leads to high removal of COD as well as reduction of grease/oil (91.3%) and total organic carbon (95.9%) in POME treatment.	Faisal and Unno (2001)

Membrane separation anaerobic treatment		
Ultrasonic membrane anaerobic system	High COD removal efficiency (98.5%) of POME in a short period of time with 79% methane recovery.	Abdurahman *et al*. (2013)
Hybrid anaerobic treatment		
Up-flow anaerobic sludge fixed film reactor	Removal efficiency of 85% at 23.15g COD/l per day were achieved with a shorter start-up period (26 days) was reported.	Najafpour *et al*. (2006)

Source: Alade *et al*., (2011); Wu *et al*., (2010)

2.13 Potential of Biogas from POME

According to Al Seadi *et. al*. (2008), the composition of biogas varies depending on the substrates as well as condition of the process. Typically, CH_4 and CO_2 content accounts for 50-75% and 20-45% volume of biogas. Biogas also contains trace of N_2, ammonia (NH_3), oxygen (O_2), H_2 and hydrogen sulphide (H_2S). Composition of biogas as suggested by Al Seadi *et al.*(2008) is given in Table 2.8 below:

Table 2.8: Composition of biogas

Compound	Chemical symbol	Content (vol%)
Methane	CH_4	50-75
Carbon dioxide	CO_2	25-45
Water vapour	H_2O	2-7
Hydrogen	H_2	1-2
Oxygen	O_2	<2
Ammonia	NH_3	<1
Hydrogen sulphide	H_2S	<1

Source: Al Seadi *et al*., (2008).

POME has become a promising source of biogas that can boost up the renewable energy sector in Malaysia. Based on the average 3 tonnes of POME generated per tonne of CPO produced (Chin *et al.*, 2013), it is estimated approximately 58 million tonnes of POME was generated in 2013 (Table 2.9). It is expected approximately 588 kilo tonnes of CH_4 will be generated if all the POME are treated anaerobically (Table 2.9).

Table 2.9: Estimation biomethane production from POME based on CPO production of Malaysia in 2013

Parameter	Unit	Value
CPO production	tonnes	19,216,459
POME generated[a]	m^3	57,649,377
COD level in POME[b]	mg/l	51,000
COD converted[c]	tonnes	2,352,094
CH4 produced[d]	tonnes	588,023

[a] Assuming that 3 m^3 POME generated per tonnes CPO produced (Chin *et al.*, 2013)
[b] Mean value of COD of POME (MPOB, 2014)
[c] Assuming digester efficiency is 80% (Chin *et al.*, 2013)
[d] Theoretical methane conversion factor is 0.25 kg CH_4 per kg COD (Bhattacharya *et al.*, 2005).

Biogas captured from POME could be used as a replacement for diesel used in package boilers or high-pressure boilers (Chin *et al.*, 2013). The biogas generated could also be used in biomass boiler as co-combustion fuel. Reduction in the reliance on diesel and biomass as boiler fuel can then provide addition revenue to palm oil mills (Tong and Jaafar, 2006).

2.14 Anaerobic co-digestion

Co-digestion which is also termed as "co-fermentation" is a waste treatment in which different wastes are mixed and treated together in a single digester (Agdag and Sponza, 2007). Co-digestion treatment has been widely used due to its numeral benefits. For example acceleration in biodegradation of solid wastes (Hartmann and Ahring, 2005), increase in stabilization phase (Lo *et al.*, 2010) as well as increase in biogas production (Jingura and Matengaifa, 2009).

Several studies have documented that mixtures of agricultural, municipal and industrial wastes can be treated efficiently and successfully together (Martín-González *et al*., 2011; Cavinato *et al.*, 2010; Gomez *et al*., 2006; Sosnowski *et al*., 2003). Agdag and Sponza (2007) reported the co-digestion of municipal wastes with industrial wastes yielded higher

methane gas compared to digestion of municipal wastes alone. Similarly, Fezzani and Cheikh (2010) recorded the highest CH_4 generated when olive mill wastewater and olive mill solid wastes was co-digested.

To prevent a poor start-up of anaerobic digester, various molecular techniques have been applied to determine the active microbial populations present in the anaerobic digester. Application of DGGE and FISH in co-digester of dairy and fish wastes discovered the presence of *Methanosaeta* and *Bacteroidetes* (Regueiroa *et al*., 2012). Ziganshin *et al*. (2013) reported the usage of 16S rRNA gene-based, clone library sequencing, terminal restriction fragment length polymorphism (T-RFLP) and pyrosequencing detected presence of *Clostridia*, *Bacteroidetes*, *Methanomicrobiales* and *Methanosarcinales* in anaerobic co-digestion of various agricultural wastes materials. Although, many researches on co-digestion anaerobic digestion have been carried out, very limited literature is found on co-digestion of POME and the microbial community structure in the digester.

2.15 Identification of microbial population

Culture-based methods are vital in investigating microbial population. However, not all organisms are cultivable, and many of them remain "unculturable". According to Oliver (2005), although these organisms are viable in the environment, they are not able to grow under laboratory condition. Thus, application of molecular techniques in isolation and characterization of environmental microbial population is highly recommended.

Various molecular techniques have been established for characterizing the diversity of microorganism population. One of the techniques is amplification of polymerase chain reaction (PCR) of conserved gene method (Rastogi and Sani, 2011). PCR amplification of

conserved region such as 16S rRNA has been widely applied such as identification of bacteria with ambiguous profiles, slow-growing bacteria and routine identification (Woo *et al*., 2008; Janda and Abbott, 2007).

16S rRNA is commonly used for bacteria identification as these genes are ubiquitous and are a highly conserved region (Hugenholtz, 2002). By comparing 16S rRNA gene sequences, bacteria can be differentiated between particular taxa or strains (Saachi *et al*., 2002). Janda and Abbott (2007) also reported that 16S rRNA is normally applied due to its large gene sequence (1,500 bp) which is suitable for statistical validation especially when identifying a new species.

DeSantis *et al.* (2007) reported that the most widely used method to analyse PCR products is to clone and then sequence the individual gene fragments. The cloning-and-sequencing method was used to identify microbial diversity in mining-impacted soil of former uranium mine site of South Dakota, USA (Rastogi *et al*., 2010). Sekiguchi *et al.* (2002) reported the PCR-based methodology was applied to determine the bacterial community structure along the Changjiang River, China. Besides that, clone libraries of 16S rRNA gene technique was used to analyse the bacterioplankton consortia in surface water of coastal California (Cottrell and Kirchman, 2000). 16S rRNA gene analysis has also successfully detected a wide variety of microbial in the anaerobic packed-bed reactor degrading organic solid wastes (Sasaki *et al.*, 2007).

Chapter 3

Molecular Identification of the Microbial Population in Palm Oil Mill Effluent (POME)

(This chapter has been accepted by the *Journal of Palm Oil Research*)

3.1 INTRODUCTION

Elaeis guineensis, or more commonly known as oil palm is one of the major crops in Malaysia and Indonesia. Malaysia produces approximately 89 million tonnes of FFB per year (Ishak *et al*., 2014). However, oil extraction process requires huge amount of water and it has been estimated that more than 50% of the water ends up as POME (Ahmad *et al*., 2003). Approximately 53 million m^3 of POME is produced every year (Lorestani, 2006). Although POME is non-toxic, it is identified as major source of aquatic pollutions when discharged untreated into nearby water system due to high concentration of organic matter, total solid, oil and grease, COD as well as BOD (Rupani *et al*, 2010).

Many attempts to treat POME are currently being employed worldwide. One of the treatments introduced is the ponding system. The ponding system which is also known as waste stabilization pond has been used in Malaysia since 1982 (Rupani *et al*., 2010). However, ponding systems have some disadvantages such as the need for large areas, long HRT, bad odour and difficulties in maintaining liquid distribution (Rupani *et al*., 2010). Due to these limitations, anaerobic treatment of POME using newer technologies such as anaerobic digesters offer more attractive solutions for CH_4 gas production and clean development mechanism (CDM). Anaerobic digester which have been studied for the treatment of POME at laboratory scale include up-flow anaerobic sludge blanket (UASB),

up-flow anaerobic sludge fixed-film (UASFF), continuous stirred tank reactor (CSTR) and membrane technology (Poh and Chong, 2009; Najafpour *et al*., 2006).

One of the key factors in determining the efficiency of anaerobic digesters is the optimal composition of the bacterial community involved in the anaerobic degradation process, as the roles of the microbial consortia in this process are still not completely understood. Traditionally, microbial communities in anaerobic sludge were identified either through light microscopic observation (Jenkins *et al*., 1993; Eikelboom, 1975) or culture-dependent techniques (Seiler and Blaim, 1982; Ueda and Earle, 1972). However, identification and characterization of culturable cells may only represent ≤ 0.1-1% of total microbial community (Steven *et al*., 2007). Thus, culture-independent methods are required as these can identify various other uncultured organisms (Vaz-Moreira *et al*., 2011). Culture-independent techniques extract and analyse total nucleic acid which theoretically represent the whole microbial consortia from environment samples (Spiegelman *et al*., 2005).

In order to provide an optimum condition for microbial propagation and to monitor the microbial activities which could contribute to greater CH_4 production, it is critical to have an accurate understanding of the microbial population of the POME. In this study, the aim of the present work was to determine the microbial community in POME by using 16S rDNA clone library and traditional culture-based techniques.

3.2 MATERIALS AND METHODS

3.2.1 Samples collection

The POME samples were collected from the anaerobic pond from Bau Palm Oil Mill (BAPOM), Kuching, Sarawak. The samples were stored in sealed container immediately after collection and preserved at 4 °C in order to avoid biodegradation due to microbial activities.

3.2.2 Isolation of bacteria from POME

The POME samples were cultured on DVS agar (Savant *et al*., 2002) by spreading 100 µl of the POME liquid on DVS agar. The DVS medium has the following composition (per L of distilled water): 6.0 g NaCl, 0.8 g $CaCl_2.2H_2O$, 1.0 g $MgCl_2.6H_20$, 10.0 g Peptone, 10.0 g Tryptone, 25.0 g CH_3CO_2K, 3.0 g KH_2PO_4, 3.0 g K_2HPO_4, 15.0 g NH_4NO_3, 20.0 g Yeast and 30.0 g agar. Grown colonies were selected and purified by streaking on DVS agar plate. All cultures were incubated at 50 °C in anaerobic jar (Oxoid, USA)

3.2.3 DNA extraction and PCR amplification of 16S rRNA gene of isolated bacteria

Colonies from the DVS agar plates were selected and used for colony PCR (Fukui and Sawabe, 2007). A small amount of colony was picked using autoclaved 10 µl tips. The colony was suspended in 10 µl sterile distilled water and vortexed for 30 seconds. The suspension (3 µl) was added into the PCR mixture which contained 2.5 µl of 10X PCR buffer (Fermentas, Canada), 1 µl of dNTPs (Fermentas, Canada), 1 µl of each primer:

forward primer (10f: 5'-AGT TTG ATC TGG CTC AGA TTG-3', 10 pmol/ml^{-1}) and reverse primer (1100r: 5'-GGG TTG CGC TCG TTG-3', 10 pmol/ml^{-1}) (Miqueletto *et al*., 2011), 0.5 µl of Taq polymerase (5 U/µl) (Fermentas, Canada) and 14.0 µl of sterile distilled water. The PCR amplifications were performed using LabCycler System (Sensoquest, Germany). The PCR amplification conditions included an initial denaturation step of 5 minute at 95 °C, followed by 30 cycles of 30 seconds at 96 °C, 1 minute at 54 °C, and 1 minute at 72 °C; and a final extension of 7 minutes at 72 °C. The PCR products were purified according to the manufacturer's instruction (Mo Bio Laboratories, USA). Purified colony PCR products were sequenced.

3.2.4 Genomic DNA extraction and purification

Total genomic DNA through direct extraction method was extracted from 0.25 g of POME sludge using Power Soil^{TM} DNA Isolation Kit (Mo Bio Laboratories, USA), according to the manufacture instruction. The DNA obtained was confirmed through electrophoresis in 1% agarose gel and amplified using the primer set 10f (5'-AGT TTG ATC TGG CTC AGA TTG-3') and 1100r (5'-GGG TTG CGC TCG TTG-3') (Miqueletto *et al*., 2011) to generate amplicons of approximately 1100 bp in size. PCR reactions mixtures were shown in Table 3.1

Table 3.1: The PCR reagents of 25µl volume reaction

PCR reagent	Quantity per reaction
10 X PCR buffer (Fermentas, Canada)	2.5 µl
25 mM MgCl2 (Fermentas, Canada)	2.0 µl
10 mM dNTPs (Fermentas, Canada)	1.0 µl
10 pmol/µl of Primer 10f (Bio Basic, Canada)	1.0 µl
10 pmol/µl of Primer 1100r (Bio Basic, Canada)	1.0 µl
Taq DNA polymerase (Fermentas, Canada)	0.5 µl
Sterile distilled water	14.0 µl
Template DNA	3.0 µl
Total final volume	25.0 µl

The PCR amplifications were then performed using LabCycler System (Sensoquest, Germany) with an initial denaturation step of 5 minute at 95 °C, followed by 30 cycles of 30 seconds at 96 °C, 1 minute at 54 °C, and 1 minute at 72 °C; and a final extension of 7 minutes at 72 °C. PCR products were purified according to the manufacturer's instruction.

3.2.5 Cloning 16S rDNA

Purified PCR fragments from direct extraction technique were ligated into pGEM-T Easy vector according to the manufacture's instruction (Promega, USA) and transformed into *Escherichia coli* (*E. coli*) XL-1 blue using the heat shock method (Sambrook and Russell, 2006). White colonies were randomly selected from the agar plates and plasmids were extracted using a plasmid extraction kit (Promega, USA). The extracted plasmids were re-amplified through PCR reaction and sent to 1st BASE, Kuala Lumpur for sequencing.

3.2.6 DNA sequencing and Phylogenetic analysis

The sequences obtained were compared to known 16S rRNA sequences in GeneBank database by using basic logical alignment tool (BLAST). Closely related sequences were aligned with PCR sequences using the program CLUSTALW and further edited manually. Phylogenetic tree were constructed by neighbour-joining method (Saitou and Nei, 1987) using MEGA ver 5.0 (Tamura *et al.*, 2011). Phylogenetic tree was evaluated by bootstrap analysis based on 100 resamplings of neighbour-joining dataset.

3.3 RESULTS AND DISCUSSION

16S rRNA region was successfully amplified and cloned into pGEM-T Easy vector. From 9 clones screened, only 3 (Isolate C, E and H) were successfully cloned with the PCR fragment size of 1100 bp. Upon cloning, the extracted plasmids from the successful clones were re-amplified, purified and sequenced (Figure 3.1). As for the isolates A and D from solid agar, PCR product of 1100 bp was successfully amplified (Figure 3.1). Table 3.2 shows the result of the DNA sequencing.

