# Bacterial and methanogenic archaeal community changes during the treatment of palm oil mill effluent and biological indicators for final discharge

パーム油排水処理過程の細菌および古細菌群集構造変化と生物学的指標菌の特定

DIANA MOHD NOR

APRIL 2019

Department of Biological Functions and Engineering

Graduate School of Life Sciences and System Engineering

Kyushu Institute of Technology

# Bacterial and methanogenic archaeal community changes during the treatment of palm oil mill effluent and biological indicators for final discharge

パーム油排水処理過程の細菌および古細菌群集構造変化と生物学的指標菌の特定


Name: **Diana Mohd Nor**

Student number: **14899042**

Supervisor: **Dr. Norhayati Ramli (UPM)**

**Assoc. Prof. Dr. Toshinari Maeda (KYUTECH)**

Co-supervisor: **Prof. Dato' Dr. Mohd Ali Hassan**

**Prof. Dr. Kenji Sakai**

**Kyushu Institute of Technology**

**Graduate School of Life Science and Systems Engineering**

**Department of Biological Functions and Engineering**

## ABSTRACT

The sustainable practice in the palm oil industry which supplies the most demanded edible oil in the world, has a long way to be well developed. One of the most challenging problems is the management of wastewater generated from the oil palm processing, known as palm oil mill effluent (POME). The most popular treatment method applied to treat POME in the palm oil mills is anaerobic ponding system. Bioconversion of POME to generate methane gas via anaerobic digestion involves a consortium of microbes which are responsible in several steps of the biodegradation process. However, the biodegradation potential of the microorganisms in the full-scale treatment system in the palm oil mill is yet to be explored. POME is also known to have the adverse environmental effects if it is not properly treated, including contamination of land and aquatic ecosystem and the loss of biodiversity. A proper treatment is needed to ensure POME can be discharged into the nearby river water or land according to the requirement set by the authority. However, the current monitoring system using physicochemical characterisation is not sensitive enough to indicate the actual source of contamination in the water bodies.

In general, a detailed evaluation of the compositions of the bacterial communities in the POME final discharges obtained from four different palm oil mills and composition of bacterial community during the anaerobic treatment of POME were elucidated in this study using PCR-denaturing gradient gel electrophoresis (DGGE) and high-throughput MiSeq approaches, aided by advanced bioinformatics analysis in analysing the bacterial community structures. The correlation relationships were also carried out which allow deeper understanding of the interactions between the shift of bacterial

community compositions and the changes of physicochemical properties of POME, including pH, temperature, biochemical oxygen demand ($BOD_5$) and chemical oxygen demand (COD). The compounds analyses were also done to correlate the biodegradation potential of bacteria during the treatment of POME.

The findings demonstrated a significant difference of bacterial species richness and evenness among the four POME final discharges. However, the bacterial community compositions in the different final discharges exhibited almost similar patterns in that the phylum *Proteobacteria* was dominant in all the samples. Interestingly, the proposed bioindicators to indicate the river water contamination due to POME final discharge, the *Alcaligenaceae* and *Chromatiaceae* families, were found to be present in all the four final discharges despite the different characteristics of the mills and the different biotreatment processes used by them. In addition, both bioindicators were also shown to be strongly and positively correlated with the concentration of $BOD_5$, hence make them reliable bioindicators to indicate the river water contamination due to POME final discharge.

Furthermore, in order to elucidate the biodegradation potential of microorganisms in the POME treatment, a thorough analysis of bacterial and archaeal communities in different stages of POME treatment was carried out which comprised of anaerobic, facultative anaerobic and aerobic processes, including the mixed raw effluent (MRE), mixing pond, holding tank and final discharge phases. The bacterial and archaeal communities were shown to be shifted according to their biodegradation potential and the changes of physicochemical properties of POME. Based on the data obtained, the following biodegradation processes were suggested to take place in the different

treatment stages: (1) *Lactobacillaceae* (35.9%) dominated the first stage that contributed to high lactic acid production; (2) higher population of *Clostridiaceae* in the mixing pond (47.7%) and *Prevotellaceae* in the holding tank (49.7%) contributed to the higher acetic acid production; (3) the aceticlastic methanogen *Methanosaetaceae* (0.6–0.8%) played a role in acetic acid degradation in the open digester and closed reactor for methane generation; (4) *Syntrophomonas* (21.5–29.2%) might be involved in fatty acids and acetic acid degradations by syntrophic cooperation with hydrogenotrophic methanogen, *Methanobacteriaceae* (0.6–1.3%); (5) phenols and alcohols detected in the early phases but not in the final discharge indicated the successful degradation of lignocellulosic materials. A sustainable palm oil industry could be developed with better POME pollution management by adopting a reliable and accurate monitoring system. To our knowledge, this is the first study reported on the biodegradation mechanisms involved in the different stages of the full-scale treatment of POME.

# CONTENTS


| Contents | | | Page |
|---|---|---|---|
| Abstract | | | ii |
| List of tables | | | viii |
| List of figures | | | ix |
| List of abbreviations | | | xi |
| **CHAPTER** | | | |
| **1** | **INTRODUCTION** | | 1 |
| **2** | **LITERATURE REVIEW** | | 5 |
| | 2.1 | Palm Oil Industry | 5 |
| | | 2.1.1 Palm Oil Industry in Malaysia | 5 |
| | | 2.1.2 Crude Palm Oil Processing | 8 |
| | 2.2 | Palm Oil Mill Effluent | 12 |
| | | 2.2.1 Characteristics of Palm Oil Mill Effluent | 12 |
| | | 2.2.2 Treatment of Palm Oil Mill Effluent | 14 |
| | | 2.2.3 Palm Oil Mill Effluent Final Discharge | 18 |
| | 2.3 | Biochemical Reactions Involved in the Treatment of Palm Oil Mill Effluent | 21 |
| | | 2.3.1 Hydrolysis | 25 |
| | | 2.3.2 Acidogenesis | 26 |
| | | 2.3.3 Acetogenesis | 27 |
| | | 2.3.4 Methanogenesis | 28 |
| | 2.4 | Assessment of Bacterial Community Composition | 30 |
| | | 2.4.1 Assessment of Bacterial Community using Molecular Fingerprinting Techniques | 31 |
| | | 2.4.2 Assessment of Bacterial Community using High-throughput Sequencing | 35 |
| | | 2.4.3 Statistical Analysis, Data Representation and Visualisation of High-throughput Data | 38 |
| | 2.5 | Bioindicator for Environmental Monitoring | 42 |
| | | 2.5.1 Microbial Indicators for Environmental Monitoring | 43 |
| | | 2.5.2 Advantages and Disadvantages of Bioindicators | 47 |
| | 2.6 | Concluding Remarks | 48 |
| **3** | **GENERAL MATERIALS AND METHODS** | | 50 |
| | 3.1 | Analyses of Physicochemical Characteristics | 50 |
| | | 3.1.1 pH and Temperature | 50 |
| | | 3.1.2 Biochemical Oxygen Demand Concentration | 51 |
| | | 3.1.3 Chemical Oxygen Demand Concentration | 52 |

3.2 Genomic DNA Extraction of Palm Oil Mill Effluent Final Discharge 52
3.3 Analysis of Bacterial Community by PCR-Denaturing Gradient Gel Electrophoresis 54
3.3.1 PCR Amplification of 16S rRNA Gene 54
3.3.2 PCR Purification 55
3.3.3 Denaturing Gradient Gel Electrophoresis 55
3.3.4 Analysis of PCR-DGGE Gel Images 56
3.3.5 Sequencing 56
3.4 Metagenomic Analysis using Illumina MiSeq Sequencing Platform 56
3.4.1 16S rRNA Gene Amplification and Illumina MiSeq Sequencing 57
3.4.2 Bioinformatics Analysis of High-throughput Sequencing Data 58
3.4.3 Taxonomy and Relative Abundance of Bacteria and Archaea 59
3.4.4 Diversity Analyses 60
3.5 Statistical Analyses 60

**4 BACTERIAL COMPOSITION AND DIVERSITY ASSESSMENT IN PALM OIL MILL EFFLUENT FINAL DISCHARGE TREATED BY DIFFERENT BIOTREATMENT PROCESSES FOR THE DETERMINATION OF BIOINDICATORS** 61
4.1 Introduction 61
4.2 Materials and Methods 62
4.2.1 Location of Study Area 62
4.2.2 Sample Collection 65
4.2.3 Physicochemical Characterisation 65
4.2.4 Nutrient Composition Analysis 66
4.2.5 Genomic DNA Extraction of Palm Oil Mill Effluent Final Discharge 66
4.2.6 Bacterial Community Analysis by PCR-Denaturing Gradient Gel Electrophoresis 67
4.2.7 High-throughput Illumina MiSeq Sequencing and Bioinformatic Analysis 67
4.2.8 Principal Coordinate Analysis 67
4.3 Results and Discussion 68
4.3.1 Physicochemical Characteristics of Palm Oil Mill Effluent Final Discharge 68

4.3.2 PCR-DGGE Analysis of Bacterial Community Structure 74
4.3.3 High-throughput Sequencing Analysis 79
4.3.4 Principle Coordinate Analysis to Correlate Bcterial Community Composition with the Pollutant Properties 86
4.4 Concluding Remarks 88

**5 SHIFT OF BACTERIAL AND METHANOGENIC ARCHAEAL POPULATIONS IN THE DIFFERENT STAGES DURING THE TREATMENT OF PALM OIL MILL EFFLUENT** 89
5.1 Introduction 89
5.2 Materials and Methods 91
5.2.1 Samples Collection 91
5.2.2 Physicochemical Characterisation 91
5.2.3 Compounds Characterisation 95
5.2.4 Genomic DNA Extraction of Palm Oil Mill Effluent Sludge and Final Discharge 96
5.2.5 Bacterial Community Analysis by PCR-Denaturing Gradient Gel Electrophoresis 97
5.2.6 Illumina MiSeq Sequencing and Bioinformatic Analysis 97
5.3 Results and Discussion 98
5.3.1 Physicochemical Characteristics of Palm Oil Mill Effluent 98
5.3.2 PCR-DGGE Analysis of Bacterial Community Structure 106
5.3.3 Next Generation Sequencing (Illumina MiSeq) Analysis 115
5.4 Concluding Remarks 129

**6 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES** 130
6.1 Conclusions 130
6.2 Recommendations for Future Studies 131

**REFERENCES** 133
**APPENDICES** 152
**LIST OF PUBLICATIONS** 155
**ACKNOWLEDGEMENTS** 157

## LIST OF TABLES


| Table | | Page |
|---|---|---|
| 2.1 | Physicochemical characteristics of POME originated from different sources. | 13 |
| 2.2 | Several methods applied for the treatment of POME with the comparisons of economical, environmental and operational aspects. | 18 |
| 2.3 | Characteristics of raw POME and POME final discharge with the respective standard discharge limits set by the DOE of Malaysia. | 20 |
| 2.4 | Microorganisms isolated from POME that involved in the biodegradation processes. | 22 |
| 2.5 | Molecular fingerprinting techniques applied to assess the composition of microbial community in various types of wastewater. | 33 |
| 2.6 | Various types of wastewater analysed using high-throughput platform to reveal microbial diversity. | 36 |
| 2.7 | The properties and applications of different Illumina platforms for an amplicon sequencing. | 37 |
| 2.8 | Potential bacterial indicators to indicate different types and sources of contamination in the water bodies. | 45 |
| 4.1 | Capacity of FFB, anaerobic retention time and total retention time of POME for the treatments A, B, C, and D. | 63 |
| 4.2 | The physicochemical characteristics of POME final discharge obtained from four different typical palm oil mills. | 68 |
| 4.3 | Percentage composition of nutrients in the different POME final discharges obtained from four different typical palm oil mills. | 74 |
| 4.4 | Phylogenetic sequence affiliation and similarity to the closest relative of amplified 16S rRNA gene sequences excised from DGGE gels. | 77 |
| 4.5 | Number of raw reads, high-quality reads, OTU and alpha-diversity indices measured as Shannon-Weaver index (H') and Evenness (E') in the POME final discharge obtained from different palm oil mills. | 81 |
| 5.1 | Estimated methane production from POME based on 60.1% COD removal in one unit of the anaerobic (closed) reactor with the capacity of 1800 $m^3$. | 104 |
| 5.2 | Phylogenetic sequence affiliation and similarity to the closest relative of amplified 16S rRNA gene sequences excised from DGGE gels for A: MRE, B: mixing pond, C: holding tank, D: reactor (closed), E: digester (open), F: facultative, G: algae, H: final discharge. | 112 |
| 5.3 | Microbial alpha-diversity characteristics for POME samples from each stage of treatment. | 118 |
| 5.4 | Presence of alcohols and phenolics as main degradation compounds from lignocellulosic materials in the different stages during the full-scale treatment of POME. | 124 |

## LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| 2.1 | Expansion of oil palm cultivation in Malaysia from 1960 until 2017. | 6 |
| 2.2 | Oil palm planted area in Malaysia. | 7 |
| 2.3 | Total production of crude palm oil and crude palm kernel oil in Malaysia. | 8 |
| 2.4 | Number of palm oil mills (●) and capacity of operating FFB ( ) at palm oil mills in Malaysia from 1980 to 2010. | 9 |
| 2.5 | Schematic diagram of typical palm oil extraction process. | 11 |
| 2.6 | Typical configuration of ponding system for the treatment of POME. | 15 |
| 2.7 | The target of BOD concentration in the general treatment system of POME in the palm oil mill. | 16 |
| 2.8 | Scheme of an anaerobic degradation process for the treatment of POME. | 24 |
| 4.1 | Locations of four selected typical palm oil mills in Peninsular Malaysia. | 63 |
| 4.2 | Process flow schematics of the different stages of full-scale treatment of POME in four typical palm oil mills. | 64 |
| 4.3 | The concentrations of $BOD_5$ and COD of the POME final discharges obtained from four different palm oil mills with y-axis on a log scale. | 72 |
| 4.4 | Representative of 16S rRNA region amplified by PCR separated on 1.6% agarose gel. | 76 |
| 4.5 | The DGGE fingerprint profiles for POME final discharges obtained from different palm oil mills. | 76 |
| 4.6 | Relative abundance of the bacterial phyla (>2%) in the POME final discharge obtained from four different palm oil mills. | 83 |
| 94.7 | Heatmap of the relative abundance of 22 bacterial orders from the total *Proteobacteria* in the POME final discharge obtained from different palm oil mills. | 85 |
| 4.8 | PCO ordinations of phylum *Proteobacteria* based on relative abundance of OTU, physicochemical properties ($BOD_5$ and COD) and nutrients compositions (phosphorus, ammonium, nitrogen, nitrate and potassium) of POME final discharges obtained from four different palm oil mills. | 87 |
| 5.1 | Sampling points of the different stages of POME treatment at FELDA Pasoh Palm Oil Mill, Negeri Sembilan, Malaysia. | 93 |
| 5.2 | The profiles of pH and temperature in the different stages of POME treatment. | 99 |
| 5.3 | The changes of BOD5 (●) and COD ( ) throughout the different stages of POME treatment. The error bars represent standard deviations of experiments performed in twelve months. | 102 |

| | | |
|---|---|---|
| 5.4 | Monthly production of biogas in commercial scale reactors (four units) with the capacity of 1800 m3 each in the studied palm oil mill in 2015. | 104 |
| 5.5 | The changes of TOC concentration throughout the different stages of POME treatment. | 106 |
| 5.6 | Representative of 16S rRNA regions amplified by PCR separated on 1.6% agarose gel electrophoresis with the approximate size of 161 bp. | 107 |
| 5.7 | DGGE fingerprint profiles (i) and normalized-DGGE images (ii) respectively for A: mixed raw effluent, B: mixing pond, C: holding tank, D: reactor (closed), E: digester (open), F: facultative, G: algae, H: final discharge for one year monitoring period. | 110 |
| 5.8 | Summary of analysed sequences in the different stages of POME treatment. | 117 |
| 5.9 | Relative abundance of the bacterial and archaeal communities categorized at the taxonomic family level in the different stages of a full-scale treatment of POME that were summarized from three sampling months (January, June and December 2015). | 120 |
| 5.10 | Relative abundance of *Lactobacillaceae*, *Veillonellaceae*, *Prevotellaceae* and *Clostridiaceae* in relation to the concentrations of acetic, lactic and propionic acids detected in the MRE, mixing pond, holding tank and closed reactor. No acids were detected in the open digester and the subsequent stages of POME treatment. | 121 |
| 5.11 | Schematic flow process diagram of biodegradation in the different stages during the treatment of POME catalysed by dominant bacteria and archaea populations for the generation of methane as end-product. | 128 |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACE | Abundance-based Coverage Estimator |
| APHA | American Public Health Association |
| ARDRA | Amplified ribosomal DNA restriction analysis |
| BOD | Biochemical oxygen demand |
| bp | Base pair |
| BPA | Bisphenol A |
| CaCl2 | Calcium chloride |
| $CO_2$ | Carbon dioxide |
| CCA | Canonical correspondence analysis |
| COD | Chemical oxygen demand |
| CPO | Crude palm oil |
| ddNTPs | Dideoxy nucleoside triphosphates |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| DO | Dissolved oxygen |
| DOE | Department of Environment |
| $FeCl_3$ | Ferric chloride |
| FELDA | Federal Land Development Authority |
| FFA | Free fatty acid |
| FFB | Fresh fruit bunch |
| g | Gram |
| $H_2$ | Hydrogen |
| HNA | High nucleic acid |
| HRT | Hydraulic retention time |
| IC | Inorganic carbon |
| kg | Kilogram |
| LNA | Low nucleic acid |
| LH-PCR | Length heterogeneity polymerase chain reaction |
| $m^3$ | Cubic metre |

| | |
|---|---|
| mg/L | Milligram per litre |
| $MgSO_4$ | Magnesium sulphate |
| min | Minutes |
| mL | Millilitre |
| mm | Millimetre |
| MPOB | Malaysian Palm Oil Board |
| MPOC | Malaysian Palm Oil Council |
| ng | Nanogram |
| nm | Nanometre |
| NGS | Next generation sequencing |
| $O_2$ | Oxygen |
| OTU | Operational taxonomic unit |
| NP | 4-Nonylphenol |
| PCR | Polymerase chain reaction |
| POME | Palm oil mill effluent |
| RDP | Ribosomal database project |
| rRNA | Ribosomal RNA |
| RISA | Ribosomal intergenic spacer analysis |
| SSCP | Single-strand conformation polymorphism |
| SCFA | Short chain fatty acids |
| TAE | Tris-acetate-EDTA |
| TEMED | Tetraetylmetylenediamine |
| TOC | Total organic carbon |
| TSS | Total suspended solids |
| T-RFLP | Terminal restriction fragment length polymorphism |
| VSS | Volatile suspended solids |
| wt/vol | Weight per volume |
| µL | Microlitre |

# CHAPTER 1

# INTRODUCTION

The oil palm industry is an active contributor to the nation's economic growth with high annual export profit of palm products which was estimated at RM 78 billion just in the year 2017 (Kushairi et al., 2017). The production of palm oil will continue to increase to meet the global demand for oil and fats products. Although the oil palm industry appears to be one of Malaysia's most structured agro-industry, the milling process produces massive colloidal wastewater during the oil extraction process at the palm oil mill, known as palm oil mill effluent (POME). The traditional milling process normally employs steam for the sterilisation of fruit, which finally lead to the formation of POME (Law et al., 2016). Typically, 1 tonne of crude palm oil production requires approximately 5 to 7 tonnes of water which over 50% of it ends up as POME (Wang et al., 2015). Due to the higher discharge of POME into the receiving water bodies, the oil palm industry has been claimed as one of the largest water polluters in Malaysia.