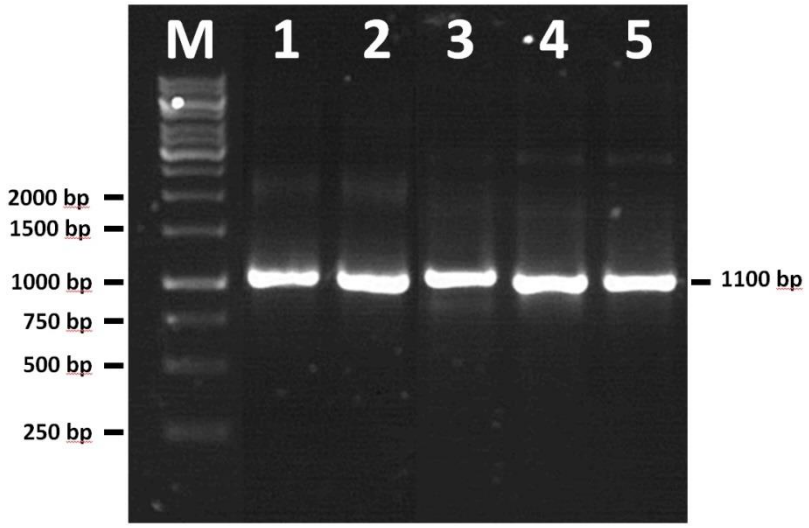

Figure 3.1: Agarose gel electrophoresis of amplified 16S rRNA region of the isolates. The extracted plasmid from 16S rRNA clone library and PCR products of isolated bacteria from solid agar which were successfully amplified (1100 bp). Lane M, 1kb ladder (Fermentas); Lane 1, 2 and 3, PCR products amplified by using extracted plasmid from isolates C, E and H respectively. Lane 4 and 5, DNA template of bacteria A and D isolated from DVS agar.

Table 3.2: Sequences homology of the isolates

Isolates	Bacteria	Gen Bank Database	Sequences homology (%)
A	*Bacillus thermoamylovorans*	FN397520	98
C	Uncultured *Thermoanaerobacteriaceae* bacterium	AM408569	99
D	*Bacillus coagulans*	AB830332	99
E	Uncultured *Thermoanaerobacteriaceae* bacterium	AM408569	99
H	Uncultured *Thermoanaerobacteriaceae* bacterium	AM408569	99

In order to further understand the microbial community in POME, the isolates were included in the phylogenetic tree and *Kluyveromyces lactis* was used as the outgroup (Figure 3.2). The sequences obtained in this study have been deposited in the GenBank database under accession numbers KF539415-KF539419.

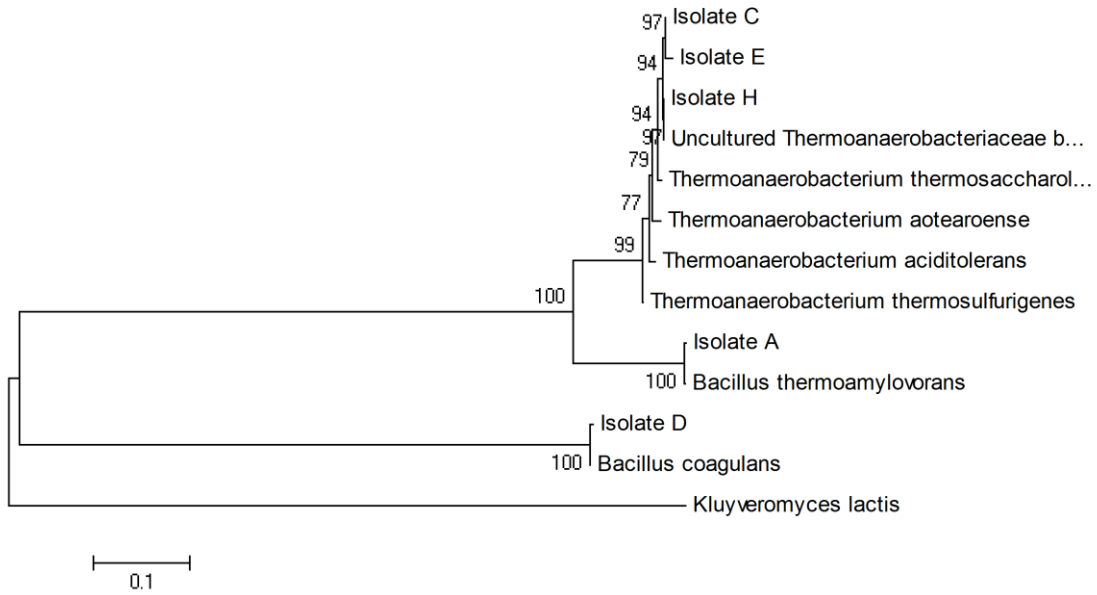

Figure 3.2: Dendrogram of partial sequence of 16S rDNA from POME, grouped by class. The dendrogram was constructed by the neighbour-joining method. The number at the nodes of the tree indicates bootstrap value of each node out of 100 bootstrap resampling. The scale bar represents 0.1 substitutions per base position.

From the phylogenetic tree, all five isolates were classified in the phylum of *Firmicutes*. Three of the *Firmicutes* (C, E and H) were clustered in the class of *Clostridia* while isolates A and D were assigned to the class of *Bacilli*. Isolate C, E and H were closely associated with *Thermoanaerobacterium* sp. from the class *Clostridia* with 99% similarity to the uncultured *Thermoanaerobacteriaceae* bacterium clone THPB-7 that had been previously found in environment sample taken from anaerobic sequencing batch reactor (AM408569, NCBI database). *Thermoanaerobacter* sp. had also been reported in POME sludge (Khemkhao *et al*., 2011; O-Thong *et al.*, 2011), paper mill and waste water from breweries (Suihko *et al*., 2005; Sommer *et al*., 2004). *Thermoanaerobacter* sp. is known to

be associated with the fermentation of glucose into ethanol, acetic acid, butyric acid, H_2 and CO_2 (Koskinen *et al*., 2008; Shin and Youn, 2005; Lynd *et al*., 2002).

The sequences of isolates from solid agar (isolate D and A) were identified as member of the bacterial genera *Bacillus*, *Bacillus coagulan* and *Bacillus thermoamylovorans* with 99% and 98% similarities respectively. The presence of *Bacillus* genus bacteria in sludge and agriculture wastes was also shown in previous findings (Ivanov *et al*., 2004; Vossoughi *et al*., 2001). It is known that *B. thermoamylovorans* and *B. coagulans* are capable of producing ethanol, acetate and lactate from glucose utilization (Tay *et al*., 2002). Similar results were also reported by other researchers (Kotay *et al*., 2007; Pantamas *et al*., 2003).

B. coagulans, *B. thermoamylovorans*, *Thermoanaerobacter* sp. are thermophilic bacteria. Metabolically, they are facultative and/or strict anaerobes and moderately acidophile (Kublanov *et al*., 2007; De Vecchi and Dargo, 2006; Combet-Blanc *et al*., 1995). These characteristics enable the bacteria to survive in POME which is acidic with pH in between 4 to 5 (Madaki and Lau, 2013). This is supported by previous findings regarding isolation of these bacteria from acidic and/or extreme environments (Longo *et al*., 2010; Koskinen *et al*., 2008; Kublanov *et al*., 2007).

Both *Bacillus* sp. and *Thermoanaerobacter* sp. were successfully identified through culture-dependent and 16S rDNA cloning method respectively. Steven *et al*. (2007) stated that combination of both of the techniques should produce a more complete characterization of microbial diversity. Through culture-dependent method, only the better adapted microbes to the culture conditions were being screen (Vaz-Moreira *et al*., 2011).

For this reason, culture-independent techniques such as 16S rRNA clone library were used to detect a different portion of bacteria population. These results show that the necessity of using both culture-based and culture-independent techniques to evaluate the microbial diversity of a complex ecosystem.

Bacillus sp. and *Thermoanaerobacter* sp. played an important role in anaerobic digestion. Miah *et al*. (2005) stated that aerobic thermophilic bacteria especially *Bacillus* sp. produced extracellular enzyme which act as catalyst in improving the degradation efficiency of organic matter in sludge. The ability of *Thermoanaerobacter* sp. to utilise complex carbohydrate such as xylan, cellulose as well as simple sugar into volatile fatty acid and H_2 have also been documented (Ueno *et al*., 2006; Cann *et al*., 2001). Furthermore, *Thermoanaerobacter* sp. could degrade primary alcohol under thermophilic condition (Ben-Bassat *et al*., 1981). It has also been reported that ethanol degradation under thermophilic condition is an important steps in methanogenesis reaction (Stams and Zehnder, 1990).

The capability of *B. coagulans*, *B. thermoamylovorans*, and *Thermoanaerobacter* sp. in utilising different substrates (Su and Xu, 2014; Chang *et al*., 2008; Kublanov *et al*., 2007) holds a promising future in the studies of anaerobic digestion treatment of POME. The ability to convert various substrates can help to improve hydrolysis process in anaerobic digestion of POME by employing co-digestion. Digestion of waste rich in nitrogen concentration such as POME (Baharuddin *et al*., 2010) together with high carbon content waste will balance the carbon to nitrogen (C/N) ratio (Lehtomaki *et al*., 2007). Additionally, co-digestion increases the digestion, stabilization as well as biogas yield (Lo *et al*., 2010; Jingura and Matengaifa, 2009).

Although *Bacillus* sp. and *Thermoanaerobacter* sp. were successfully isolated from POME, it only represented a small community of microorganisms presence in POME as acidogenesis, acetogenesis and methanogenesis bacteria were not isolated. According to Shima *et al*. (2002), volatile fatty acid produced are utilised by methanogens to synthesis CH_4 gas in the presence of sulphur reducer (Demirel and Scherer, 2008). Thus further characterization on methanogens in POME should be carried out to understand the microbial population in POME.

3.4 CONCLUSION

The microbial community of the POME was reflected in the sequencing results of the 16S rRNA clone library and traditional culture-based technique. This study showed that *Thermoanaerobacter* sp. from the *Clostridia* class was isolated through 16S rRNA clone library while *B. coagulans* and *B. thermoamylovorans* were isolated using traditional culture-based technique with high sequence similarities (>90%) found in both isolation methods.

ACKNOWLEDGMENT

This project was funded by a research grant from Universiti Malaysia Sarawak (Top-Down grant: 01(TD01)/764/2010(01)) and L18403 F07 00 CMS AGROTECH.

Chapter 4

Identification of Methane Producing Bacteria from Palm Oil Mill Effluent (POME) with Solid Cud from Ruminant Stomach

(This chapter has been published in *Journal of Biochemistry, Microbiology and Biotechnology*, 2 (1): 23-26 (2014).

4.1 INTRODUCTION

CPO production in Malaysia has been increasing continuously over the years, from 4.1 million tonnes in 1985 to 6.1 million tonnes in 1990. Production further increased by 11.29% to 18.9 million tonnes in 2011 (MPOB, 2011). However, the increase of production leads to generation of huge quantities of wastes. During oil extraction process, about 50% of water used results in POME while others are lost as steam, mainly through sterilizer exhaust, piping leakages as well as wash water (Ahmad *et al*., 2003). POME contains suspended solids and total dissolved solids in the range of 18,000 mg l^{-1} and 40,000 mg l^{-1} respectively (Ma, 2000). Both solids which are known as Palm Oil Mill Solids (POMS) consist of 3.6, 0.9 and 2.1 mg l^{-1} of total nitrogen, phosphorus and potassium respectively which results in bad odors and consider as source of ground pollution (Yaser *et al*., 2007).

POMS can be applied as fertilizer as it has high nutrient value (Rupani *et al*., 2010). However, during rainy season, the drying process of POMS becomes difficult as the rate of drying become slower. Due to this limitation, anaerobic treatment of POMS such as anaerobic digester offer more attractive solutions for biogas production and clean development mechanism (CDM).

Anaerobic digestion involves a wide variety of microbial community. Due to the complex microbial ecology involved, anaerobic digestion process is often treated as 'black box' (Supaphol *et al*., 2011). However, with the development of culture-independent molecular techniques research on microbial population has greatly expanded (Demirel and Scherer, 2008). The application of molecular biology techniques such as 16S rRNA clone library is important for understanding and clarifying complex reaction which occurred in anaerobic digester.

Recently, application of mixing different wastes in a single anaerobic digester known as co-digestion has been widely applied due to its numeral benefits (Lin *et al*., 2011; Bouallagui *et al*., 2009). Applications of mixing different waste in anaerobic digester adjust the moisture content and pH, improve nutrient content, widen the range of bacterial strains and increase the biogas yields (Esposito *et al*., 2012; Khalid *et al*., 2011).

Usage of waste such as solid cud from ruminant stomach as co-mixture is of high interest as ecosystem of ruminant animal such as cow is a highly evolved natural anaerobic system (Weimer *et al*., 2009). According to Weimer *et al.* (2009), cattle represent the greatest evolutionary of cellulosic biomass utilization. Approximately 70% of the bacterial cells in the rumen are attached to the solid cud (Forsberg and Lam, 1977). In addition, the solid cud contains the nutritional requirements of the microbial population. The entire polysaccharides in plant cells such as cellulose, most hemicellulose, pectin, starch and fructans are readily hydrolysed and fermented into volatile fatty acids by one or more microorganisms in ruminant stomach (Van Soest, 1994). Mengel *et al.* (2001) stated that

cytoplasmic contents of forages also contain substantial amount of protein, lipid and nucleic acid which are fermented into ammonia, glycerol and major volatile fatty acids.

In order to produce higher biogas yield, inoculum source is crucial for optimization of inoculum ratio. Lin *et al*. (2011) reported inoculum would affect the concentration of ammonia and volatile fatty acid. Both concentrations and environmental factors are the main factor that causes changes in microbial population dynamics of anaerobic digestion of mixed wastes (Supaphol *et al*., 2011). Nevertheless, researches on the microbial community in anaerobic co-digestion process in different ratio have been reported (Adebayo *et al*., 2014; Zhao *et al*., 2014; Boulanger *et al*., 2012).

In this study, the aim of the present work was to determine the methane producing bacteria community in POMS with solid cud from ruminant stomach at different ratio using 16S rRNA clone library techniques.

4.2 MATERIAL AND METHODS

4.2.1 Samples Collection

POMS was collected from the anaerobic pond from Bau Palm Oil Mill (BAPOM), Kuching, Sarawak. The solid cud from the first compartment of cow's stomach was collected from a slaughter house located at Ladang Lapan, Kuching. Both samples were stored in sealed container immediately after collection and preserved at 4 °C in order to avoid biodegradation due to microbial activities.

EFB was collected from BAPOM and was dried at 60 ˚C prior shredding. Dried EFB was shredded using Cutting Mills SM100 (Retsch, Germany) cutting mill with 1.0 mm sieve size and stored in a tightly closed container at room temperature.

4.2.2 Anaerobic vessel set up

Co-mixture with different ratio (Table 4.1) were incubated at 50 °C in a 2 L vessel with initial starter of 400 ml. Sampling for both ratio were conducted every 4 weeks interval during 12 weeks of incubation.

Table 4.1: Different ratio of POMS and solid cud in co-digestion

Ratio	Co-digestion mixture
1:2	POMS: Solid cud
2:1	POMS: Solid cud

4.2.3 DNA Extraction and PCR Amplification

Bacterial DNA of both ratio of co-mixture were extracted using Power Soil^{TM} DNA Isolation Kit (Mo Bio Laboratories, USA) and amplification of 16S rRNA region was amplified using Met86F (5'-GCT CAG TAA CAC GTG G-3') and Met1340R (5'-CGG TGT GTG CAA GGA G-3') primers (Wright and Pimm, 2003) to produce amplicon of approximately 1300 bp in sizes. PCR of the 16S rRNA was run in 25 µl reactions comprising 50-100 ng of DNA, 10X Taq DNA polymerase buffer (Fermentas, Canada), 0.5 µl of 10 mM dNTP mix (Fermentas, Canada), 2.5 µl of 25 mM $MgCl_2$ (Fermentas, Canada), 0.5 µl of each primer and 0.2 µl of 5 U AmpliTaq DNA polymerase (Fermentas, Canada). Table 4.2 showed the PCR amplification parameters.