The huge generation of POME from the palm oil industry has become a serious issue that not only affects the industry, but also people and the environment. POME is brownish as it contains appreciable amounts of organic matter which eventually increases both the biochemical oxygen demand (BOD) and the chemical oxygen demand (COD) that originate from steam extraction process (Bala et al., 2015; Saeed et al., 2015). Without a proper treatment, POME will inflict serious environmental pollution. The anaerobic digestion applied for the treatment of POME could increase the rate of biodegradation (Poh and Chong, 2009), in addition to the conventional

POME treatment using the ponding system. However, if it is not being managed efficiently, the discharge of treated or partially treated POME into a nearby river could lead to severe environmental pollution (Rupani et al., 2010).

Therefore, it is important to have a reliable assessment method to indicate the source of contamination due to POME final discharge. Current assessment via determination of the physicochemical properties of the affected river water is considered inaccurate as they may contain pollutants originated from other anthropogenic sources such as from the residential areas and agricultural practices. The use of an indicator could be regarded as a potential approach for assessing the specific cause of pollution in the effluent receiving river water (Zhang et al., 2014). The *Alcaligenaceae* and *Chromatiaceae* have been proposed in the previous study to be used as potential bioindicators to indicate the river water contamination due to POME final discharge. Both of them which were reported to be present in the effluent receiving river but not in the unpolluted river water were also determined to be originated from the POME final discharge (Sharuddin et al., 2017).

Nevertheless, the aim of POME treatment is alike which is to reduce the polluting power of this wastewater below the effluent discharge standard before it is being released into the environment, but different biotreatment processes have been employed in the different palm oil mills, generating different properties of POME final discharge. The generation of waste from palm oil mills is also dependent on the amount of fresh fruit bunch (FFB) processed (Liew et al., 2017). Hence, it remains unclear how these different biotreatment processes and capacities could affect the result of the previous study which determined that *Alcaligenaceae* and *Chromatiaceae* were the

bioindicators. Since the bacteria are highly sensitive to disturbances and could react differently depending on the properties of pollutants, environmental factors and treatment processes, it is important to assess the practical relevance of the bioindicator for monitoring the river water quality.

In addition, as emphasis is placed on the concept of biodegradability in biological treatment of POME, utilisation of vast microbial consortia in the pond and anaerobic treatment systems in the mills (Choong et al., 2018; McHugh et al., 2003) needs to be looked into more thoroughly. Up to date, insights into the bacterial and archaeal communities and key players catalysing a complex series of biochemical reactions to reduce the polluting power of POME in the treatment system are still unclear. Therefore, the substrates causing inefficient treatment of POME are not ascertained. Digestions problems may also arise from insufficient understanding of the biodegradation mechanism which in turn resulting in failure of the treatment system.

Current studies have focused largely on the microbial community structure of POME in various bioreactor configurations (Rana et al., 2017) with little attention given to the bacterial and archaeal consortia involved in the biodegradation in the different stages of full-scale treatment of POME. The management of POME remains unclear and challenging without understanding the biodegradative pathways involved throughout the treatment. Therefore, to ensure a sustainable industrial practice, it is essential to have more knowledge on the bacterial population, diversity and how they are related to the biodegradation process and severity of pollution.

The current conventional culturing methods used in analysing the bacterial community structure is inadequate as most of the important environmental bacterial species are unculturable (Lu et al., 2015; Rani et al., 2008). However, with the application of molecular phylogenetic methods and the emergence of next generation sequencing (NGS), it is now possible to obtain a view on the bacterial community that was previously inaccessible (Tan et al., 2015). The integration of PCR-denaturing gradient gel electrophoresis (DGGE) approach and high-throughput MiSeq serves as valuable tools to study the bacterial community composition and structure (Yu et al., 2015). The databases containing information regarding the relative abundance and activity of bacterial communities could provide thorough insights on the bacterial community composition in the POME final discharges taken from different biotreatment processes of POME, as well as the bacterial and archaeal diversity and composition in the different stages of POME treatment. The biodegradation potential of bacterial community throughout the treatment process could also be obtained by correlating the shift of bacterial community with the changes of physicochemical properties of POME.

This study aimed to assess bacterial community changes in the POME final discharges generated from different biotreatment processes, as well as during the full-scale treatment of POME. The specific objectives of this research were:

I. To assess the presence of bioindicators in the POME final discharge generated by different biotreatment processes and correlate to the changes of pollutant properties;

II. To assess the changes of bacterial and methanogenic archaeal communities in the different stages during the treatment of POME.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Palm Oil Industry

Over several decades, palm oil has been known as the most edible vegetable oil consumed by people around the world. The oil palm, *Elaeis guineensis*, is the most productive oil producing plant which was first brought to Malaysia from West Africa in 1876 and commercialized in 1970. Developing countries such as Indonesia, Malaysia, Nigeria and Thailand are among the major palm oil producers and supplies in the world. Now, with over 17 million tonnes annual CPO production, Malaysia is ranked as the second largest producer of CPO after Indonesia (Laurance et al., 2010)

### 2.1.1 Palm Oil Industry in Malaysia

In Malaysia, oil palm cultivation begun in 1917 and it was started to develop in the subsequent years. The year 2017 has marked 100 years of oil palm cultivation in Malaysia (Zunaira and Hanim, 2017). In years 1960s, statistics on oil palm cultivation show Malaysia has had only 54,000 hectares of oil palm plantations. Since then, the oil palm cultivation area has increased dramatically. Oil palm plantations have occupied 3.38 million hectares of Malaysian lands in years 2000s (Abdullah, 2003; Awalludin et al., 2015). The recent statistics showed the increasing of 0.96% of oil palm plantation from 4.85 million hectares in 2010 to 5.81 million hectares in 2016 (MPOB, 2016) as shown in **Figure 2.1**. The oil palm plantation expanded quickly

under the tropical climate in Malaysia due to the stable all-year-round temperature ranging from 25°C to 33°C with rain and high intensity of sunlight (Ali et al., 2012).

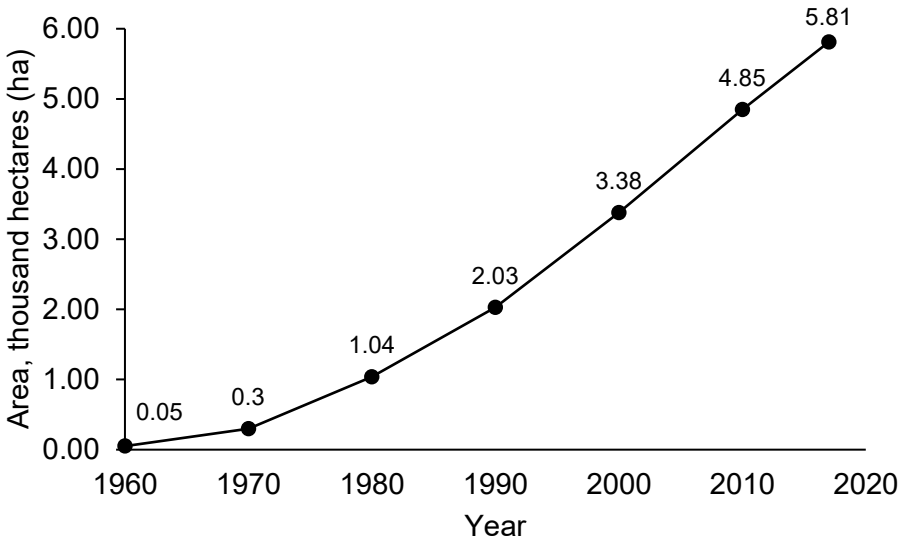



**Figure 2.1 Expansion of oil palm cultivation area in Malaysia from 1960 until 2016** (Sources: Abdullah (2003); MPOB (2016)).

The increment of oil palm cultivation area was resulted from the government incentive to meet the strong demand of palm oil global production and to fulfil the growing global need for oils and fats sustainably. **Figure 2.2** shows the oil palm planted area in Malaysia where Peninsular Malaysia showed the largest oil palm planted area with 2.70 million hectares (46.6%). Sarawak overtook Sabah as the largest oil palm planted state in 2017 with 1.56 million hectares or 26.8% of the total Malaysian oil palm planted area and Sabah with 1.55 million hectares (26.6%) (MPOB, 2016).

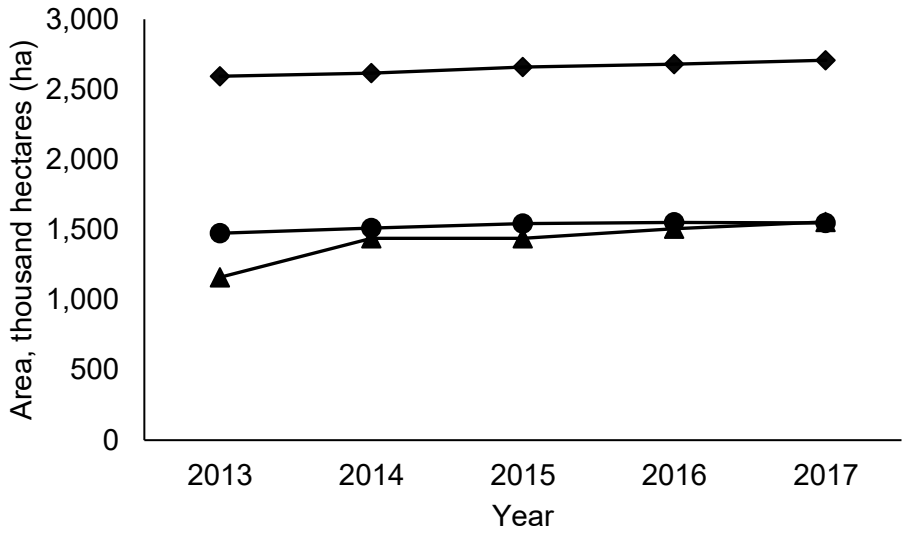



**Figure 2.2** **Oil palm planted area in Malaysia; ♦: Peninsular Malaysia, ●: Sabah and ▲: Sarawak** (Source: MPOB (2017)).

The same expanding trend can also be observed in the production of CPO (**Figure 2.3**). In overall, Malaysia recorded an increase in palm oil production. However, the El-Nino weather phenomenon during the second half of 2015 and into the first half of 2016 had impacted the Malaysian oil palm industry performance in 2016 (MPOB, 2016). Nevertheless, the palm oil production showed starling performance with significant increased following recovery from the impact of the El-Nino phenomenon in 2017. The oil palm industry has becomes a strong contributor to the nation's economic growth with annual high export revenues of palm products which was over RM 77.85 billion in the year 2017 (MPOB, 2017).

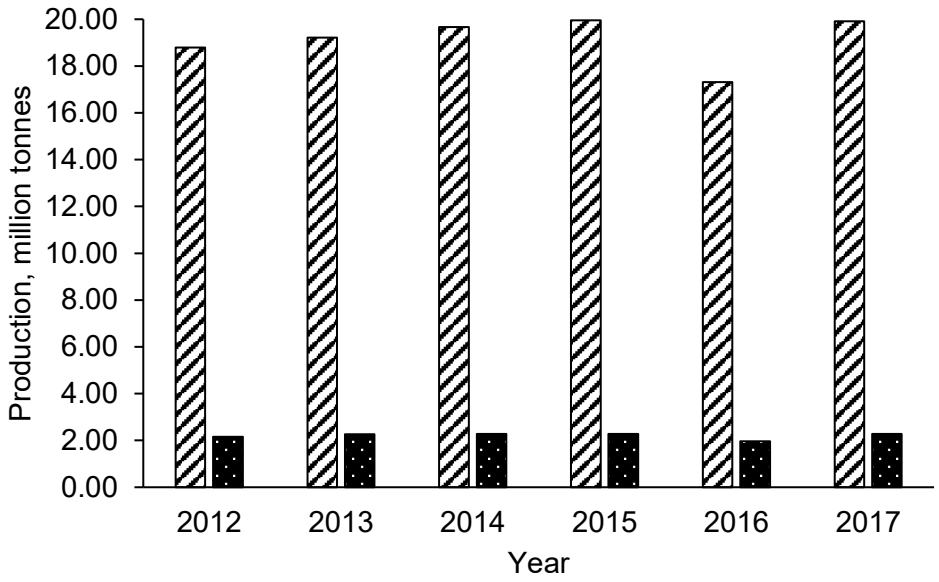



**Figure 2.3 Total production of crude palm oil (▨) and crude palm kernel oil (■) in Malaysia** (Source: MPOB (2017)).

### 2.1.2 Crude Palm Oil Processing

Palm oil processing is carried out in the palm oil mills to extract the oil from fresh fruit bunch (FFB). After harvesting, FFB needs to be processed as soon as possible in order to prevent a rapid increase of free fatty acids (FFA) which could affect the quality of the CPO. Therefore, most of the palm oil mills are generally located in the plantations area to facilitate the timely transportation and to ensure an effective processing of FFB. The increased number of palm oil mills has resulted in the increased amounts of FFB to be processed year by year (**Figure 2.4**). Up to year 2017, Malaysia recorded a total of 454 palm oil mills in operation, with 244 mills operated in Peninsular Malaysia and the remaining 210 mills in Sabah and Sarawak (MPOB, 2017).

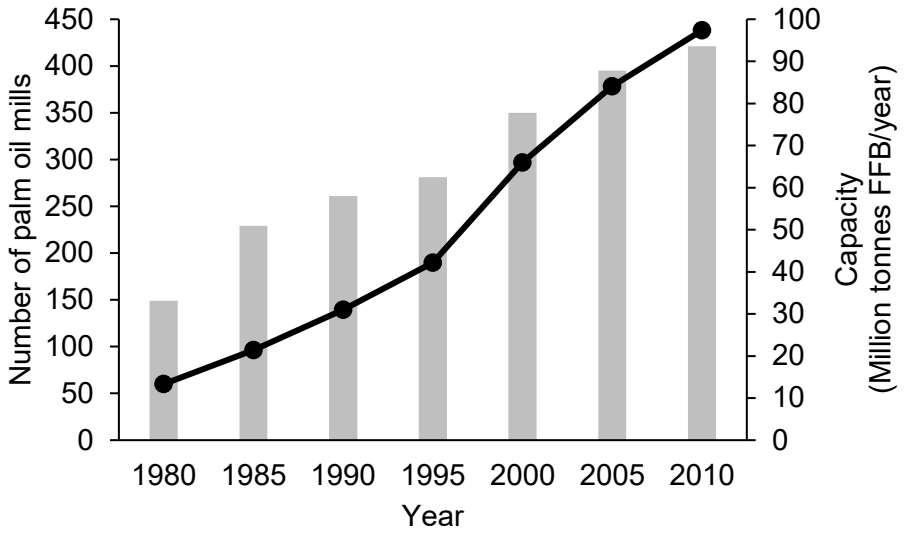



**Figure 2.4 Number of palm oil mills (●) and processing capacity of FFB ( ) at palm oil mills in Malaysia from 1980 to 2010** (Sources: http://www.mpob.gov.my/en/palm-info/environment/520-achievements (accessed on 29.06.2018) and http://bepi.mpob.gov.my/index.php/en/statistics/sectoral-status.html (accessed on 29.06.2018)).

Of the total palm oil mills in Malaysia, Federal Land Development Authority (FELDA) which is a Malaysian government agency owned 72 palm oil mills that producing 2.66 million metric tonnes of CPO annually, more than any other producer worldwide, hence making FELDA the world's largest producer of CPO (Felda Group Venture, 2017). The FELDA palm oil mills were divided into three categories based on mill's operation capacity which are 30, 40 and 60 tonnes of FFB processing per hour.

In the process of CPO extraction, the wet palm oil milling process is commonly applied in Malaysia. Excessive volumes of water and steam are required to remove dirt and for sterilisation in the different steps of the wet process. The huge quantity of water is typically obtained from an adjacent freshwater resource such as a river, which requires very little treatment and pumping costs. The palm oil milling process, describing the

sources and quantities of water, its subsequent POME generation, as well as the products and by-products is presented in **Figure 2.5**. After several steps of CPO extraction process, a sludge of two layers comprising of oil and water are formed in the recovery tank. The oil layer which is the lighter phase of the sludge is drained to the clarification tank where the CPO is skimmed off. The CPO is then purified, vacuum dried and stored in the CPO storage tank, waiting to be transported to the refinery. The heavier aqueous layer known as POME is discharged into the wastewater treatment plant (Ahmed et al., 2015).

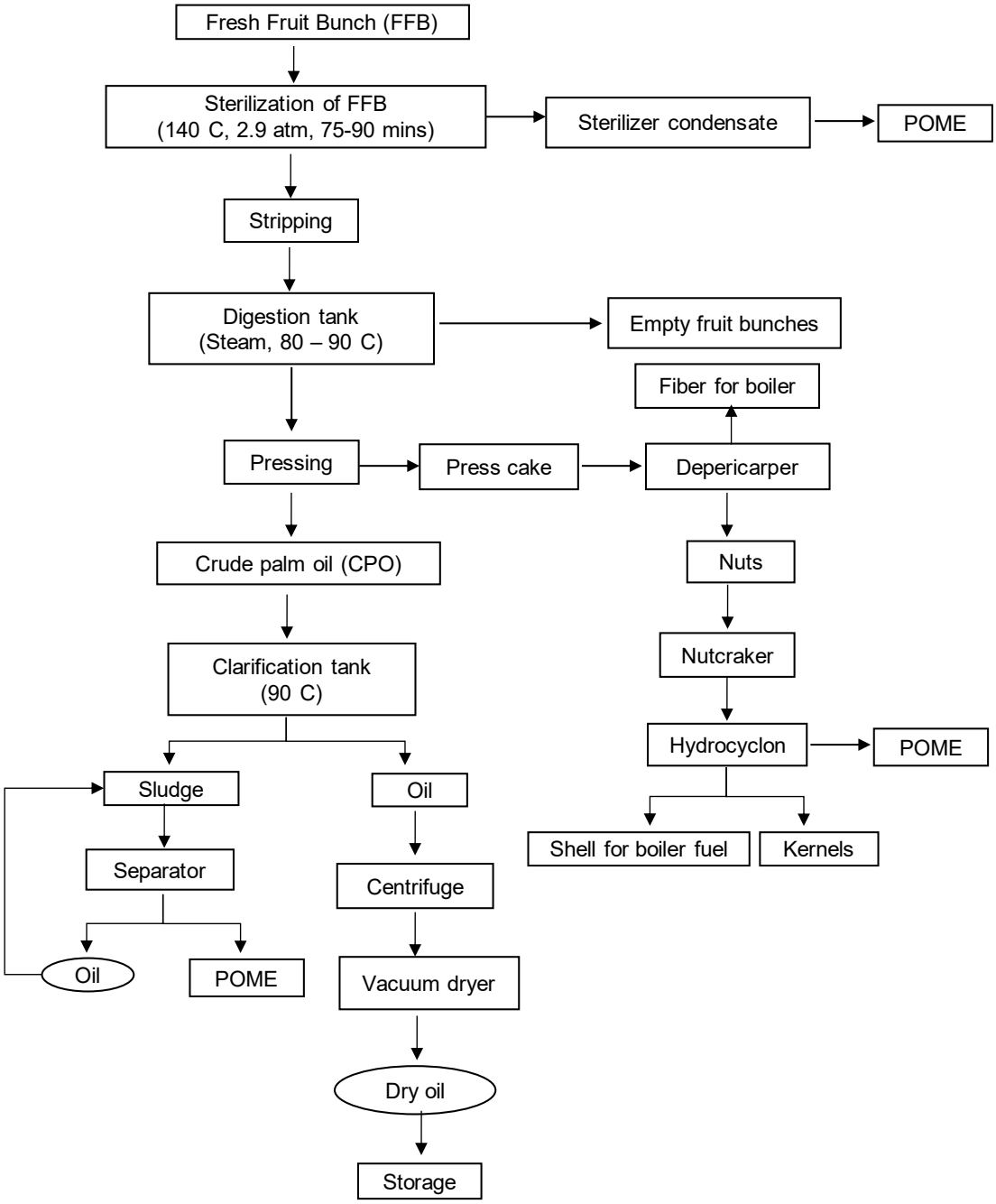



**Figure 2.5** **Schematic diagram of typical palm oil extraction process** (Source: Ahmed et al. (2015)).

## 2.2 Palm Oil Mill Effluent

POME is the liquid waste generated by palm oil industry which considered as the most expensive and difficult waste to manage by the mill operators due to the large volumes generated (Madaki and Seng, 2013). Due to this reason, as the easiest and cheapest method for disposal of POME, the raw or partially treated POME is still being released into the environment. Nevertheless, the discharging of POME into the environment had caused the alarming rise of pollution particularly to the aquatic ecosystem.