Table 4.2: PCR amplification reaction

Step Cycle	Temperature/Time	
Initial denaturation	95 ^{o}C (10 min)	
Denaturation	94 ^{o}C (40 seconds)	34 cycles
Anneling	54 ^{o}C (50 seconds)	
Elongation	72 ^{o}C (90 seconds)	
Final elongation	72 ^{o}C (10 min)	

4.2.4 Cloning 16S rRNA

PCR products of both co-mixture (1:2 and 2:1) were purified using UltraClean soil DNA kit (Mo Bio Laboratories, USA) according to the manufacturer's instruction. 16S rRNA clone libraries were constructed by ligating the purified PCR fragments into pGEM-T Easy vector as described by the supplier (Promega, USA). Plasmid DNA was transformed into *Escherichia coli* XL-1 blue using the heat shock method (Sambrook and Russell, 2006). Plasmid containing the PCR insert were identified using blue/white screening on Luria-

Bertani Agar (LBA) which contained 100 µgml^{-1} ampicillin (Fisher Scientific, USA) and 80 µgmL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Thermo Scientific, USA). White colonies were randomly selected from the LBA plates and plasmids were extracted using a plasmid extraction kit (Promega, USA). The extracted plasmids were re-amplified through PCR reaction using primer T7 and Sp6 and sent to 1st BASE, Kuala Lumpur for sequencing.

4.2.5 Phylogenetic analysis

The sequences obtained were trimmed and further analysed using the Bellerophon software (Huber *et al*., 2004) to remove chimeric rRNA clones. Sequences similarities with 16S rRNA sequences in GeneBank^{TM} database were conducted using the basic logical alignment tool (BLAST). PCR sequences were aligned with closely related sequences using CLUSTALW program in the MEGA 5.0 software package. MEGA 5.0 (Tamura *et al*., 2011) was also used to a construct neighbour-joining tree, which was bootstrap resampled 1000 times.

4.3 RESULTS AND DISCUSSION

The 16S rRNA regions were successfully amplified from the DNA extracted from the different ratio (1:2 and 2:1) of co-mixture. From the 24 clones screened (6 from both Week 0 and Week 4 of 1:2 ratio and 6 from both Week 0 and Week 4 of 2:1 ratio) only 12 clones contained the correct size of DNA insert (1,300 bp) (Figure 4.1). No amplification of PCR products for Week 8 and 12 of both co-mixtures were generated.

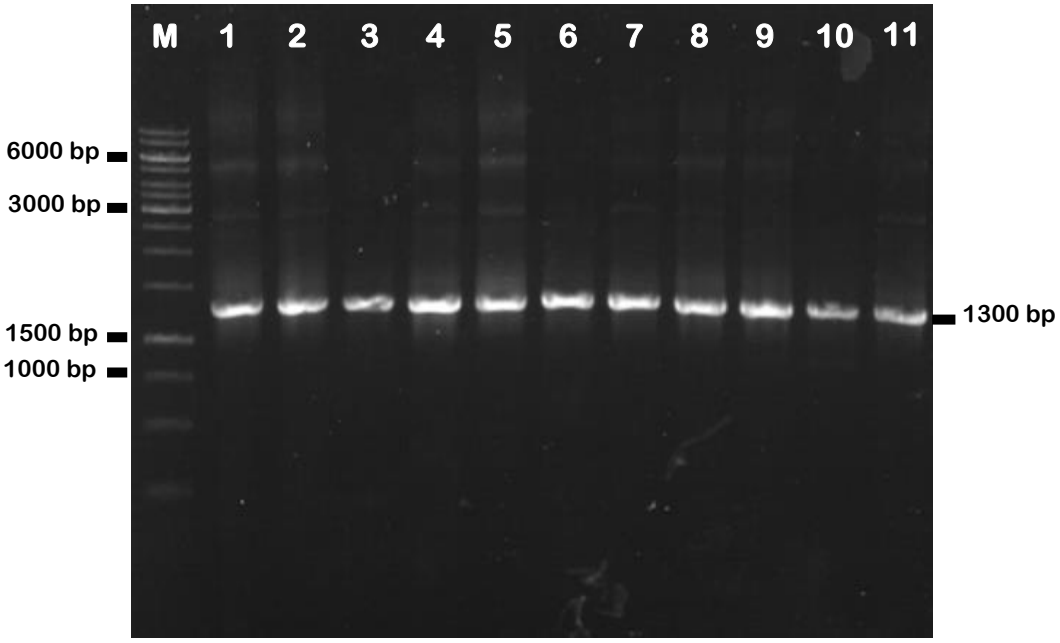

Figure 4.1: Agarose gel electrophoresis of amplified 16S rDNA region. The extracted plasmid from 16S rDNA clone library which were successfully amplified (1300 bp). Lane M, 1kb ladder (Fermentas); Lane 1, 2 and 3, PCR products of mixed sample with 1:2 ratio on Week 0 amplified using extracted plasmid from transformed bacteria; Lane 4,5, and 6, PCR products of mixed sample with 2:1 ratio on Week 0 amplified using extracted plasmid from transformed bacteria; Lane 7,8, and 9, PCR products of mixed sample with 1:2 ratio (Week 4) amplified using extracted plasmid from transformed bacteria; Lane 10 and 11, PCR products of mixed sample with 2:1 ratio (Week 4) were amplified by using extracted plasmid from transformed bacteria

The diversity and phylogeny of the isolates were investigated by constructing phylogenetic tree with *Kluyveromyces lactis* as the outgroup (Figure 4.2). The sequences obtained in this study have been deposited in the GenBank database under accession numbers KJ522696-KJ522706

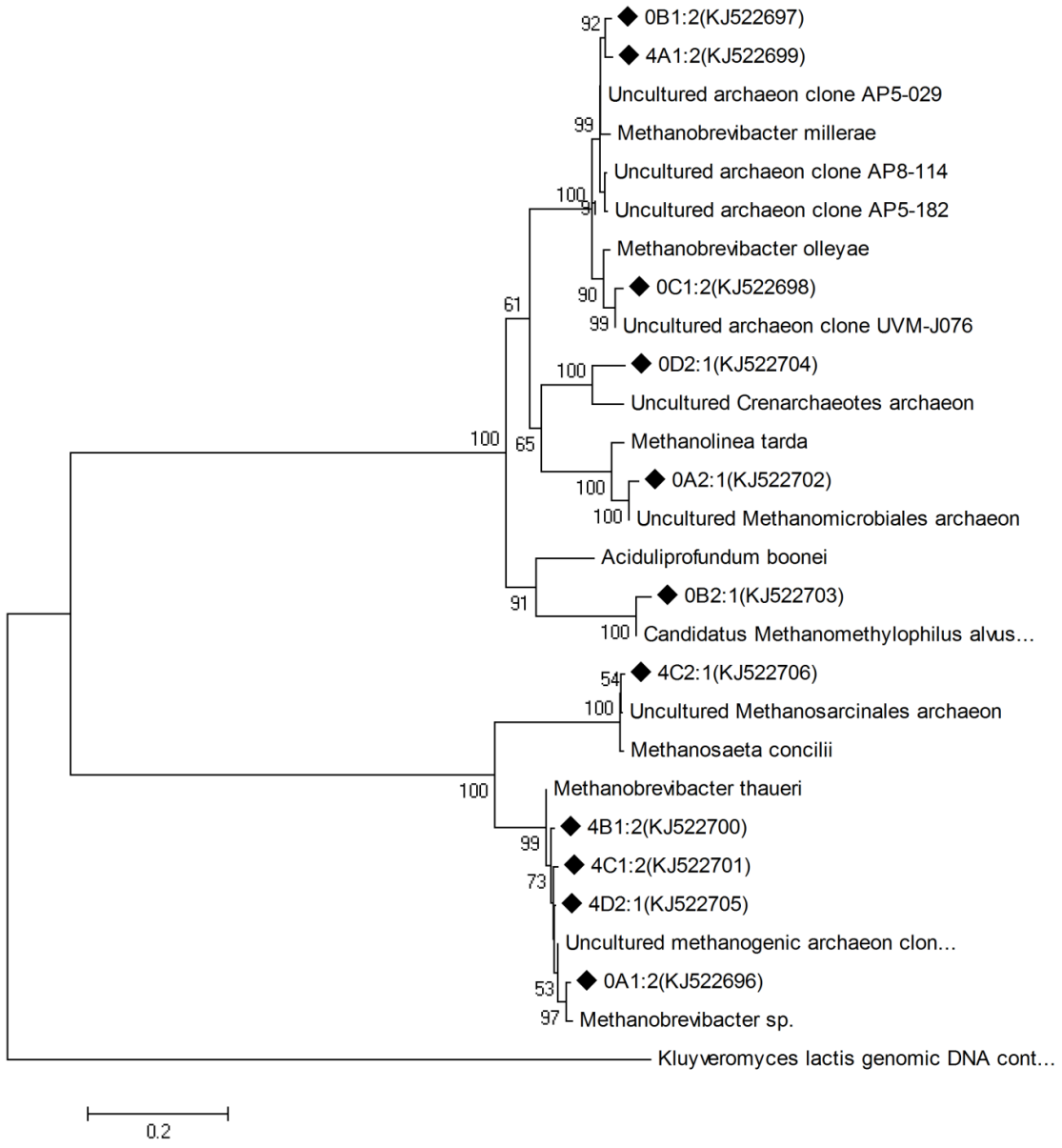

Figure 4.2: Dendrogram of partial sequence of 16S rRNA of clone libraries from different ratio (1:2 and 2:1) co-mixture. The number at the nodes of the tree indicates bootstrap value of each node out of 1000 bootstrap resampling. The scale bar represents 0.2 substitutions per base position.

From the phylogenetic tree, 66.6% of the clones isolated displayed 95% or greater genus-level sequence homology to species belonging to *Methanobrevibacter*. Within this genus, 41.6% (5/12) of all clones had 97.0% or greater species-level sequence similarity to *M. millerae*. In contrast, only 0.083% of library clones were identified as *M. olleyae*, *M. arboripilus* and *M. thaueri* respectively.

The other four clone libraries were divided into three different phylogenetic groups. Two of the clones showed 90% or greater sequences homology belonging to *Methanosaeta concilii* while the remaining clones were identified as *Methanolinea tarda* and *Aciduliprofundum boonei* respectively with 97% sequence similarities.

Majority of the clones from co-mixture of 1:2 (anaerobic sludge: solid cud) ratio in Week 0 and Week 4 belonged to the genus *Methanobrevibacter*. This might be due to the large amount of solid cud from ruminant stomach used as co-mixture. Based on the analysis of rumen archaea in rumen content, *Methanobrevibacter* spp. have been identified as the most abundant methanogens in the rumen (Janssen and Kirs, 2008). The presence of *Methanobrevibacter* sp. had also been reported in ovine and bovine content (Wright *et al*., 2004a) and dairy cow (King *et al*., 2011). In addition, Singh *et al*. (2013) also reported the presence of *Methanobrevibacter* sp. in ruminal fluid of buffalo. Clones from environment samples which show genus-level sequence similarity of more than 95% to *Methanobrevibacter* sp. are most abundant in gastrointestinal samples from herbivores (Hook *et al*., 2010; Wright *et al*., 2007; Whitford *et al*., 2001).

In contrast, different methanogens such as *Methanosaeta concilii, Methanolinea tarda* and *Aciduliprofundum boonei* were found in co-mixture (2:1 ratio) which contains larger volume of anaerobic sludge in Week 0. The presence of *Methanosaeta* sp., *Methanolinea* sp., and *Aciduliprofundum* sp., in sludge and agriculture wastes were also shown in previous findings (Wright and St-Pierre, 2012; Tabatabaei *et al*., 2009, Imachi *et al*., 2008). *Aciduliprofundum boonei* which was present in the anaerobic digester of 2:1 mixture, is believed to be a novel methanogenic archaeal that is distantly related with *Thermoplasmatales* (Spang *et al*., 2013). However, in Week 4, only *Methanobrevibacter*

millerae and *Methanoseata concilii* were detected. Changes in microbial population might due to the larger volume of POME used in the co-mixture. *Methanosaeta concilii* is reported to be the most abundant methanogens in POME anaerobic digestion (Tabatabaei *et al.,* 2009). According to Karakashev *et al.* (2006), the only change observed during incubation of samples dominated by *Methanosaetaceae,* was the elimination of subdominant populations as shown in Table 4.3.

Table 4.3: Microbial population in anaerobic digester

Weeks	Clones	Bacteria Species
0	0A1:2	*Methanobrevibacter millerae*
	0B1:2	*Methanobrevibacter millerae*
	0C1:2	*Methanobrevibacter ruminantium*
	0A2:1	*Methanolinea tarda*
	0B2:1	*Aciduliprofundum boonei*
	0C2:1	*Methanobrevibacter arboriphilus*
	0D2:1	*Methanosaeta concilii*
4	4A1:2	*Methanobrevibacter millerae*
	4B1:2	*Methanobrevibacter millerae*
	4C1:2	*Methanobrevibacter millerae*
	4C2:1	*Methanosaeta concilii*
	4D2:1	*Methanobrevibacter millerae*

Methane (CH_4) can be produced either through aceticlastic conversion or acetate oxidation (Karakashev *et al.*, 2006). Aceticlastic conversion is only carried out by *Methanosarcinaceae* or *Methanosaetaceae*. The family *Methanosaetaceae* has a high affinity for acetate compared to *Methanosarcinaceae* (Tabatabaei *et al.*, 2009). This methanogens is one of the main species responsible for the conversion of acetate to CH_4. Species within this family use acetate as their main carbon source, which is metabolized into CH_4 and CO_2. Tabatabaei *et al.* (2009) also reported that the presence of *Methanosaeta* species helped in improving the granulation process which results in a more stable bioreactor performance.

Acetate oxidation is performed by acetate-oxidizing bacteria in syntrophic association with hydrogenotrophic methanoges such *Methanolinea* and *Methanobrevibacter* (Sakai *et al.*, 2012). Acetate in the digester which is converted into H_2 and CO_2 is utilized by hydrogenotrophic methanogens for CH_4 production (Yamamoto *et al*., 2014). Although acetate is not converted to CH_4, acetate is needed for the growth of hydrogenotrophic methanoges.

4.4 CONCLUSION

Methanogens population in co-mixture was reflected from 16S rRNA clone library in this study. Four types of methanogens: *Methanobrevibacter* sp., *Methanosaeta concilii*, *Methanolinea tarda* and *Aciduliprofundum boonei* were present in the co-mixture of anaerobic sludge with solid cud. With the knowledge of methanogens community in co-mixture, a better understanding in enhancing biogas production using anaerobic digester can be achieved in reduction of greenhouse gases emission.

ACKNOWLEDGEMENT

This project were funded by a research grant from UNIMAS (Top-Down grant: 01(TD01)/764/2010(01)).

Chapter 5

Biogas Production from Empty Fruit Bunch (EFB) via Co-mixture of Palm Oil Mill Effluent (POME) sludge and Ruminant Solid Cud

(This chapter will be submitted to Pertanika Journal of Science and Technology)

5.1 INTRODUCTION

The apparent instability and scarcity of fuel throughout the world has lead to the exploration of alternative sources of renewable energy such as liquid biofuel and biogas which are more sustainable and eco-friendly (Micky *et al*., 2014; Vincent *et al*., 2014; Vincent *et al*., 2011). These gases can be generated from various sources via anaerobic digestion, and are important for nutrient recycling and renewable energy production (Neves *et al*., 2006; Murto *et al*., 2004). Biogas consists of methane (CH_4), carbon dioxide (CO_2) and some trace of gases generated from degradation of biomass or organic matter under anaerobic condition (Umar *et al*., 2013) which involves four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Lam and Lee, 2011).

Anaerobic digestion is deemed the most suitable method in treating organic wastes such as wastewater sludge, industrial wastewater, food waste, animal manure and POME (Khalid *et al*., 2011; Sialve *et al*., 2009; Najafpour *et al*., 2006; Berndes *et al*., 2003). Of those organic wastes, POME generated from the sterilization and clarification of palm oil mill processing is of high interest as it contains high amount of organics (15,000-100,000 mg/l) (Hassan *et al.*, 2004) Furthermore, more than 50 million tonnes of POME is generated annually with increasing trend (Yacob *et al*., 2006). However, long retention periods and

low removal efficiencies of organic compounds (Park *et al.*, 2005) with low yield of biogas production during anaerobic digestions often make this process unattractive.

Among the approaches to improve digestion efficiency of organic matter is by employing co-digestion or co-culturing procedures using two or more starting inocula and substrates (Labatut *et al.*, 2011; Mata-Alvarez *et al*., 2000). This is supported by Alvarez *et al.* (2010) that documented higher biogas production during co-digestion of different substrates in the same anaerobic digestion vessels, as compared to single mixture. Goberna *et al.* (2010) also discovered that up to 337% more biogas was produced via the co-digestion of cattle excreta and oil mill wastes.

Although there are several studies that have documented on the production of biogas via co-digestion processes using different types of starter inocula, very limited literature is found on the co-digestion of EFB using co-mixture of POME and solid cud from ruminant stomach. Although diets such as forage, concentrates and mixture of forage and concentrate was the major factor in determining relative abundance of microbial in ruminant, *Prevotella*, *Butyrivibrio*, *Ruminococcus* as well as *Methanobrevibacter* are known as the core bacteria community in ruminants (Henderson *et al*., 2015; Leahy *et al*., 2010). POME has also been documented to consist of a variety of microorganisms such as *Escherichia coli*, *Desulfovibrio*, *Pseudomonas* sp., *Bacillus* sp., *Methanosaeta concilli* (Ohimain *et al*., 2012; Tabatabaei *et al*., 2009). As not much is known on the interactions between these methanogen rich waste and how efficient their mixture is to produce biogas, investigation of biogas production from co-digestion of POME sludge with solid cud from ruminant stomach in different mixing ratio on EFB as compared to mono-digestion of

POME and solid cud by using anaerobic digester under thermophilic condition was carried out.

5.2 MATERIAL AND METHODS

5.2.1 Samples collection

POME sludge was collected from the anaerobic pond of Bau Palm Oil Mill (BAPOM) situated at Kuching, Sarawak. The POME sludge was kept in a tightly closed container. The solid cud from the first compartment of a ruminant stomach was obtained from a slaughter house located at Ladang Lapan, Kuching. Both samples were transported immediately to Microbiology Laboratory of the Faculty Resource Science and Technology, Universiti Malaysia Sarawak after collection and preserved at 4 °C in order to avoid biodegradation due to microbial activities (Tang *et al*., 2008).

EFB was also collected from BAPOM and was dried at 80 ˚C for 2 days prior to shredding. The dried EFB was then shredded using Cutting Mills SM100 (Retsch, Germany) cutting mill with 1.0 mm sieve size and stored in a tightly closed container at room temperature.

5.2.2 Experimental digester and design

The experimental design as shown in Figure 5.1 was conducted by using lab-scale anaerobic digesters from 2 L Schott bottles with initial starter inoculum of 400 ml. The first two digester (A1 and A2), were inoculated with POME sludge and solid cud from ruminant stomach respectively. The next three digesters B1, B2 and B3 were inoculated with different ratio of POME sludge and solid cud (Table 5.1) and incubated at 50 ˚C for 4 weeks. Each treatment was performed in duplicate. All of the treatments were fed three times a week with 10% (w/v) EFB in 100 ml of DVS media. DVS medium (Savant *et al*.,

2002) consists of (l^{-1} distilled water): 6.0 g NaCl, 0.8 g $CaCl_2.2H_2O$, 1.0 g $MgCl_2.6H_2O$, 5.0 g Peptone, 5.0 g Tryptone, 5.0 g CH_3CO_2K, 3.0 g KH_2PO_4, 3.0 g K_2HPO_4, 5.0 g, NH_4NO_3 and 5.0 g yeast. The pH of all reactor mixtures were maintained at 7.0±0.3 using 1.0 N of HCl or 1.0 N of NaOH (Lutoslawki *et al*., 2011). The amount of biogas produced by each digester was recorded daily using the water displacement method (Umar *et al*., 2013).

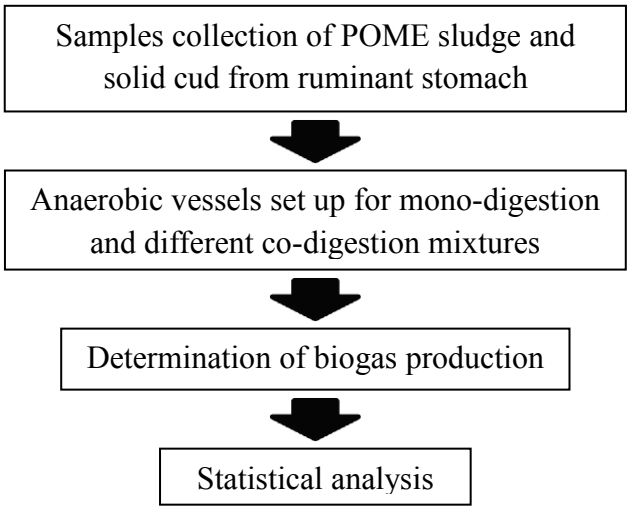

Figure 5.1: Flow chart of the experimental design.

Table 5.1: Different ratio of POME and solid cud in co-digestion mixture

Digesters	Ratio	Co-digestion mixture
B1	1:1	POME: Solid cud
B2	1:2	POME: Solid cud
B3	2:1	POME: Solid cud

5.2.3 Statistical analysis

The experiment data was statistically evaluated using analysis of variance (ANOVA) to determine the significant differences between mono-digestion and co-digestion mixture. In all cases, the significant level of $p< 0.01$ was used.

5.3 RESULTS AND DISCUSSION

The initial pH of the mono-digestion using POME sludge was recorded at pH 8.23, while the pH value for solid cud was 7.15. Throughout the 4 weeks of incubation period, the pH was adjusted to its optimum value of 7.00±0.30. However, the pH reading for all co-mixtures; POME: solid cud (1:1), (1:2) and (2:1) were within optimum range of 6.67, 7.23 and 7.43, respectively, throughout the incubation period without adjustment.

The pH of an anaerobic digestion is an important indicator in monitoring the performance of an anaerobic digester and should be maintained throughout the anaerobic digestion process. The optimal range of pH to attain maximal biogas production in anaerobic digestion is 6.50-7.50 (Liu *et al*., 2008). Kangle *et al*. (2012) mentioned that initially pH will decrease with the production of volatile fatty acid. However, methanogenic bacteria would initially consume the volatile fatty acid, thus increasing the pH value and stabilizing the digester performance.

Application of solid cud as co-mixture as well as EFB as substrate during feeding can be used as an alternative in maintaining the stability of digester. Wastes which are high in organic content provide buffering capacity and wide range of nutrients (Esposito *et al*., 2012). However, those wastes have low carbon/nitrogen (C/N) ratio (Baharuddin *et al*., 2011; Cuetos *et al*., 2010; Edstro¨m *et al*., 2003). Usages of plant materials with high carbon content as co-digester counter balance the C/N ratio of feedstock, thereby decreasing concentration of nitrogen and risk of ammonia inhibition (Cuetos *et al*., 2008; Lehtomaki *et al*., 2007).

The daily biogas production during the 4-weeks incubation period of the mono-digestion of POME sludge and solid cud from ruminant stomach as well as co-digestion of POME sludge and solid cud in different ratio on EFB are shown in Figure 5.2. Mono-digestion of POME sludge and solid cud produced biogas on day 1 with 251.0 cm^3/d and 150.5 cm^3/d respectively. Co-digestion from the mixing ratio of POME: solid cud (1:1), (1:2) and (2:1) were measured, and their peak volumes were 163 cm^3/d, 350 cm^3/d and 291 cm^3/d on day 8 respectively (Figure 5.2). Although both of the digestion of single substrate (POME sludge and solid cud) produced biogas earlier than co-digestion, the biogas production from all the treatments started to decrease after day 16. These results indicated that biogas production from co-digestion of POME and solid cud on EFB is slower compared to the mono-digestion set. This probably occurred due to environmental changes for the microorganism consortium which was obtained from original environment (POME sludge from anaerobic pond and solid cud from first compartment of ruminant stomach) and introduced into new environment (2 L anaerobic digester). The different bacteria in co-digestion mixture required some time to acclimatize with each other before consuming the organic matter for their growth (Alwari *et al*., 2011).

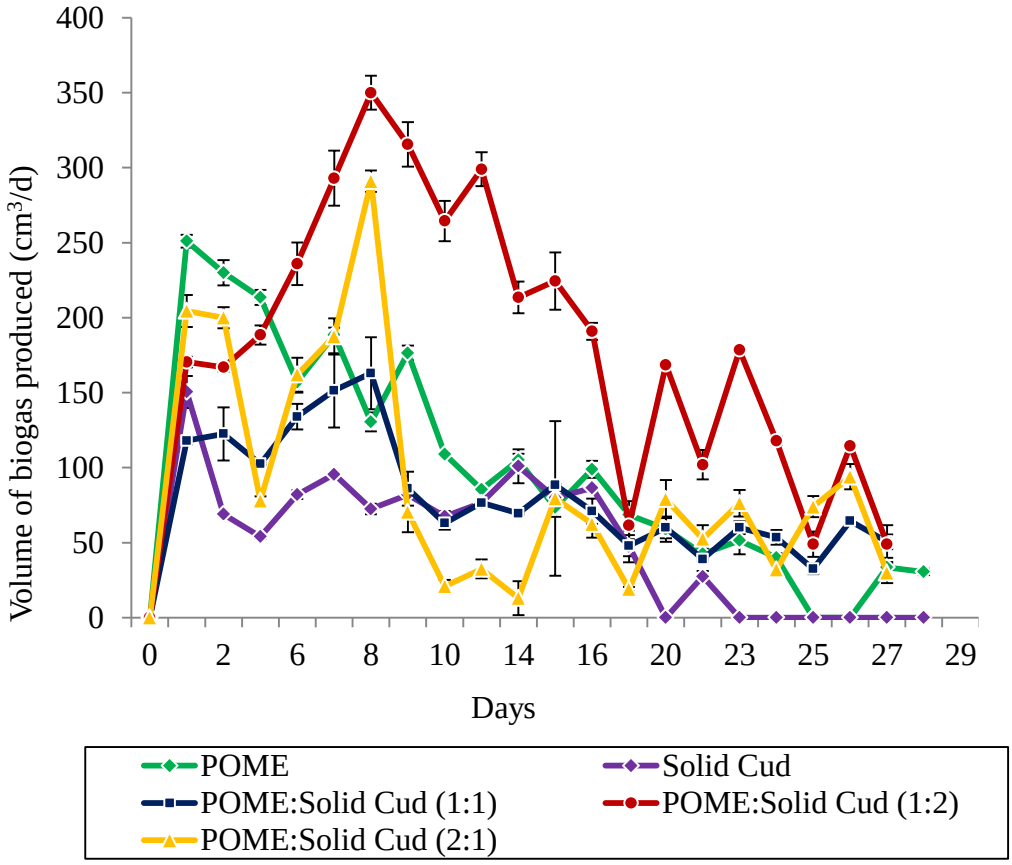

Figure 5.2: Daily biogas production of mono-digestion of POME sludge and solid cud as well as co-digestion of POME sludge with solid cud in different ratios (1:1), (1:2) and (2:1) on EFB.

The cumulative biogas production by the co-digestion of POME sludge and solid cud at different ratio on EFB is shown in Figure 5.3. POME: solid cud (1:2) showed the highest biogas production in all treatments which was 42.87% and 70.91% more compared to single digestion of POME sludge (2,144.5 cm^3) and solid cud (1,092 cm^3). POME: solid cud (1:2) also demonstrated 55.94% and 50.48% higher production than POME: solid cud (1:1) (1,654 cm^3) and (2:1) (1,858.5 cm^3) respectively. Although POME: solid cud (1:1) and (2:1) produced less biogas compared to mono-digestion of POME sludge, both co-digestion mixtures generated 33.97% and 41.25% more than single treatment of solid cud. These results showed that addition of solid cud into POME sludge treatment improved biodegradability and biogas production compared to single substrate treatment. Similar

results were documented by other researchers as well (Lee *et al*., 2013; Lehtomaki *et al*., 2007; Mata-Alvarez *et al*., 2000). Gelegenis *et al*. (2007) reported that higher biodegradability of carbohydrates (mainly constituents of whey) in co-digestion of whey with manure led to higher biogas production. Similarly Wu *et al*. (2010) recorded an increase in the volume of biogas produced when swine manure was co-digested with crop residue, wheat straw and oat.

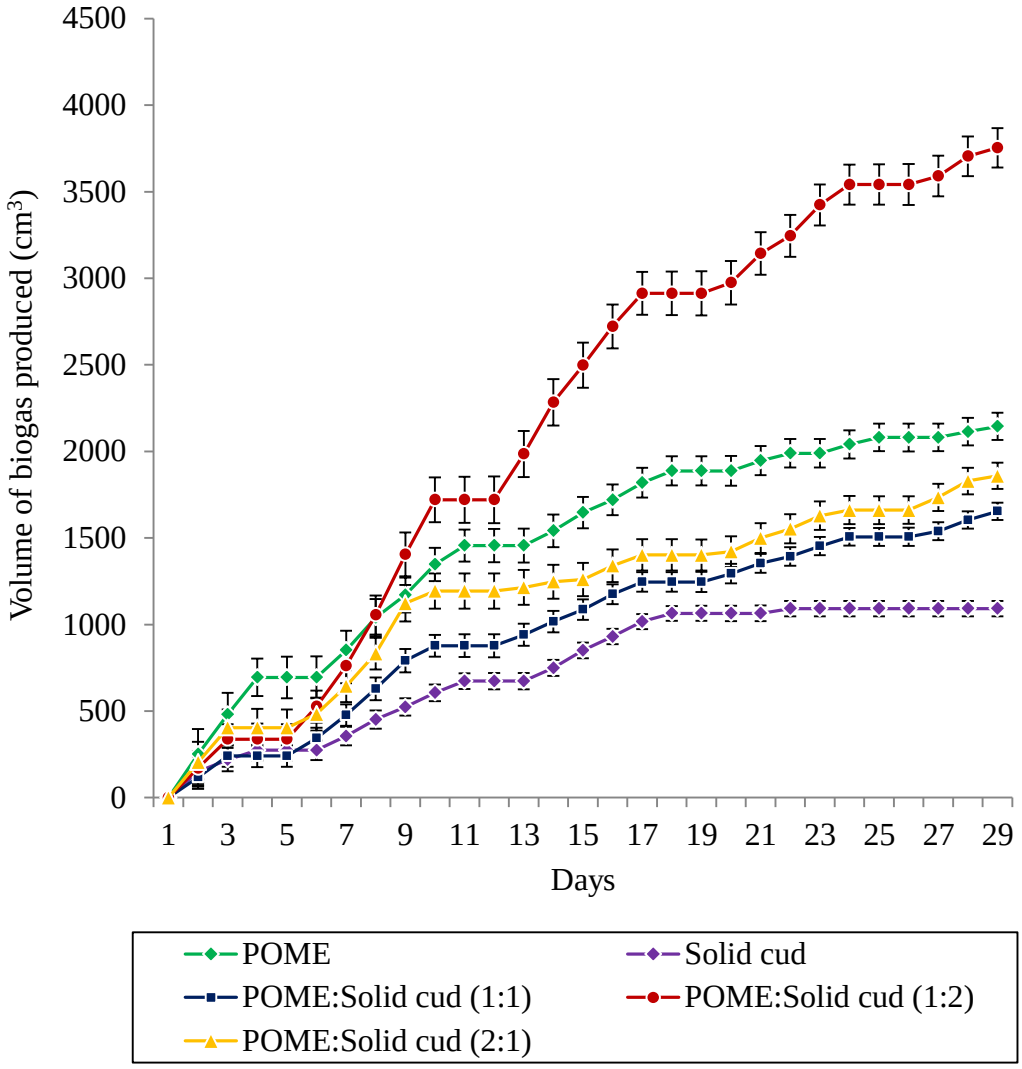

Figure 5.3: Cumulative biogas production of mono-digestion of POME sludge and solid cud as well as co-digestion of POME sludge with solid cud in different ratios (1:1), (1:2) and (2:1) on EFB.