### 2.2.1 Characteristics of Palm Oil Mill Effluent

The raw POME is produced from the sterilisation and clarification stages in the form of highly concentrated dark brown colloidal slurry of water at the temperature of 80°C to 90°C (Ahmad et al., 2005), containing 95 – 96% of water, 0.6 – 0.7% of oil and grease and 4 – 5% of total solids (Ahmad et al., 2006). The generation of POME can create odour problems and nuisance to the community living nearby the palm oil, as well as cause the river water pollution. The refined characteristics of POME were extracted from several literatures as shown in **Table 2.1**. The characteristics of POME may vary by different batches, days and factories, depending on the several factors including the oil palm cropping seasonal and palm oil mill operations (occasional public holidays, closure of the mill, operation and quality control of individual mills) (Wu et al., 2010; Yacob et al., 2005).

**Table 2.1 Physicochemical characteristics of POME originated from different sources.**

| Characteristics | Unit | POME | | | | |
|---|---|---|---|---|---|---|
| | | Poh and Chong (2014) | Kim et al. (2013) | Malakahmad et al. (2014) | Ahmad et al. (2011a) | Fang et al. (2011) |
| Chemical oxygen demand (COD) | $mgL^{-1}$ | 322580 ± 9500 | 49200 ± 6200 | 50450 ± 850 | 65000 | 97000 |
| Biochemical oxygen demand (BOD) | $mgL^{-1}$ | 1700 ± 2500 | - | 24850 ± 3540 | 40000 | - |
| Total solids | $mgL^{-1}$ | - | 46200 ± 5900 | - | 45000 | 67300 |
| Suspended solids | $mgL^{-1}$ | 15000 ± 1350 | 15200 ± 1800 | 12053 ± 1467 | - | 40600 |
| Volatile solids | $mgL^{-1}$ | - | 37000 ± 4600 | - | 26300 | 57300 |
| Volatile suspended solids | $mgL^{-1}$ | - | 14400 ± 1800 | - | 20000 | 34500 |
| Total Kjeldahl nitrogen | $mgL^{-1}$ | 852 ± 107 | 1000 ± 120 | 749 ± 35 | 890 | 3200 ± 550 |
| Oil and grease | $mgL^{-1}$ | 6100 ± 1094 | - | 2038.5 ± 212.5 | 1500 | - |
| Temperature | °C | 47 ± 4.5 | - | - | 55.5 | - |
| pH | - | 4.7 ± 0.2 | 4.3 ± 0.2 | 4.42 ± 0.1 | 4.5 | - |

### 2.2.2 Treatment of Palm Oil Mill Effluent

Due to its high organic load, POME is regarded as one of the most polluting agro-industrial residues (Poh and Chong, 2009), therefore it is a great challenge to treat them. The increase in palm oil production has raised the question of how to efficiently manage the increasing volume of POME while preserving the environment. The selection of the POME wastewater treatment system is largely influenced by the operating and maintenance costs, land availability and mill location. The first factor plays a greater role in the treatment system selection.

More than 85 percent of palm oil mills in Malaysia use the low-cost weighing system to treat POME because of cost-effective treatment, low capital, ease of handling and simplicity (Liew et al., 2015). Previously, the open ponding system was the popular choice which generally consists of cooling and mixing, anaerobic, facultative and aerobic ponds (**Figure 2.6**). The cooling and mixing pond stabilizes the POME temperature and pH before anaerobic digestion. In the anaerobic stage, a wide variety of organic materials could be stabilised and concurrently produces methane from the digestion process. In the meantime, the subsequent facultative and aerobic ponds are required to further reduce the organic content of wastewater before it is discharged into the rivers.

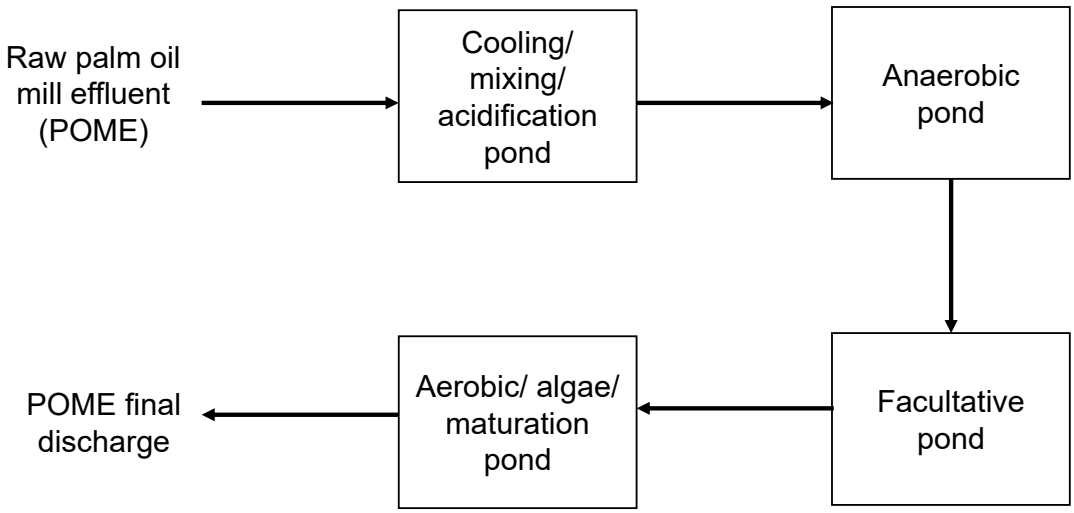



**Figure 2.6** **Typical configuration of ponding system for the treatment of POME** (Source: Zainal et al. (2017)).

Open ponding system has been shown to successfully reduce the concentration of pollutants such as BOD (100 – 610 mg/L), COD (100 – 1725 mg/L) and ammoniacal nitrogen (100 – 200 mg/L) (Chin et al., 1996; Zahrim et al., 2014). However, the ponding system has limitations of long hydraulic retention time (HRT) and require large land space. In addition, the final discharge of POME usually does not meet the limits set by the Malaysian Department of the Environment (DOE). The BOD target of the different stages of POME treatment is illustrated in **Figure 2.7**.

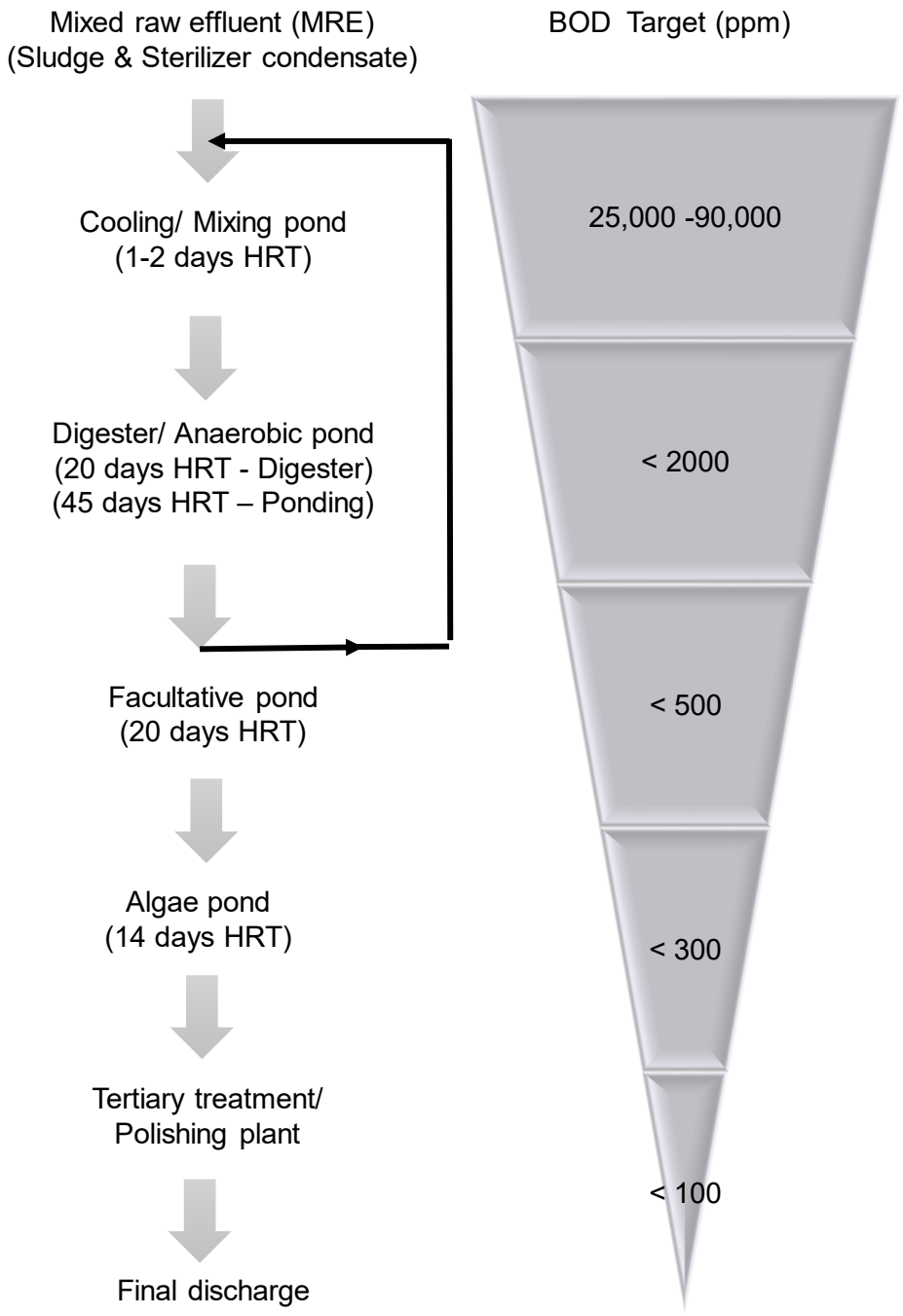



**Figure 2.7** **The target of BOD concentration in the general treatment system of POME in the palm oil mill** (Adapted from Zahrim et al. (2014)).