The ANOVA results showed that total biogas production of co-digestion of POME: solid cud on EFB during 4 weeks incubation is significantly higher ($p<0.01$) than mono-digestion of POME sludge or solid cud (Figure 5.4). These results indicated that co-digestion of POME sludge and solid cud on EFB improve biogas yield.

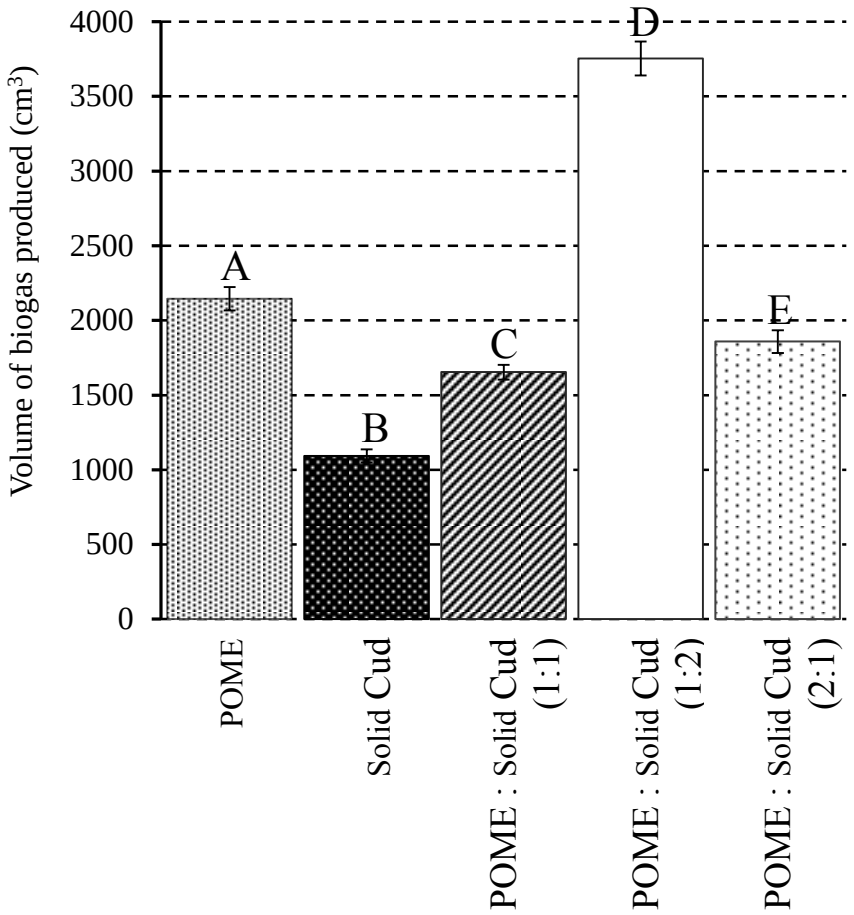

Figure 5.4: Total biogas production of mono-digestion of POME sludge and solid cud as well as co-digestion of POME sludge with solid cud in different mixing ratios (1:1), (1:2) and (2:1). The ANOVA test was conducted to determine the differences between the inoculum with significant difference of $p<0.01$. Values with the same letter indicate no significant difference.

This study has shown that the use of solid cud and EFB improves the biogas production of POME sludge anaerobic digestion. Plant biomass composed of cellulose (40-50%), hemicellulose (20-40%), lignin, as well as protein, pectin, soluble non-structural materials

and inorganic materials (Chandra *et al*., 2012). Although cellulose and hemicellulose are difficult to degrade, hydrolysis of both structures occurs rapidly due to the breakdown of the structures by the ruminants' ability to shred the solid cud into smaller pieces. In addition, presence of macronutrient and trace elements in plants such as phosphorus and iron respectively enhance the microbial growth and act as buffering agent in the digester (Demirel and Scherer 2011). Complementary characteristics of co-substrate such as substrate with low nitrogen content, increase production of biogas. The carbon/nitrogen ratio for solid cud was reported to be between 15 and 22 which were within the optimum ratio for anaerobic digestion (Lehtomaki *et al*., 2008). These also reduce problems linked with the accumulation of intermediate volatile compounds and high ammonia concentration (Castillo *et al*., 2006).

In addition, presence of hydrogenotrophic methanogen such as *Methanobrevibacter* sp. in solid cud from ruminant stomach enhances the production of methane gas. High content of cellulose and hemicellulose in solid cud favours the production of substrates that enhance sympathetic conditions for hydrogenotrophic methanogens. Henderson *et al.* (2015), reported 77% of archaea presences in solid cud were hydrogenotrophic methanogens. In addition, methane produced through aceticlastic reaction was rare due to the slow growth rate of aceticlastic methanogens such as *Methanosarcina* spp. and *Methanosaeta* spp.

Usage of more than a type of wastes in anaerobic digestion improves the balance of nutrients, synergistic effect of microorganisms which increases the digestion rate as well as biogas production (Lo *et al*., 2010; Jingura and Matengaifa, 2009; Yen and Brune, 2007; Hartmann and Ahring, 2005). Several promising results have also been obtained by co-digesting cattle manure with agriculture waste (Cavinato *et al.*, 2010; Lehtomaki *et al*.,

2007), manure with cheese whey (Kacprzac *et al.*, 2010), municipal solid wastes with wastes from sewage treatment plants (Martin-Gonzalez *et al*., 2010), municipal solid wastes with slaughter house wastes (Cuetos *et al*., 2008), municipal solid waste with agriculture waste (Samani *et al*., 2008), decanter cake from oil palm with frozen food wastewater and rubber block wastewater (Kaosol and Sohgrathok, 2012), and POME with refined glycerine wash water (Sulaiman *et al*., 2009) and ruminant fluid (Alrawi *et al*., 2011).

5.4 CONCLUSION

The present work has shown that co-digestion of POME sludge with solid cud on PO-EFB shows promising result compared to mono-digestion of POME sludge and solid cud at 50 ˚C. The highest cumulative biogas production on PO-EFB was produced from co-digestion of POME with solid cud (1:2) with a total of 3,754 cm^3. Co-mixture of POME: solid cud (1:2) improved the biogas yield by 42.87% and 70.91% compared with single digestion of POME sludge and solid cud respectively.

ACKNOWLEDGEMENT

This project was funded by a research grant from UNIMAS (Top-Down grant: 01(TD01)/764/2010(01)).

Chapter 6

CONCLUSION AND RECOMMENDATIONS

Anaerobic digestion has great potential in treating palm oil mill wastes and generating biogas. However, the diversity of the microbial consortia in anaerobic digestion is often neglected due to the complicated microbial ecology. Therefore, the current study was performed to conduct culture-dependent and culture-independent molecular technologies to understand the reactions occurring in biogas digester. The results showed that, *Bacillus coagulans, Bacillus thermoamylovorans* and *Thermoanaerobacter* sp. from POME were successfully isolated and identified. These bacterial groups play important roles in the hydrolysis and production of volatile fatty acid during anaerobic digestion process. The abilities of these bacteria to digest a variety of substrates such as xylan and cellulose coupled with the methanogenesis capacities of other bacteria further improved the biogas yield. This finding suggested the application of agriculture wastes which is high in carbon content such as solid cud of ruminant stomach could improve hydrolysis stage which is a limiting factor during anaerobic digestion. Besides, usage of solid cud from ruminant stomach as co-mixture is of high interest as ruminants represent the greatest evolutionary of cellulosic biomass utilization.

Application of different ratio of solid cud from ruminant stomach in anaerobic digestion showed changes in microbial dynamic. *Methanobrevibacter* spp. which were identified in co-mixture of POME sludge: solid cud (1:2) were present from Week 0 until Week 4. However, the types of methanogens species present in co-mixture POME sludge: solid cud (2:1) were reduced to *Methanosaeta concilii* and *Methanobrevibacter millerae* after 4 weeks of incubation. The present results showed that different ratio of POME sludge: solid

cud changed the concentration of ammonia and volatile acid. This effect caused different methanogenesis pathways were being carried out in different ratio of co-digestion. This study also revealed no methanogen was detected after 4 weeks of incubation, indicating that the addition of solid cud from ruminant stomach managed to reduce the incubation period of anaerobic digestion of POME from 12 weeks to 4 weeks.

In addition, application of solid cud of ruminant stomach and EFB into anaerobic digester of POME also improved the production of biogas. Biogas production of POME: solid cud (1:2) was 42.87% and 70.91% more compared to mono-digestion of POME and solid cud respectively. POME: solid cud (1:2) also showed 55.94% and 50.48% higher yield than POME: solid cud (1:1) and (2:1) respectively. This result confirmed that anaerobic co-digestion improve anaerobic digestion by reducing digestion time and increasing biogas yield. This study also proved that co-digestion of mixtures stabilizes the performance of the anaerobic digestion. Usage of solid cud from ruminant stomach and EFB which are low in nitrogen content stabilizes the pH of the digester without further adjustment. Chemical compositions of solid cud also improve the microbial growth and act as buffering agent in the digester. The applications of co-mixtures further improve the C/N ratio and decrease the concentration of nitrogen, thus increase the production of biogas. In addition, presence of hydrogenotrophic methanogen such as *Methanobrevibacter* sp. in solid cud from ruminant stomach enhances the production of methane gas as methane produced through aceticlastic reaction was rare due to the slow growth rate of aceticlastic methanogens.

The numerous benefits of co-digestion would simplify the economical requirements for the application of the wastes (POME, solid cud from ruminant stomach and EFB) into biogas. Application of co-digestion not only reduces the amount of wastes produced in the palm oil

industry but also increase the production of biogas which could boost up the national renewable energy sector.

This work has provided evidences on the presence of bacteria in POME, methanogenic population in different ratio of anaerobic co-digestion of POME: solid cud, performance of single substrate (POME and solid cud) digestion and co-digestion of different ratio of POME: solid cud. Further study on the detection of amount of methane produced from the biogas is recommended. The information gathered can be used to further improve the performance of anaerobic treatment of POME. Study on the microbial communities change during the anaerobic digestion by using more advanced molecular techniques such as polymerase chain reaction-denaturating gradient gel electrophoresis (PCR-DGGE) and fluorescence in situ hybridization (FISH) is also recommended in order to optimize the anaerobic digestion. In addition, study on different diets on microbial community in ruminants is recommended as feed compositions influence the microbial community structure in ruminants.

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APPENDIX

>IsolateC [organism=Uncultured Thermoanaerobacteriaceae bacterium] UMAS SW1,16S ribosomal RNA, partial sequence

AGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGAA
GTGAGTACTACGGTACGAACTTAGCGGCGGACGGGTGAGTAACGCGTGGACAATCTACCCT
GTAGACTGGGATAACACCTCGAAAGGGGTGCTAATACCGGATAATGTCGAGAAGCGGCATC
GCTTTTCGAAGAAAGGAGAGAATCCGCTATAGGAGGAGTCCGCGTCCCATTAGCTAGTTGG
TGAGGGTAACGGCCCACCAAGGCGACGATGGGTAGCCGGCCTGAGAGGGTGAACGGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGTGCAATG
GGGGAAACCCTGACACAGCAACGCCGCGTGAGCGAAGAAGGCCTTCGGGTTGTAAAGCTCA
ATAGTATGGGAAGATAATGACGGTACCATACGAAAGCCCCGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCACGTAGGC
GGCTATATAAGTCAGGTGTGAAAAACCTGGGCTTAACCGAGGGTATGCATCTGAAACTATA
TAGCTTGAGTCAAGGAGAGGAGAGCGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATC
AGGAAGAATACCAGTGGCGAAAGCGGCTCTCTGGACTTGAACTGACGCTGAGGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGG
TGTGGGTTAGTATAATCCGTGCCGGAGTTAACGCAATAAGTATCCCGCCTGGGGAGTACGG
CCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACAGAATCGAGTAGAAAT
ACTTGAGTGCCTCGTAAGAGGAGCTGTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC

>IsolateE [organism=Uncultured Thermoanaerobacteriaceae bacterium] UMAS SW3, 16S ribosamal RNA, partial sequence

ACGAACGCTGGCGGCGTGTCCTAACACATGCAAGTCGAGCGAAGTGAGTACTACGGTACGA
ACTTAGCGGCGGACGGGTGAGTAACGCGTGGACAATCTACCCTGTAGACTGGGATAACACC
TCGAAAGGGGTGCTAATACCGGATAATGTCGAGAAGCGGCATCGCTTTTCGAAGAAAGGAG
AGAATCCGCTATAGGAGGAGTCCGCGTCCCATTAGCTAGTTGGTGAGGGTAACGGCCCACC
AAGGCGACGATGGGTAGCCGGCCTGAGAGGGTGAACGGCCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGTGCAATGGGGGAAACCCTGACACAG
CAACGCCGCGTGAGCGAAGAAGGCCTTCGGGTTGTAAAGCTCAATAGTATGGGAAGATAAT
GACGGTACCATACGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGG
GCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCACGTAGGCGGCTATATAAGTCAGGTG
TGAAAAACCTGGGCTTAACCGAGGGTATGCATCTGAAACTATATAGCTTGAGTCAAGGAGA
GGAGAGCGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAATACCAGTGGC
GAAAGCGGCTCTCTGGACTTGAACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGGTGTGGGTTAGTATAATCC
GTGCCGGAGTTAACGCAATAAGTATCCCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACCCGA
AGAACCTTACCAGGGCTTGACATCCACAGAATCGAGTAGAAATACTTGAGTGCCTCGTAAG
AAGAACTGTGAAACAAGGTGGTGCATGGTTGTCGCCAGCTCCTGTCCTGAAGATGTTGGGT
TAAGTCCCGCAACGAGGCGCAACCC

>IsolateH [organism=Uncultured Thermoanaerobacteriaceae bacterium] UMAS SW4, 16S ribosomal RNA, partial sequence

AGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGAA
GTGAGTACTACGGTACGAACTTAGCGGCGGACGGGTGAGTAACGCGTGGACAATCTACCCT
GTAGACTGGGATAACACCTCGAAAGGGGTGCTAATACCGGATAATGTCGAGAAGCGGCATC

GCTTTTCGAAGAAAGGAGAGAATCCGCTATAGGAGGAGTCCGCGTCCCATTAGCTAGTTGG
TGAGGGTAACGGCCCACCAAGGCGACGATGGGTAGCCGGCCTGAGAGGGTGAACGGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGTGCAATG
GGGGAAACCCTGACACAGCAACGCCGCGTGAGCGAAGAAGGCCTTCGGGTTGTAAAGCTCA
ATAGTATGGGAAGATAATGACGGTACCATACGAAAGCCCCGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCACGTAGGC
GGCTATATAAGTCAGGTGTGAAAAACCTGGGCTTAACCGAGGGTATGCATCTGAAACTATA
TAGCTTGAGTCAAGGAGAGGAGAGCGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATC
AGGAAGAATACCAGTGGCGAAAGCGGCTCTCTGGACTTGAACTGACGCTGAGGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGG
TGTGGGTGAGGAATCATCCGTGCCGGAGTTAACGCAATAAGTATCCCGCCTGGGGAGTACG
GCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACAGAATCGAGTAGAAA
TACTTGAGTGCCTCGTAAGAAGAGCTGTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGT

>IsolateA [organism=Bacillus thermoamylovorans] UMAS SW5, 16S ribosomal RNA, partial sequence
TTGCTTTTTTTGTTGGTTAAGCGGCGGGACGGGTGGAGTAACACCGTGGGGTAACCCTGCC
CTGTAAGACCGGGGATAACTCCCGGGAAACCGGGTGCTAATACCGGGATAGATTATCTTTC
CGCCCTGGAGAGATAAGGAAAAGATGGCTATTTGCCATCACTTTACAGATGGGCCCCGCGG
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTC
GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCGGAGGAAATGCCGGTACCTTGAC
GGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGT
GAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAG
GAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAGGCGGCTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCC
TTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTC
GGGGACAGGATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT

>IsolateD [organism=Bacillus coagulans strain] UMAS SW6, 16S ribosomal RNA, partial sequence
CTGTCACTCTGTCCCCCGAAGGGGAAGGCCCCTGTCTCCAGGGAGGTCAGAGGATGTCAAG
ACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC
CCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATG
CGTTAGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTA
CAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTA
CACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGCCTCCCAGTTTCCAATGACCGCTTGC
GGTTGAGCCGCAAGATTTCACATCAGACTTAAGAAGCCGCCTGCGCGCGCTTTACGCCCAA
TAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGT
GGCTTTCTGGCCGGGTACCGTCAAGGCGCCGCCCTGTTCGAACGGCACTTGTTCTTCCCCG
GCAACAGAGTTTTACGACCCGAAGGCCTTCTTCACTCACGCGGCGTTGCTCCGTCAGACTT
TCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGT
CCCAATGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGGTGAGCCGTTA

CCCCCGCCAACTAAGCTAATGCGCCGCGGGGCCCATCTGTAAGTGGCAGCCGAAGCCGCCT
TTCCTTTTTCCTCCATGCGGAGAAAAAAACTATCCGGTATTAGCCCCGGTTTCCCGGCGTT
ATCCCGGTCTTACAGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTAACCTTTTAA
AAGCAAGC

>0A1:2 [organism=Uncultured Methanobrevibacter sp.] [clone=P01-A] UMAS SW7,16S ribosomal RNA, partial sequence
GGTGTTGCAAAGGGACCAGGACGTTTCCCCGGGCAATAGTGATACGCGATTACTACGCATT
CCAGCTTCATGAGAACGAGTTACAGTCCTCAATCCGAACTACGACTAAGTTTAGAGGATTA
CCTCCACCTTTCGGTGTCGGAACCCATTGTCTCAGCCATTGTAGCCCGCGTGTTGCCCAGA
GGATTCGGGGCATACGGACCTACCGTCGTCCACTCCTTCCTCCTATTTATCATAGGCGGTC
CCCTTAGTGTGCCCATCGTCCAAAAAAGGACATGCTGGTAACTAAGGGCGTGGGTCTCGCT
CGTTGCCTGACTTAACAGGACGCCTCACGGTACGAGCTGACGGCGGCCATGCACCTCCTCT
CAGCTAGTCAAGCAAAGTCATCAACCTGGCTATCATACAGCTGTCGCCTCTGGTGAGATGT
CCGGCGTTGAATCCAATTAAACCGCAGGCTCCACGCGTTGTGGTGCTCCCCCGCCAATTCC
TTTAAGTTTCAGTCTTGCGACCGTACTTCCCAGGCGGCGGACTTAACAGCTTCCCTTCGGC
ACTGGAGCAGCTCAAAGCCACCCCAACACCAAGTCCGCATCGTTTACAGTTAGGACTACCC
GGGTATCTAATCCGGTTCGCGCCCCTAACTTTCGTCCCTCACCGTCAGAACCGTTCCAGTT
AGACGCCTTCGCAACAGGCGGTCCTCCCAGGATTACAGAATTTCACCTCTACCCTGGGAGT
ACCTCTAACCTCTCCCGGTCTCAAGTCTAATAGTATCTCCAGCAATTCCCACAGTTAAGCT
GCAGGATTTCACCAGAGACTTATTAAACCGGCTACGGACGCTTTAGGCCCAATAAAAATTG
CTACCACTAGAGCTGCCGGTGTTACCGCGGCGGCTGGCACCGGTCTTGCCCAGCTCTTATT
CCAAAAGCTCTTTACACTAATGAAAAGCCATCCCGTTAAGAATGGCACTTGGGATCCCCCC
GTCGCGATTTCTCACATTGCGGAGGTTTCGCGCCTGCTGCGCCCCGTAGGGCCTGGAACCT
TGTCTCAGGTTCCATCTCCGGGCTCTTGCTCTCACAACCCGTACCGATCAACGGCTTGGTA
AGCCATTACCTAACCAACTACCTAATCGGCCGCAGACCCATCCTTAGGCGAAAAAACATTT
AAACAAAGAACCATTACAGGAAAAATTGCCTATCCAGTATTATCCCCAGTTCCCAGGGTTC
CCCGTCCAAGGGG

>0B1:2 [organism=Uncultured Methanobrevibacter millerae] [clone=P01-B] UMAS SW8,16S ribosomal RNA, partial sequence
TGCCCAAGAAACACCGGGGGATACCTACCCTTAGGACCGGGATAACCCTGGGAAACTGGGG
ATAATACTGGATAGGCAATTTTTCCTGCAATGGTTCTTTGTTTAAATGTTTTTTCGCCTAA
GGATGGGTCTGCGGCCGATTAGGTAGTTGGTTAGGTAATGGCTTACCAAGCCGTTGATCGG
TACGGGTTGTGAGAGCAAGAGCCCGGAGATGGAACCTGAGACAAGGTTCCAGGCCCTACGG
GGTGCAGCAGGCGCGAAACCTCCGCAATGTGAGAAATCGCGACGGGGGGGATCCCAAGTGCC
ATTCTTAACGGGATGGCTTTTCATTAGTGTAAAGAGCTTTTGGAATAAGAGCTGGGCAAGA
CCGGTGCCAGCCGCCGCGGTAACACCGGCAGCTCTAGTGGTAGCAATTTTTATTGGGCCTA
AAGCGTCCGTAGCCGGTTTAATAAGTCTCTGGTGAAATCCTGCAGCTTAACTGTGGGAATT
GCTGGAGATACTATTAGACTTGAGACCGGGAGAGGTTAGAGGTACTCCCAGGGTAGAGGTG
AAATTCTGTAATCCTGGGAGGACCGCCTGTTGCGAAGGCGTCTAACTGGAACGGTTCTGAC
GGTGAGGGACGAAAGTTAGGGGCGCGAACCGGATTAGATACCCGGGTAGTCCTAACTGTAA
ACGATGCGGACTTGGTGTTGGGGTGGCTTTGAGCTGCTCCAGTGCCGAAGGGAAGCTGTTA
AGTCCGCCGCCTGGGAAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCGGGGGAGCA
CCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCACCAGAGGCGA
CAGCTGTATGATAGCCAGGTTGATGACTTTGCTTGACTAGCTGAGAGGAGGTGCATGGCCG
CCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTT
AGTTACCAGCATGTCCTTTTTTGGATGATGGGCACACTAAGGGGACCGCCTATGATAAATA
GGAGGAAGGAGTGGACGACGGTAGGTCCGTATGCCCCGAATCCTCTGGGCAACACGCGGGC
TACAATGGCTGAAACAATGGGTTCCGACACCGAAAGGTGGAGGTAATCCTCTAAACTTAGT

CGTAGTTCGGATTGAGGACTGTAACTCGTTCTCATGAAGCTGGAATGCGTAGTAATCGCGT
ATCACATTCGGGGTCCCCC

>0C1:2 [organism=Uncultured Methanobrevibacter olleyae] [clone=P01-C] UMAS SW9,16S ribosomal RNA, partial sequence
GGGCCCCCCCCCGCGGCGGGAGCTCCCCGGGGCGCCGGGGACCCACNNGGTTATGTCCAGG
AAAACAGGGGTGATACTTCCCTTAGGACCGGGATAACCCTGGGAAACTGGGGCTAATACTG
GATAGATGATTTTTCCTGGAATGGTTTTTTGTTTAAATGTTTTTTCGCCTAAGGATGGGTC
TGCGGCAGATTAGGTAGTTGGTTAGGTAATGGCTTACCAAGCCTATGATCTGTACGGGTTG
TGAGAGCAAGAGCCCGGAGATGGAACCTGAGACAAGGTTCCAGGCCCTACGGGGCGCAGCA
GGCGCGAAACCTCCGCAATGTGAGAAATCGCGACGGGGGGATCCCAAGTGCCATTCTTAAC
GGGATGGCTTTTCTTAAGTGTAAAAAGCTTTTGGAATAAGAGCTGGGCAAGACCGGTGCCA
GCCGCCGCGGTAACACCGGCAGCTCTAGTGGTAGCTGTTTTTATTGGGCCTAAAGCGTTCG
TAGCCGGTTTGATAAGTCACTGGTGAAATCCTGTAGCTTAACTGTGGGAATTGCTGGTGAT
ACTGTTGAACTTGAGGTCGGGAGAGGTTAGCGGTACTCCCAGGGTAGAGGTGAAATTCTGT
AATCCTGGGAGGACCACCTGTGGCGAAGGCGGCTAACTGGAACGAACCTGACGGTGAGGGA
CGAAAGCTAGGGGCGCGAACCGGATTAGATACCCGGGTAGTCCTAGCCGTAAACGATGCGG
ACTTGGTGTTGGGATGGCTTTGAGCCGCTCCGGTGCCGAAGGGAAGCTGTTAAGTCCGCCG
CCTGGGAAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCGGGGGAGCACCACAACGC
GTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCACCAGGAGCGACAGCTGTAT
GATTACCAGGCTGATGACCTTGTTTGACTAGCTGAGAGGAGGTGCATGGCCGCCGTCAGCT
CGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAG
CATGTCCTTTTTTGGATGATGGGCACACTAAGGGGACCGCCTATGATAAATAGGAGGAAGG
AGTGGACGACGGTAGGTCCGTATGCCCCGAATCCTCTGGGCAACACGCGGGCTACAATGGC
TGAAACAATGGGTTCCGACACCGAAAGGTGGAGGTAATCCTCTAAACTTAGTCGTAGTTCG
GATTGAGGACTGTAACTCGTTCTCATGAAGCTGGAATGCGTAGTAATCGCGTATCACTATT
GGCCGGTGAAACGTCCTGCCCCCTTTTGCACACCCCGAA

>4A1:2 [organism=Uncultured Methanobrevibacter millerae] [clone=P41-A] UMAS SW10,16S ribosomal RNA, partial sequence
TGCCCAGAACACACGGGGGATACCTACCCTTAGGACCGGGATAACCCTGGGAAACTGGGGA
TAATACTGGATAGGCAATTTTTCCTGCAATGGTTCTTTGTTTAAATGTTTTTTCGCCTAAG
GATGGGTCTGCGGCCGATTAGGTAGTTGGTTAGGTAATGGCTTACCAAGCCGTTGATCGGT
ACGGGTTGTGAGAGCAAGAGCCCGGAGATGGAACCTGAGACAAGGTTCCAGGCCCTACGGG
GTGCAGCAGGCGCGAAACCTCCGCAATGTGAGAAATCGCGACGGGGGGATCCCAAGTGCCA
TTCTTAACGGGATGGCTTTTCATTAGTGTAAAAAGCTTTTGGAATAAGAGCTGGGCAAGAC
CGGTGCCAGCCGCCGCGGTAACACCGGCAGCTCTAGTGGTAGCAATTTTTATTGGGCCTAA
AGCGTCCGTAGCCGGTTTAATAAGTCTCTGGTGAAATCCTGTAGCTTAACTGTGGGAATTG
CTGGAGATACTATTAGACTTGAGACCGGGAGAGGTTAGAGGTACTCCCAGGGTAGAGGTGA
AATTCTGTAATCCTGGGAGGACCGCCTGTTGCGAAGGCGCCTAACTGGAACGAACCTGACG
GTGAGGGACGAAAGCTAGGGGCGCGAACCGGATTAGATACCCGGGTAGTCCTAGCCGTAAA
CGATGCGGACTTGGTGTTGGGGTGGCTTTGAGCTGCTCCAGTGCCGAAGGGAAGCTGTTAA
GTCCGCCGCCTGGGAAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCGGGGGAGCAC
CACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCACCAGAGGCGAC
AGCTGTATGATAGCCAGGTTGATGACTTTGCTTGACTAGCTGAGAGGAGGTGCATGGCCGC
CGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTA
GTTACCAGCATGTCCTTTTTTGGATGATGGGCACACTAAGGGGACCGCCTATGATAAATAG
GAGGAAGGAGTGGACGACGGTAGGTCCGTATGCCCCGAATCCTCTGGGCAACACGCGGGCT
ACAATGGCTGAAACAATGGGTTCCGACACCGAAAGGTGGAGGTAATCCTCTAAACTTAGTC
GTAGTTCGGATTGAGGACTGCAACTCGTTCTCATGAAGCTGGAATGCGTAGTAATCGCGGA
TCACTATTGCCCGGGAATAATCCCTGCCCTCTTGCACCAC

>4B1:2 [organism=Uncultured Methanobrevibacter millarae] [clone=P41-B] UMAS SW11,16S ribosomal RNA, partial sequence