Among the various treatment methods of POME, anaerobic digestion is considered more efficient as compared to the other methods due to higher treatment effectiveness and the potential of the energy recovery (Wu et al., 2010). According to Ma et al. (1999), the end product of the anaerobic digestion of POME is a mixture of biogas, mainly comprises of methane and carbon dioxide in a ratio of 65:35 and traces of $H_2S$. Approximately, 28 $m^3$ of biogas can be obtained from 1 tonne of POME (Quah and Gillies, 1984). However, due to the variations in POME treatment practices, the methane emission may differ. As reported by Shirai et al. (2003) and Yacob et al. (2005), 35–45% of methane was generated from the anaerobic treatment of POME.

In addition, the anaerobic digestion of organic waste has advantages over aerobic treatment process due to its high organic removal rates and low energy requirement. Anaerobic digestion is also considered as the primary biological treatment process for high organic strength wastewater since it produces less sludge compared to aerobic process (Angenent et al., 2004). The advantages and limitations of several methods for the treatment of POME were listed in **Table 2.2**.

**Table 2.2** **Several methods applied for the treatment of POME with the comparisons of economical, environmental and operational aspects** (Adapted from Choong et al. (2018)).

| Treatment method | Pros | Cons |
|---|---|---|
| **Anaerobic pond** | Low cost<br>Reliable, stable, simple | Large area required, uncaptured greenhouse gases (GHGs)<br>Long HRT |
| **Aerobic**<br>*E.g.: Rotating biological reactor, Activated sludge reactor* | | Very energy intensive<br>Inefficient treatment<br>Requires incorporation of other treatment systems |
| **Anaerobic digestion high-rate closed system**<br>*E.g.: Continuous stirred tank reactor, Upflow anaerobic sludge blanket reactor, Anaerobic fluidized bed reactor* | Captured GHGs, higher treatment efficiency | High cost<br>Relatively more sophisticated |
| **Chemical treatment**<br>*E.g.: Coagulation and flocculation, floatation* | | Extra cost required<br>Inefficient treatment<br>Requires pre-treatment |
| **Physical treatment**<br>*E.g.: Sedimentation, Centrifugation* | Low cost | Inefficient treatment<br>Requires pre-treatment |

### 2.2.3 Palm Oil Mill Effluent Final Discharge

During oil palm processing, substantial amount of water is consumed, thus generating large volume of raw POME amounting 43.29 million $m^3$/year (based on calculation from 2.5 $m^3$ raw POME generated) (MPOB, 2016). According to Wu et al. (2009),

approximately 5.0 to 7.5 tonnes of water would be required to process 1 tonne of CPO and the remaining 50% of this water would end up as POME. Hence, more attention is given to the generation of POME which usually being discharged into the nearby river, either in the form of raw or treated POME that causes an alarming rise in aquatic pollution (Rupani et al., 2010).

It is the mill's responsibility to regularly monitor the quality and quantity of the POME final discharge and ensuring the treated POME meets the standard regulations. Laws and regulations have been enacted in order to prohibit and halt the environmental problem caused by POME. Under the Environment Quality Act of Malaysia 1974, the Environmental Quality (Prescribed Premises) (Crude Palm Oil) Order 1977 and the Environment Quality (Prescribed Premises) (Crude Palm Oil) Regulations 1977, necessary legal instruments were provided for the control of effluent discharged from palm oil mills (Latif et al., 2014).

The DOE of Malaysia proposes more stringent regulations due to the pollution potential of POME final discharge and the failure of many industries to comply with the discharge standard. (Bello and Abdul Raman, 2017). One of the main provisions of the new regulation is that POME should be well treated and obeys the limits in respective discharge standard set by the DOE as presented in **Table 2.3**. Recently, the DOE announced more stringent regulation to reduce the BOD discharge limit from 100 mg/L down to 50 mg/L and 20 mg/L, depending on the location of palm oil mill (Julaidi, 2014; Tabassum et al., 2015). The stringent regulations may continue to evolve as the government and public efforts to protect and sustain the environment.

**Table 2.3 Characteristics of raw POME and final discharge with the respective standard discharge limits set by the DOE of Malaysia.**

| Parameters | Average value (Raw POME) | Average value (POME final discharge) | Current standard discharge limit (DOE, 1982) | Future standard discharge limit (DOE, 2015) |
|---|---|---|---|---|
| COD (mg/L) | 51 000 | 800 | 100 | NA |
| $BOD_3$ (mg/L) | 25 000 | 200 | 100 | 20 |
| pH | 9.0 | 4.2 | 5.0 – 9.0 | 5.0 – 9.0 |
| Temperature (°C) | 85 | 25 | 45 | 45 |
| Colour (ADMI) | 10 000 | 500 | 200 | 100 |
| Total suspended solid (mg/L) | 18 000 | 130 | 400 | 200 |
| Total nitrogen (mg/L) | 750 | 127 | 200 | 150 |
| Ammoniacal nitrogen (mg/L) | 35 | - | NA | NA |
| Total volatile solids (mg/L) | 34 000 | - | NA | NA |
| Oil and grease (mg/L) | 4 000 – 6 000 | - | 50 | 5 |
| Manganese (mg/L) | 2.0 | - | 10 | 10 |
| Zinc (mg/L) | 2.3 | - | 10 | 10 |
| Copper (mg/L) | 0.8-0.9 | - | 10 | 10 |
| Iron (mg/L) | 180 | - | 50 | 50 |
| Phosphorus (mg/L) | 180 | - | NA | NA |
| Potassium (mg/L) | 2270 | - | NA | NA |
| Magnesium (mg/L) | 615 | - | NA | NA |
| Boron (mg/L) | 7.6 | - | NA | NA |

Notes: NA: not available, ADMI: American Dye Manufactures Institute (Source: Zainal et al. (2017))

## 2.3 Biochemical Reactions Involved in the Treatment of Palm Oil Mill Effluent

A series of biochemical reactions involved during the treatment of POME in which the organic material is decomposed through the metabolic pathways of naturally occurring microorganisms. Various types of microorganisms were successfully isolated from POME that were functioned in different biodegradation processes as listed in **Table 2.4**. Anaerobic digestion can be used to process any carbon-containing material with various degrees of degradation. However, the implementation of anaerobic digestion technology for the treatment of POME is not straightforward. Anaerobic degradation is a complex process and performed by a well-organised community of microorganisms. Some of the microbial groups involved are slow-growing and sensitive to the changes in operating conditions. This can cause instability during both the start-up and operation of the anaerobic process (Yacob et al., 2006). In addition, to make the biogas process more attractive from a commercial point of view and to facilitate the increased integration into our energy supply systems, these instability problems must be overcome in an economically viable way.

**Table 2.4 Microorganisms isolated from POME that involved in the biodegradation processes** (Adapted from Tan et al. (2015)).

| Type of bacteria | Function/Role | Carbon/ Energy Source | Reference |
|---|---|---|---|
| **Hydrolytic bacteria** | | | |
| *Clostridium* sp.<br><br>*Pseudomonas* sp. | Produce cellulose and xylanase for hydrolytic purpose | Cellulose Hemicellulose | Khemkhao et al. (2015)<br>Elijah et al. (2013) |
| *Bacillus* sp.:<br>- *B. licheniformis*<br>- *B. firmus* | Degrade plant dry matter | Lignocellulosic biomass | Zainudin et al. (2013) |
| *Cellulomonas* sp. | Synthesize acid from glucose | Cellulose | Hii et al. (2012) |
| *Micrococcus luteus*<br>*Stenotrophomonas maltophilia* | Break down lipids into fatty acids and glycerol | Cellulose, Lipids | Bala et al. (2014a) |
| **Acidogenic/Fermentative bacteria** | | | |
| *Thermoanaerobacterium* sp.:<br>- *T. thermosaccharolyticum*<br>- *T. aotearoense*<br>- *T. polysaccharolyticum*<br>- *T. aciditolerans* | Produce $H_2$ and butyric acid | Celullose | Mamimin et al. (2012) |
| *Clostridium* sp.:<br>- *C. butyricum*<br>- *C. paraputrificum*<br>- *C. beijerinckii PS-3* | | Celullose Carbohydrates | Chong et al. (2009); Yossan et al. (2012) |
| *Enterobacter* sp.:<br>- *Enterobacter asburiae*<br>- *Enterobacter cloacae* | Produce $H_2$ gas from carbohydrates and fatty acids | Fatty acids, carbohydrates | Wong et al. (2014) |
| **Acetogenic Bacteria** | | | |
| *Clostridium thermocellum* | Produce acetate and hydrogen | Cellulose | Nitipan et al. (2014) |
| *Syntrophomonas* sp.*:*<br>- *Syntrophomonas sapovorans* | Degrade fatty acids in association with methanogens | Fatty acids | Ahmad et al. (2011b) |

| | | | |
|---|---|---|---|
| - *Syntrophomonas curvata*<br>- *Syntrophus aciditrophicus* | | | |
| **Sulphate-reducing Bacteria** | | | |
| *Clostridium botulinum* | Reduce sulphate | Carbohydrates | Saidudu (2014) |
| *Desulfovibrio aerotolerans* | Decompose fatty acids | Fatty acid | Wong et al. (2014) |
| *Desulfotomaculum* sp. | Produce $H_2$ gas and acetate during fermentation | Fatty acid, glucose | Nitipan et al. (2014) |
| **Methanogens (Archaea)** | | | |
| *Methanobacterium* sp. | Convert $H_2$ into methane | Fatty acid, carbon | Poh and Chong (2009) Poh et al. (2010) |
| *Methanospirillum hungatei* | | $CO_2$, acetate | Demirel and Scherer (2008) |
| *Methanosaeta* sp.:<br>- *M. thermophila*<br>- *M. concilii* | Convert acetate into methane | $CO_2$ | Tabatabaei et al. (2009) |
| *Methanosarcina* sp.:<br>- *M. thermophila*<br>- *M. acetivorans*<br>- *M. barkeri* | | Acetate | Walter et al. (2015) |

In an anaerobic process, the POME is degraded into methane, carbon dioxide ($CO_2$) and water which involves hydrolysis, acidogenesis (including acetogenesis) and methanogenesis (Bitton, 2005; Hassan et al., 2005). The scheme of anaerobic degradation process of POME is illustrated in **Figure 2.8**.