TTTTCCCCGGCCAATAGGTTACGCGATTACTACGCATTCCAGCTTCATGAGAACGAGTTAC
AGTCCTCAATCCGAACTACGACTAAGTTTAGAGGATTACCTCCACCTTTCGGTGTCGGAAC
CCATTGTCTCAGCCATTGTAGCCCGCGTGTTGCCCAGAGGATTCGGGGCATACGGACCTAC
CGTCGTCCACTCCTTCCTCCTATTTATCATAGGCGGTCCCCTTGGTGTGCCCATCATCCAA
AAAAGGACATGCTGGTAACTAAGGGCGTGGGTCTCGCTCGTTGCCTGACTTAACAGGACGC
CTCACGGTACGAGCTGACGGCGGCCATGCACCTCCTCTCAGCTAGTCAAGCAAAGTCATCA
ACCTGGCTATCATACAGCTGTCGCCTCTGGTGAGATGTCCGGCGTTGAATCCAATTAAACC
GCAGGCTCCACGCGTTGTGGTGCTCCCCCGCCAATTCCTTTAAGTTTCAGTCTTGCGACCG
TACTTCCCAGGCGGCGGACTTAACAGCTTCCCTTCGGCACTGGAGCAGCTCAAAGCCACCC
CAACACCAAGTCCGCATCGTTTACAGTTAGGACTACCCGGGTATCTAATCCGGTTCGCGCC
CCTAACTTTCGTCCCTCACCGTCAGAACCGTTCCAGTTAGACGCCTTCGCAACAGGCGGTC
CTCCCAGGATTACAGAATTTCACCTCTACCCTGGGAGTACCTCTAACCTCTCCCGGTCTCA
AGTCTAATAGTACCTCCAGCAATTCCCACAGTTAAGCTACAGGATTTCACCAGAGACTTAT
TAAACCGGCTACGGACGCTTTAGGCCCAATAAAAGTTGCTACCACTAGAGCTGCCGGTGTT
ACCGCGGCGGCTGGCACCGGTCTTGCCCAGCTCTTATTCCAAAAGCTTTTTACACTAATGA
AAAGCCATCCCGTTAAGAATGGCACTTGGGATCCCCCCATCGCGATTTCTCACATTGTGGA
GGTTTCGCGCCTGCTGCGCCCCGTAGGGCCTGGAACCTTGTCTCAGGTTCCATCTCTGGGC
TCTTGCTCTCACAACCCGTACCGATCAACGGCTTGGTAAGCCATTACCTAACCAACTACCT
AATCGGCCGCAGACCCATCCTTAGGCGAAAAAACATTTAAACAAAGAACCATTGCAGGAAA
AATTGCCTATCCAGTATTATCCCCAGTTCCCAGGTATCCCGCCAAGGGG

>4C1:2 [organism=Uncultured Methanobrevibacter millerae] [clone=P41-C] UMAS SW12,16S ribosomal RNA, partial sequence

ACCCCGAATGTGATACGCGATTACTACGCATTCCAGCTTCATGAGAACGAGTTACAGTCCT
CAATCCGAACTACGACTAAGTTTAGAGGATTACCTCCACCTTTCGGTGTCGGAACCCATTG
TCTCAGCCATTGTAGCCCGCGTGTTGCCCAGAGGATTCGGGGCATACGGACCTACCGTCGT
CCACTCCTTCCTCCTATTTATCATAGGCGGTCCCCTTGGTGTGCCCATCATCCAAAAAAGG
ACATGCTGGTAACTAAGGGCGTGGGTCTCGCTCGTTGCCTGACTTAACAGGACGCCTCACG
GTACGAGCTGACGGCGGCCATGCACCTCCTCTCAGCTAGTCAAGCAAAGTCATCAACCTGG
CTATCATACAGCTGTCGCCTCTGGTGAGATGTCCGGCGTTGAATCCAATTAAACCGCAGGC
TCCACGCGTTGTGGTGCTCCCCCGCCAATTCCTTTAAGTTTCAGTCTTGCGACCGTACTTC
CCAGGCGGCGGACTTAACAGCTTCCCTTCGGCACTGGAGCAGCTCAAAGCCACCCCAACAC
CAAGTCCGCATCGTTTACAGTTAGGACTACCCGGGTATCTAATCCGGTTCGCGCCCCTAAC
TTTCGTCCCTCACCGTCAGAACCGTTCCAGTTAGACGCCTTCGCAACAGGCGGTCCTCCCA
GGATTACAGAATTTCACCTCTACCCTGGGAGTACCTCTAACCTCTCCCGGTCTCAAGTCTA
ATAGTACCTCCAGCAATTCCCACAGTTAAGCTACAGGATTTCACCAGAGACTTATTAAACC
GGCTACGGACGCTTTAGGCCCAATAAAAGTTGCTACCACTAGAGCTGCCGGTGTTACCGCG
GCGGCTGGCACCGGTCTTGCCCAGCTCTTATTCCAAAAGCTTTTTACACTAATGAAAAGCC
ATCCCGTTAAGAATGGCACTTGGGATCCCCCCATCGCGATTTCTCACATTGTGGAGGTTTC
GCGCCTGCTGCGCCCCGTAGGGCCTGGAACCTTGTCTCAGGTTCCATCTCTGGGCTCTTGC
TCTCACAACCCGTACCGATCAACGGCTTGGTAAGCCATTACCTAACCAACTACCTAATCGG
CCGCAGACCCATCCTTAGGCGAAAAAACATTTAAACAAAGAACCATTGCAGGAAAAATTGC
CTATCCAGTATTATCCCCAGTTCCCAGGTTCCCCGCTAGGGG

>0A2:1 [organism=Uncultured Methanolinea tarda] [clone=P02-A] UMAS SW13,16S ribosomal RNA, partial sequence

TTNCGTTGTTCAGTAACACGGGTGGCAACTTCCCTGTGGAGGGGGATAACCCCGGAAAACT
GGGGATAATACCCCATAGGTTAGGGTGGCTGGAATGCCCCCTAGCTCAAAGGTCCGCCGCC
ACAGGATGGGTCTGCGGCCGATTAGGTTGTTGTTGGGGTAACGGCCCAACAAGCCTTTGAT
CGGTACGGGTTGTGGGAGCAAGAGCCCGGAGATGGATTCTGAGACACGAATCCAGGCCCTA
CGGGGCGCAGCAGGCGCGAAAACTTTACAATGCGAGAAATCGTGATAAGGGAACCCCGAGT
GCCCGTAAATTCGGGCTGTCCGCCAGTGCAAAAAACTGGTGAAGAAAGAGCCGGGCAAGAC
CGGTGCCAGCCGCCGCGGTAATACCGGCGGCTCGAGTGGTGGCCACTATTACTGGGCTTAA
AGCGTCCGTAGCTTGGTTGTTAAGTCTCCTGGGAAATCCATCGGCTCAACCGATGGGCGTT
CAGGAGATACTGGCAACCTAGGGACCGGGAGAGGTGAGAGGTACTCCAGGGGTAGGAGTGA
AATCCTGTAATCCTTGGGGGACCACCTGTGGCGAAGGCGTCTCACTAGAACGGCTCCGACA
GTGAGGGACGAAAGCTGGGGGAGCAAACCGGATTAGATACCCGGGTAGTCCCAGCTGTAAA
CGATGCGCGTTAGGTGTATCGGTGACCACGAGTCACCGAGGTGCCGAAGGGAAACCGTGAA
ACGTGCCGCCTGGGAAGTACGGTCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCAC
CACAACAGGTGGAGCCTGCGGTTTAATCGGACTCAACGCCGGGAAGCTCACCGGATAAGAC
AGCTGAATGATAGCCGGGTTGAAGACTCTGCTTGACTAGCTGAGAGGAGGTGCATGGCCGT
CGTCAGTTCGTACTGTGAAGCATCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCAACA
GTTGCCAGCATGTCCTCCGGGATGGTGGGGACACTGTTGGGACCGCCTCTGCTAAAGAGGA
GGAAGGAATGGGCAACGGTAGGTCAGCATGCCCCGAATTATCCGGGCTACACGCGGGCTAC
AATGGTCAGGACAATGGGTATCAACACCGAAAGGTGAAGGCAATCTCCTAAACCTGTCCTT
AGTTCGGATTGTGGGCTGCAACTCGCCCACATGAAGCTGGAATCTGTAGTAATCGCGTCTC
AAATGGCCCGGTGATTATGCCCTGCCCCCCTTGGCAACCCGGAAA

>0B2:1 [organism=Candidtus Methanomethylophilus alvus] [clone=P02-B] UMAS SW14,16S ribosomal RNA, partial sequence
GGGCCAAGGGGATAATCCAGGGGAAACTTCTGGATAATTCCCCCATAGATCATGAGATTCG
GGAATGAATTTATGGTTCAAAAGTTCCGGGCGCTTTTAGGATCCGTTTTGCGGCCTATCAA
GGTAGTAGTGGGGTGTAACGTACCCCCCTAGCCTTATTACGGGTATGGGCCTTGAGAGAGG
GAGCCCAGAGTTGGATTCTGAGACACGAATCCAGGCCCTACGGGGCGCAGCAGTCGCGAAA
AACGTCACAATGGGCGAAAGCCCGATGAGGGAATTCCTAGTGCTAGCACTTTTGTGTTAGC
TTTTCTTTAGCGTAGATAACTAGAGGAATAAGGGCTGGGTAAGACGGGTGCCAGGCCGCCG
CGGTAATACCCGCAGCCCGAGTGGTGGTCGATCTTATTGAGTCTAAAACGTTCGTAGCCGG
TCTGATAGATCCTTGGGTAAATCGGGGGGCTTAACCTTCCGAATTCCGAGGAGACCGTCAG
GCTTGGGATCGGGAGAGGTAAGAGGTACTTCAGGGGTAAGGGTAAAATCCTGTAATCCTTG
GAGGACCACCGGTGGCGAAGGCGTCTTACTAGAACGAATCCGACGGTGAGGGACGAAGCCC
TAGGTCGCAAACGGGATTAGATACCCCGGTAGTCTAGGGTGTAAACGCTGCAGACTTGGTG
TTGGAGGCCCTTCGGGGGCATTCAGTGCCGGAGAGAAGTTGTTAAGTCTGCTACTTGGGGA
GTACGTCCGCAAGGATGAAACTTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCG
TGCGGTTTAATTGGATTCAACACCGGAAAACTCACCAAGGGAGACCATCACATGAAAGCCA
GGCTAATGACTTTGCTTGATTCTTGGAGAAGTGGTGCATGGCCATCGTCAGTTCGTACTGT
AAAGCGTTCTCTTAAGTGAGATAACGAACAAGACCCTCACTTATAATTGCTAACCGGATCT
CCGGATTCGGTGCACATTATCGGGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACG
GTAGGTCAGTATGCCCTGAATCTCTTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGA
GTTCCGACACCGAAAGGTGAAGGTAATCTCGAAACCCGTCCGTAGTTCGGACTGAGGGTTG
TAACTCACCCTCACGAAGCTGGATTCCGTAGTAATCGCGAATCAAAAACTCCGCGGGAATA
TGCCCCGGTCCCTTTGCCCACCCGGA

>0D2:1 [organism=Uncultured Crenarchaeotes archaeon] [clone=P02-D] UMAS SW16,16S ribosomal RNA, partial sequence
CAGTAACACGGGGGGTAACCTGCCCTTAGGACGGGATCACCCCCCGGAAACTGGGGCTAAT
CCCCGATAGGTAAAGAACTCTGGAATGAGTCTTTGCCCAAAGGCCGTTAGAGCATGCTTCT
GGCGGTGCCTAAGGATGGGGCCGCGACCGATCAGGTTGTTGGTGAGGTAATGGCCCACCAA

GCCTATAACCGGTGCGGGCCGTGAGAGCGGGAGCCCGGAGATGGGCACTGAGACAAGGGCC
CAGGTCCTACGGGGCGCAGCAGTCGCGAAAACTTTGCAATACACGAAAGTGTGACAGGGTC
ATCCCGAGTGCCGACCGCTGAGGTTGGCTTTTACCCAGTCTAGAAAGCTGGGGGAATAAGG
AGAGGGCAAGTCTGGTGTCAGCCGCCGCGGTAATACCAGCTCTCCGAGTGGTGTGGACGTT
TATTGGGCCTAAAGCATCCGTAGCTGGCCAAACAAGTCCCCTGTTAAACCCACCGATTTAA
TCGTTGGCGTGCGGGGGATACTGCTCGGCTAGGGGACGAGAGAGGCAGACGGTATTCCCGG
GGTAGGGGTGAAATCTTGTAATCCTTGAAGGACCACCAGTGGCGAAGGCGTCTCACCAGAA
CGGACCTGACGGCAAGGGACGAAAGCTAGGGGCACGAACCGGATTAGATACCCGGGTAGTC
CTAGCCGTAAACGATACTCGCTAGGTGTCGGCCACGGTGCGACCGTTGTCGGTGCCGTAGG
GAAGCCGTGAAGCGAGCCACCTGGGAAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGG
CGGGGGAGCACCACAACGGGTGGAGCTTGCGGTTTAATTGGATTCAACGCCGGAAATCTTA
CCGGGACCGACAGCAATATGAAGGCCAGGCTGAAGACTTTGCCGGATTAGCTGAGAGGTGG
TGCATGGCCGTCGTCAGTTCGTACTGTGAAGCATCCTGTTAAGTCAGGCAACGAGCGAGAC
CCACGCCCACAGTTGCCAGCGTACTCTCTGGAGTGACGGGTACACTGTGGGGACCGCCGCT
GCTAAAGCGGAGGAAGGAATGGGCAACGGTAGGTCAGTATGCCCCGAATATCCCGGGCTAC
ACGCGAGCTACAATGGTTGGTACAATGGGTATCTACCCCGAAAGGGGACGGGAATCTCCTA
AAACCAATCTTAGTTCGGATTGAGGGCTGCAACTCGCCCTCATGAAGCTGGAATCCGTAGT
AATCGCGTTTCAACAGAACGCGGTGATACGTCCCGGCCCTTTTGGCCACCC

>4D2:1 [organism=Uncultured Methanobrevibacter thaueri] [clone=P42-A] UMAS SW17,16S ribosomal RNA, partial sequence
TCCCGAATGTGATACGCGATTACTACGCATTCCAGCTTCATGAGAACGAGTTACAGTCCTC
AATCTGAACTACGACTAAGTTTAGAGGATTACCTCCACCTTTCGGTGTCGGAACCCATTGT
CTCAGCCATTGTAGCCCGCGTGTTGCCCAGAGGATTCGGGGCATACGGACCTACCGTCGTC
CACTCCTTCCTCCTATTTATCATAGGCGGTCCCCTTAGTGTGCCCATCATCCAAAAAAGGA
CAAGCTGGTAACTAAGGGCGTGGGTCTCGCTCGTTGCCTGACTTAACAGGACGCCTCACGG
TACGAGCTGACGGCGGCCATGCACCTCCTCTCAGCTAGTCAAGCAAAGTCATCAACCTGGC
TATCATACAGCTGTCGCCTCTGGTGAGATGTCCGGCGTTGAATCCAATTAAACCGCAGGCT
CCACGCGTTGTGGTGCTCCCCCGCCAATTCCTTTAAGTTTCAGTCTTGCGACCGTACTTCC
CAGGCGGCGGACTTAACAGCTTCCCTTCGGCACTGGAGCAGCTCAAAGCCACCCCAACACC
AAGTCCGCATCGTTTACAGTTAGGACTACCCGGGTATCTAATCCGGTTCGCGCCCCTAACT
TTCGTCCCTCACCGTCAGAACCGTTCCAGTTAGACGCCTTCGCAACAGGCGGTCCTCCCAG
GATTACAGAATTTCACCTCTACCCTGGGAGTACCTCCAACCTCTCCCGGTCTCAAGTCTAA
TAGTATCTCCAGCAATTCCCACAGTTAAGCTACAGGATTTCACCAGAGACTTATTAAACCG
GCTACGGACGCTTTAGGCCCAATAAAAGTTGCTACCACTAGAGCTGCCGGTGTTACCGCGG
CGGCTGGCACCGGTCTTGCCCAGCTCTTATTCCAAAAGCTTTTTACACTAATGAAAAGCCA
TCCCGTTAAGAATGGCACTTGGGATCCCCCCATCGCGATTTCTCACATTGTGGAGGTTTCG
CGCCTGCTGCGCCCCGTAGGGCCTGGAACCTTGTCTCAGGTTCCATCTCCGGGCTCTTGCT
CTCACAACCCGTACCGATCAACGGCTTGGTAAGCCATTACCTAACCAACTACCTAATCGGC
CGCAGACCCATCCTTAGGCGAAAAAACATTTAAACAAAGAACCATTACAGGAAAAATTGCC
TATCCGGTATTATCCTCAGTTCCCAAGTTCCCCATCTTAGGGG

>4C2:1 [organism=Uncultured Methanosaeta concilii] [clone=P42-C] UMAS SW18,16S ribosomal RNA, partial sequence
ACCCCGGTCTGTTGACGCGATTACTACGGATTCCAGCTTCATGAGGGCGAGTTGCAGCCCT
CAATCCGAACTAAGATTGGTTTTAGGAGATTCCCGTCCCCTTTCGGGGTAGATACCCATTG
TACCAACCATTGTAGCCCGCGTGTAGCCCGGGATATTCGGGGCATACTGACCTACCGTTGC
CCATTCCTTCCTCCGCTTTAGCAGCGGCGGTCCCCACAGTGTACCCGTCACTCCAGAGAGT
ACGCTGGCAACTGTGGGCGTGGGTCTCGCTCGTTGCCTGACTTAACAGGATGCTTCACAGT
ACGAACTGACGACGGCCATGCACCACCTCTCAGCTAATCCGGCAAAGTCTTCAGCCTGGCC
TTCATATTGCTGTCGGTCCCGGTAAGATTTCCGGCGTTGAATCCAATTAAACCGCAAGCTC

CACCCGTTGTGGTGCTCCCCCGCCAATTCCTTTAAGTTTCAGCCTTGCGGCCGTACTTCCC
AGGTGGCTCGCTTCACGGCTTCCCTACGGCACCGACAACGGTCGCACCGTGGCCGACACCT
AGCGAGTATCGTTTACGACTAGGACTACCCGGGTATCTAATCCGGTTCGTGCCCCTAGCTT
TCGTCCCTTGCCGTCAGGTCCGTTCTGGTGAGACGCCTTCGCCACTGGTGGTCCTTCAAGG
ATTACAAGATTTCACCCCTACCCCTGAAGTACCTCTCACCTCTCCCGGCCTCGAGCCAGAC
AGTATCTCCTGAAAGCCTGACAGTTAAGCTGCCAGATTTCTCAAGAGACTTATCCGGCCGG
CTACAGACCCTTTAGACCCAATAATAACGGTTACCACTCGAGCCGCCGGTGTTACCGCGGC
GGCTGGCACCGGTCTTGCCCGGCCCTTGCTATGAAATGCTTTTTAGGCATCTCGACAGCCA
GATTTGTAACCTGGCACTCGAGGTTCCCTTATCGCTGTTGCCAGCATTGTAAAGTTTTCGC
GCCTGCTGCACCCCGTAGGGCCTGGATTCGTGTCTCAGAATCCATCTCCGGGCTCTTGCTC
TCACAACCCGTACCCGTCGTAGGCTAGTAGGTACGCTACACCCACTACTACCTGATAGGCC
GCAGACCCATCCTTAGGCGCCGGAGCTTTTAATCTCGGTGCATTCCAGCAGCCGAGACTTA
TAGGGGATTATACCAAGTTCCCGGTTACCCACCAGGG

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Identification of Methane-producing Bacteria from Palm Oil Mill Sludge (POMS) with Solid Cud from Ruminant Stomach

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History
Received: 24 February 2014
Received in revised form: 15 March2014
Accepted: 12 Mac 2014
Available online: 17 August 2014

Keywords
Palm Oil Mill Sludge (POMS), solid cud, methane producing bacteria, 16S rRNA-cloning

Abstract

Biological generation in anaerobic environments such as enteric fermentation and anaerobic waste treatment from agriculture sector are the major contributor of methane gas which has the potential as biogas. The aimed of this study was to identify methane-producing bacteria in anaerobic vessel which contained a mixture of Palm Oil Mill Sludge (POMS) and solid cud taken from the first compartment of cow's stomach (1:2 and 2:1 ratio) as co-mixture. The co-mixture was incubated at 50 °C in a 2 L vessel with initial starter of 400 ml and sampling was conducted every 4 weeks interval during 12 weeks of incubation. For specific detection of methanogens, 16S rRNA-cloning analysis was carried out. *Methanobrevibacter* sp. and *Methanosaeta* sp. were confirmed to be presence within the 2:1 ratio of co-mixture while only *Methanobrevibacter* sp. was found in 1:2 ratio of co-mixture on both Week 0 and Week 4. No methanogens were detected for both co-mixtures on Week 8 and Week 12.

Introduction

Crude palm oil (CPO) production in Malaysia has been increasing continuously over the years, from 4.1 million tonnes in 1985 to 6.1 million tonnes in 1990. The production is further increased by 11.29% to 18.9 million tonnes in 2011 [1]. However, increase of production leads to generation of huge quantities of wastes. During oil extraction process, about 50% of water used results in palm oil mill effluent (POME) while others are lost as steam, mainly through sterilizer exhaust, piping leakages as well as wash water [2]. POME contains suspended solids and total dissolved solids in the range between 18,000 mg L^{-1} and 40,000 mg L^{-1} respectively [3]. Both solids are known as palm oil mill sludge (POMS). POMS consists of 3.6, 0.9 and 2.1 mg L^{-1} of total nitrogen, phosphorus and potassium, respectively, which results in bad odors and is consider as a source of ground pollution [4].

POMS can be applied as fertilizer as it has high nutrient value [5]. However, during rainy season, the drying process of POMS becomes difficult as the rate of drying become slower. Due to this limitation, anaerobic treatment of POMS such as anaerobic digester offer more attractive solutions for biogas production and clean development mechanism (CDM).

Anaerobic digestion process involves a wide variety of microbial community. In order to produce higher biogas yield, inoculum source is crucial for optimization of inoculum ratio. In this study, the aim of the present work was to determine the methane-producing bacteria community in POMS with solid cud from ruminant stomach using 16S rRNA clone library techniques.

Material and Methods

Samples Collection

Palm Oil Mill Sludge (POMS) was collected from the anaerobic pond from Bau Palm Oil Mill (BAPOM), Kuching, Sarawak. The solid cud from the first compartment of cow's stomach was collected from a slaughter house located at Ladang Lapan, Kuching. Both samples were stored in sealed container immediately after collection and preserved at 4 °C in order to avoid biodegradation due to microbial activities.

Anaerobic vessel set up

Co-mixture with different ratio (Table 1.0) were incubated at 50 °C in a 2 L vessel with initial starter of 400 ml. Sampling for both ratio were conducted every 4 weeks interval during 12 weeks of incubation.

DNA Extraction and PCR Amplification

Bacterial DNA of both ratio of co-mixture were extracted using Power Soil ™ DNA Isolation Kit (Mo Bio Laboratories, USA) and amplification of 16S rRNA region was amplified using

Table 1: Different ratio of Poms and solid cud in co-digestion

Ratio	Co-digestion mixture
1:2	POMS: Solid cud
2:1	POMS: Solid cud

Met86F and Met1340R primers [6]. PCR of the 16S rRNA was run in 25 µl reactions comprising 50-100 ng of DNA, 10X Taq DNA polymerase buffer, 0.5 µl of 10 mM dNTP mix (Fermentas, Canada), 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of each primer and 0.2 µl of 5 U AmpliTaq DNA polymerase (Fermentas, Canada). The amplification condition included 95 °C for 10 minutes followed by 34 cycles of 94 °C for 40 seconds, 54 °C for 50 seconds and 72 °C for 90 seconds. On the 35th cycles, the final elongation step was increased to 10 minutes at 72 °C.

Cloning 16S rDNA

PCR products of both co-mixture (1:2 and 2:1) were purified accordang to the manufacturer's instruction (Mo Bio Laboratories, USA). 16S rRNA clone libraries were constructed by cloning purified PCR fragments into pGEM-T Easy vector using the heat shock method. White colonies were randomly selected from the agar plates and plasmids were extracted using a plasmid extraction kit (Promega, USA). The extracted plasmids were re-amplified through PCR reaction and sent for sequencing.

Phylogenetic analysis

The sequences obtained were trimmed and further analysed using Bellerophon program [7] to remove chimera rRNA clones. Sequences similarities with 16S rRNA sequences in GeneBank ™ database were conducted using basic logical alignment tool (BLAST). PCR sequences were combined with closely related sequences. MEGA 5 [8] was used to construct neighbour-joining tree, which was bootstrap resampled 1000 times.

Results and Discussion

16S rRNA region was successfully amplified from the DNA extracted from the different ratio (1:2 and 2:1) of co-mixture. From 24 clones screened (6 from both Week 0 and Week 4 of 1:2 ratio and 6 from both Week 0 and Week 4 of 2:1 ratio) only 12 clones contained the correct size of DNA insert (1300bp) (Figure 1). No amplification of PCR products for Week 8 and 12 of both co-mixtures.

The diversity and phylogeny of the isolates were investigated by constructing phylogenetic tree with *Kluyveromyces lactis* as the outgroup (Figure 2). The sequences obtained in this study have been deposited in the GenBank database under accession numbers KJ522696-KJ522706.

From the phylogenetic tree, 66.6% of clones isolated, displayed 95% or greater genus-level sequence homology to species belonging to *Methanobrevibacter*. Within this genus, 41.6% (5/12) of all clones had 97% or greater species-level sequence similarity to *Methanobrevibacter millerae*. In contrast, only 0.083% of library clones were identified as *Methanobrevibacter olleyae*, *Methanobrevibacter arboripilus* and *Methanobrevibacter thaueri* respectively.

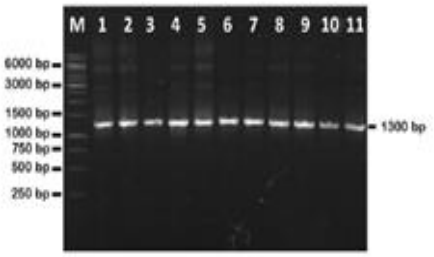

Figure 1: Agarose gel electrophoresis of amplified 16S rDNA region. Lane M, 1kb ladder (Fermentas); Lane 1, 2 and 3, PCR products of mixed sample with 1:2 ratio on Week 0 amplified using extracted plasmid from transformed bacteria; Lane 4,5, and 6, PCR products of mixed sample with 2:1 ratio on Week 0 amplified using extracted plasmid from transformed bacteria; Lane 7,8, and 9, PCR products of mixed sample with 1:2 ratio (Week 4) amplified using extracted plasmid from transformed bacteria; Lane 10 and 11, PCR products of mixed sample with 2:1 ratio (Week 4) were amplified by using extracted plasmid from transformed bacteria.

The other four clone libraries were divided into three different phylogenetic groups. Two of the clones showed 90% or greater sequences homology belonging to *Methanosaeta concilii* while the remaining clones were identified as *Methanolinea tarda* and *Aciduliprofundum boonei* respectively with 97% sequence similarities.

Majority of the clones from co-mixture of 1:2 (anaerobic sludge: solid cud) ratio belonged to the genus *Methanobrevibacter*. Presence of *Methanobrevibacter* sp. had also been reported in ovine and bovine content [9] and dairy cow [10]. In addition, Singh *et al.*[11] also reported presence of *Methanobrevibacter* sp. in ruminal fluid of buffalo. Clones from environment samples which show genus-level sequence similarity of more than 95% to *Methanobrevibacter* sp. are most abundance in gastrointestinal samples from herbivores [12, 13, 14]. In contrast, different methanogens such as *Methanosaeta concilii*, *Methanolinea tarda* and *Aciduliprofundum boonei* were found in co-mixture (2:1 ratio) which contains larger volume of anaerobic sludge. The presence of *Methanosaeta* sp., *Methanolinea* sp. and *Aciduliprofundum* sp. in sludge and agriculture wastes are also demonstrated in previous findings [15, 16, 17].

Conclusion

Methanogens population in co-mixture was reflected from 16S rRNA clone library in this study. *Methanobrevibacter* sp., *Methanosaeta concilii*, *Methanolinea tarda* and *Aciduliprofundum boonei* were presented in the co-mixture of anaerobic sludge with solid cud. With the knowledge of methanogens community in co-mixture, a better understanding in enhancing biogas production using anaerobic digester can be achieved in reduction of greenhouse gases emission.

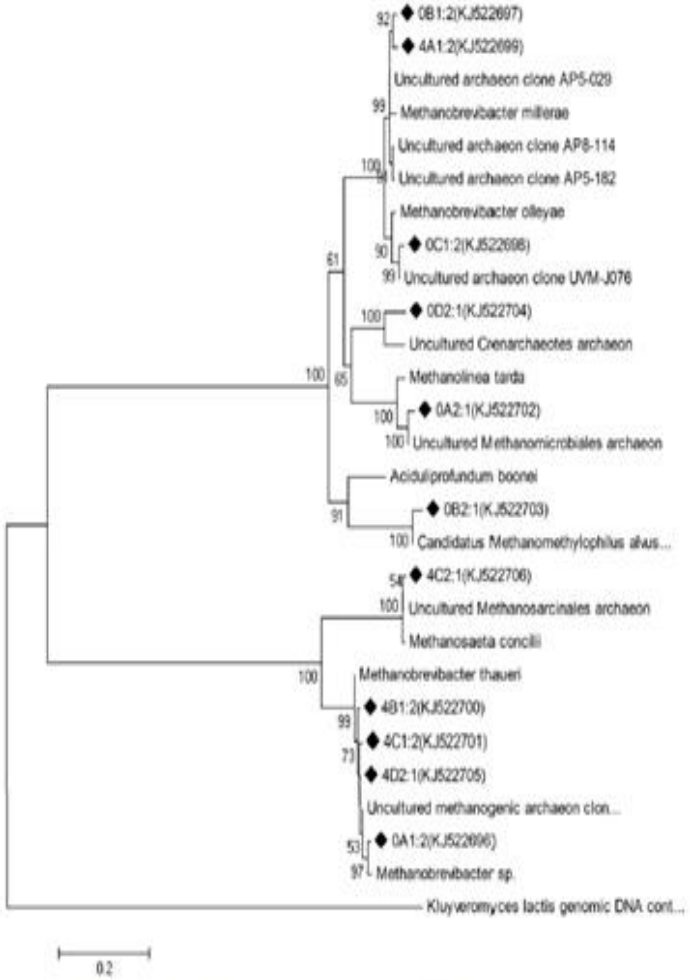

Figure. 2: Dendrogram of partial sequence of 16S rRNA of clone libraries from different ratio (1:2 and 2:1) co-mixture. The number at the nodes of the tree indicates bootstrap value of each node out of 1000 bootstrap resampling. The scale bar represents 0.2 substitutions per base position.

Acknowledgement

This project were funded by a research grant from UNIMAS (Top-Down grant: 01(TD01)/764/2010(01)) and the by the Tun Openg Sago Chair (ORC/12/2011(08)).

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