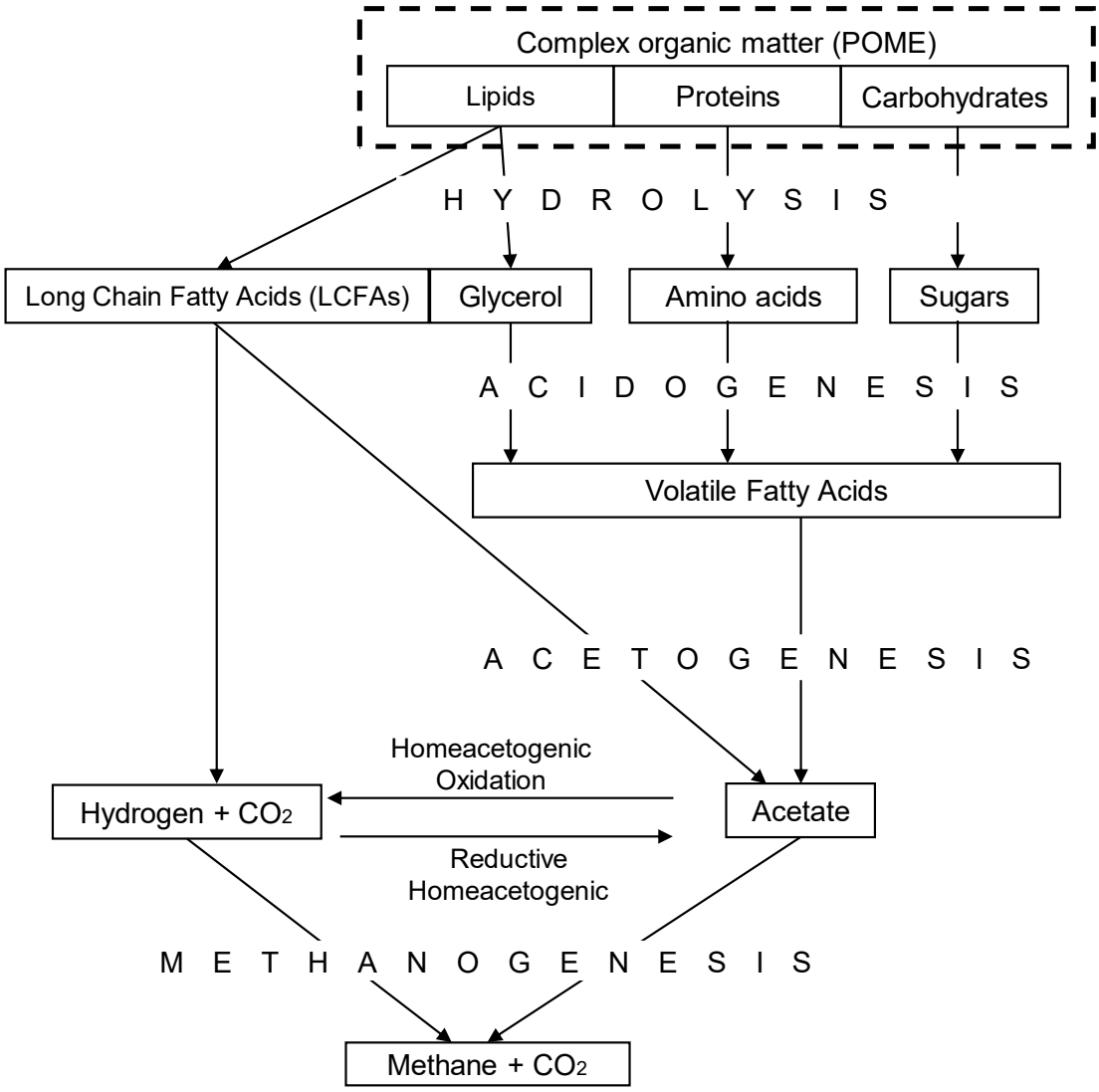



**Figure 2.8** **Scheme of an anaerobic degradation process for the treatment of POME which involves hydrolysis, acidogenesis, acetogenesis and methanogenesis** (Source: Ohimain and Izah (2017)).

### 2.3.1 Hydrolysis

The first phase in anaerobic digestion is hydrolysis, a process of converting complex substrates into simple smaller more soluble molecules. It is a slow process and is the rate-limiting reaction in the acid forming phase. Complex carbohydrates, lipids and proteins are converted into smaller soluble masses such as glucose, alcohol, amino acids and volatile fatty acids which in turn are immediately converted to shorter chain volatile fatty acids generating carbon dioxide ($CO_2$) and hydrogen ($H_2$) as by-products (Singhania et al., 2013). Several hydrolytic enzymes such as cellulases, cellobiose, xylanase, amylase, lipase and protease which are secreted by microbes attached to a polymeric substrate (Lam and Lee, 2011) enable the hydrolysis to occur.

A number of bacterial species involved in the polymer hydrolysis phase have been isolated from POME, including species belonging to the genera *Clostridium* (Khemkhao et al., 2015), *Bacillus* (Zainudin et al., 2013), *Cellulomonas* (Hii et al., 2012), *Micrococcus* and *Stenotrophomonas* (Bala et al., 2014a). As the polymer hydrolysis phase occurs extracellularly, it does not directly contribute to the energy harvesting metabolism of the involved microbial community. Thus, the bacterial species active in the polymer hydrolysis phase are also active during the acidogenesis phase in order to gain energy for cell growth and replication. The polymer hydrolysis phase of biochemical reaction is generally considered to be the primary rate limiting step for the conversion of waste feedstocks that has a high concentration of recalcitrant compounds such as cellulose (Yadvika et al., 2004).

### 2.3.2 Acidogenesis

Hydrolysis is immediately followed by the acid-forming phase known as acidogenesis. In this process, acidogenic bacteria produced acetate, propionate, butyrate and iso-butyrate (alcohol and lactate. Abundant of facultative anaerobes and strict anaerobes degrade soluble monomers from hydrolysis stage in fermentation process producing carbon dioxide ($CO_2$), hydrogen gas ($H_2$), alcohol and organic acid. Although some of end-product for example acetate, $H_2$ and $CO_2$ is been directly utilized by methanogens prior to biogas production, other intermediates such as volatile fatty acid can cause digester failure (Hattori et al., 2008; Zhang et al., 2015). The specific concentrations of products formed in this stage vary with the type of bacteria as well as with the culture conditions, such as temperature and pH. Final products of fermentation eventually become precursors for methane production (Lam and Lee, 2011).

$H_2$ is produced is produced in the fermentative pathways. The $H_2$ production is required for an anaerobic digester where $H_2$ is an important substrate for the production of methane. It is crucial to ensure the level of $H_2$ is low since the acetogenic bacteria in an anaerobic degradation process can be inhibited by the high pressure of $H_2$ (Gerardi, 2006). Besides that, acidogenesis and methanogenesis phases are in dynamic balance in an anaerobic digestion system. After the conversion of organic matters into volatile acids and $H_2$, it will be converted into methane and $CO_2$ (Kim et al., 2003).

A wide variety of microbes have been identified to participate in the acidogenesis phase, including from the phyla *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and

*Proteobacteria* (Mamimin et al., 2012; Poh et al., 2010; Tanikkul et al., 2016). In addition to the genera listed for polymer hydrolysis phase, genera of bacteria which solely participate in the acidogenesis phase including those belonging to the family *Anaerolineaceae* in the phylum *Chloroflexi* (Wen et al., 2015), the genera *Bifidobacterium* and *Paludibacter* in the phylum *Bacteroidetes* (Ambuchi et al., 2016) and thermophilic bacteria in the phylum *Thermotogae* (Khemkhao et al., 2015).

### 2.3.3 Acetogenesis

Acetogenesis occurs through carbohydrate fermentation where acetate is produced as a main product, in a combination with $CO_2$ and $H_2$. The role of $H_2$ as an intermediary product is of critical importance to anaerobic digestion reactions. Long chain fatty acids generated from hydrolysis of lipids are oxidised to acetate or propionate with the formation of $H_2$ gas. Under standard conditions, the oxidisation might happen with the presence of $H_2$ in the solution. Therefore, a low $H_2$ partial pressure is required to thermodynamically allow the conversion to occur (Ostrem, 2004). This can happen with the presence of hydrogen scavenging bacteria that consumes $H_2$. The concentration of $H_2$ measured by partial pressure is an indicator of the stable (active) of a digester.

A group of bacteria known as syntrophic acetogens are responsible for the conversion of non-acetate short chain fatty acids (SCFAs) into acetate, $CO_2$ and $H_2$. Genera such as *Desulfobulbus*, *Syntrophobacter* and *Smithella* of the order *Deltaproteobacteria* are capable of converting propionate to acetate (Stams et al., 2012), while members of

*Syntrophomonas* of the phylum *Firmicutes* are capable of converting butyrate and longer chain of SCFAs (Ahmad et al., 2011a; Hatamoto et al., 2007).

The syntrophic label for these bacteria comes from the requirement of the presence of a group of hydrogen utilizing organisms in order to preserve a favourable thermodynamic gradient for the conversion of SCFAs to acetate. This is often a hydrogen utilising methanogen, however reports have indicated that sulphate reducing bacteria are also capable of supporting syntrophic acetogenesis under conditions of high sulphate concentration (Ozuolmez et al., 2015).

### 2.3.4 Methanogenesis

Methanogenesis or methane fermentation is exclusively carried out by a specialised group of microorganisms, the archaeal methanogens. Methanogenesis is the rate-controlling portion of the process because methanogen has slower growth rate than acidogens. Methanogens are very sensitive to changes and prefer a neutral to slightly alkaline environment and hardly to survive at pH below 6.

The methanogens converts the soluble matter into methane (Ostrem, 2004). The methanogens are conceptually divided into three groups according to phylogenetic and phenotypic similarity (Anderson et al., 2009; Bapteste et al., 2005). The first group (Class I methanogens) including the orders *Methanobacteriales*, *Methanococcales* and *Methanpyrales*, while the second and third groups (Class II and Class III methanogens, respectively) including the order *Methanomicrobiales* and *Methanosarcinales*,

respectively (Anderson et al., 2009). This distinction is important as the Class I and II methanogens are both regarded as being hydrogenotrophic, in that they only use formate, and/or $CO_2$ and $H_2$ as their substrates for methanogenesis. The Class III methanogens, on the other hand, are characterised as being acetoclastic or acetotrophic, being able to use acetate and depending on the particular genus, methanol, methylamines or other one carbon compounds (Anderson et al., 2009). Although anaerobic digestion can be considered to take place in these four stages, all processes occur simultaneously and synergistically.

About 66% of methane is generated and approximately 34% of $CO_2$ is produced from acetate decarboxylation through acetoclastic pathway. According to Hattori et al. (2008), the degradation of acetate is taking place continuously via two pathways; firstly, acetate cleavage by acetotrophic methanogens which is accomplished by two genera of methanogens, *Methanosarcina*, *Methanosaeta* and syntrophic acetate oxidation (Hattori et al., 2008). Secondly, a two-step reaction in which acetate is oxidised to $H_2$ and $CO_2$ and these products afterwards are converted to methane. At this stage, *Clostridium* spp. (acetate oxidizing bacteria) and *Methanobacteriales* or *Methanomicrobiales* (hydrogenotrophic methanogens) completed the reaction (Sasaki et al., 2011).

The degradations of acetate are significantly influenced by the environmental conditions such as temperature, concentrations of ammonia and amino acids, the types of reactors and organic loading rate (Ji et al., 2013). Typical reactions of anaerobic digestion for the generation of methane are listed as follows:

$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + CO_2$ [1]

$C_2H_5OH+CO_2 \longrightarrow CH_4+ 2CHOOH$ [2]

$CH_3COOH \longrightarrow CH_4 + CO_2$ [3]

$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$ [4]

## 2.4 Assessment of Bacterial Community Composition

The vast diversity of the bacterial communities underpinning the wastewater treatment and the complexity of the interactions between different trophic groups and the environmental conditions remain poorly understood. The metabolism and lifestyle of most of the microbes also remain unknown. Conventional methods are commonly used to assess microbial communities in the environment which commonly based on cultivation, also known as traditional culture methods. These methods mostly rely on culturing microbes using growth media such as Luria-Bertani medium, nutrient agar and tryptic soy agar, followed by the identification of pure culture. Typically, the isolation of pure cultures applying several growth steps is necessary to characterise the bacteria in more detail (Sousa and Pereira, 2013).

Consequently, the conventional methods are labour-intensive and time-consuming, since the results are not available until at least 1–3 days (Braga et al., 2013; Van Belkum et al., 2013). Moreover, the culture-based methods have the limitation whereby most of the microorganisms do not able to grow on artificial media. It has been suggested that more than 99% of environmental microorganisms cannot be cultured in the laboratory and only 0.1 – 1.0% are cultivable using standard culturing

techniques (Bing-Ru et al., 2006; Hugenholtz et al., 1998; Ranjard et al., 2000; Su et al., 2012). However, with the development of the molecular fingerprinting techniques and high-throughput sequencing technologies, the complex microbial communities involved in the various wastewater treatment systems can be comprehensively investigated.

### 2.4.1 Assessment of Bacterial Community using Molecular Fingerprinting Techniques

Microbial community profiling is commonly used to evaluate and compare between different microbiomes in various types of environments. The 16S ribosomal RNA (rRNA) gene has been almost exclusively used as a marker gene in the studies of microbial communities, either community composition or population dynamics. The 16S rRNA gene is a phylogenetic marker for both bacteria and archaea. This gene is composed of nine hypervariable regions interspersed with conserved regions (Van et al., 1996). The reason it was chosen as the marker gene over other rRNA genes (i.e., 5S or 23S rRNA gene, about 120 bp and 3,000 bp, respectively) is because its length (about 1,600 bp) is long enough to reveal the phylogenetic information needed to distinguish different bacteria and archaea but short enough to be easily sequenced. In addition, the mosaic structure of both hypervariable regions and the conserved regions allow the design of specific probes and primers at different taxonomic levels.

Furthermore, because of the common acceptance of 16S rRNA genes as the marker for phylogenetic studies of microbiomes, several large public databases dedicated to

16S rRNA genes have been constructed. These databases including Greengenes (http://greengenes.lbl.gov/), Silva (http://www.arb-silva.de/), and Ribosomal Database Project (RDP) (http://rdp.cme. msu.edu/) greatly facilitate archiving and analysing the phylogenetic of 16S rRNA genes.

A variety of 16S rRNA-based techniques have been developed and applied in the different studies of microbial ecology in the wastewater as listed in **Table 2.5**. Among various molecular fingerprinting techniques, a combination of PCR amplification of 16S rRNA genes with denaturing gradient gel electrophoresis (DGGE) is widely used by many researchers where the technique is well-established for investigating the microbial community compositions in various types of wastewater treatment systems, mainly due to its technical simplicity, rapidness and low cost (Shin et al., 2010; Zamanzadeh et al., 2013). Nelson et al. (2011) in their study claimed that the DGGE profiles concurred with the detailed profiles of microbial communities in several digesters that were determined using 454 pyrosequencing. DGGE can also be a useful tool to identify representative samples to be further analyses by deep sequencing, especially when it involves a large number of samples.

**Table 2.5 Molecular fingerprinting techniques applied to assess the composition of microbial community in various types of wastewater.**

| Methods | Type of wastewater | References |
|---|---|---|
| Denaturing gradient gel electrophoresis (DGGE) | Industrial wastewater | Yao et al. (2010) |
| | Sewage treatment plant | Li et al. (2013) |
| Random amplified polymorphic DNA (RAPD) | Dye industry | Kapley et al. (2007) |
| Terminal restriction fragment length polymorphism (T-RFLP) | Olive mill effluent | Bertin et al. (2006) |
| | Full-scale wastewater treatment plant | Wells et al. (2011) |
| | Coking effluent | Felföldi et al. (2010) |
| Length heterogeneity PCR (LH-PCR) | Pulp and paper mill | Tiirola et al. (2003) |
| Single-strand conformation polymorphism (SSCP) | Treated effluents from agriculture, food processing and petrochemical industries, pulp and paper plant, breweries, slaughterhouses and municipal waste | Leclerc et al. (2004) |
| Amplified ribosomal DNA restriction analysis (ARDA) | Wastewater treatment plant | Gich et al. (2010) |
| Ribosomal intergenic spacer analysis (RISA) | Municipal wastewater | Lapara et al. (2011) |

DGGE gel is made up of polyacrylamide solution containing a DNA denaturant of urea and formamide at two different gradients. In PCR-DGGE approach, the PCR products are obtained from environmental DNA using a set of primers for a specific molecular marker. The GC clamp is attached to the 5' end of one of the primers, in order to avoid the total denaturation which causes the PCR products to migrate without sticking on the gel as a single-stranded DNA (Muyzer, 1999). At the end, as the sequence variation among different PCR amplicons determines the melting behavior, hence, different sequences will stop migrating after reaching a certain position in the gel. The DGGE fingerprint profiles can then be further analysed by referring to the banding patterns which include the number, position, and intensity of the bands in a DGGE gel to estimate the number and relative abundance of dominant species in the sample (Tan et al., 2000).

Various studies have implemented the PCR-DGGE method for the assessment of microbial community structure in the different types of wastewater. For instance, PCR-DGGE is used in the characterisation of microbial community composition in a full-scale domestic (Ding et al., 2011) and industrial wastewater treatment system (Moura et al., 2009), pharmaceutical wastewater (LaPara et al., 2002), sewage treatment plant (Hesham et al., 2011) and olive mill waste (Vivas et al., 2009). Besides that, PCR-DGGE has also been implemented to study the impact of wastewater treatment towards microbial diversity such as in the anaerobic digestion (Shi et al., 2013; Shin et al., 2010), aerobic (Yang et al., 2008) and constructed wetland (Ibekwe et al., 2003; Moura et al., 2009; Zhong et al., 2014).

However, the major limitation of DGGE lies on the difficulty to obtain sequence information of the 16S rRNA gene fragments. To putatively identify the bands on DGGE gels, a set of amplicons from known species can serve as references, but such identification is not reliable and was only used many years ago (De Souza et al., 2004). As for DGGE, although individual DNA bands in the gel could be excised out, re-amplified and sequenced directly or after cloning, it has been reported that one band could contain more than one DNA sequences, making it difficult to reliably identify the bacteria or methanogens represented by individual bands (Muyzer, 1999). Nevertheless, despite the DGGE limitation, this method can still be used to obtain a snapshot of the microbial communities from large number of samples (Kirk et al., 2004).

### 2.4.2 Assessment of Bacterial Community using High-throughput Sequencing

High-throughput sequencing involves large scale sequencing that allows an in-depth investigation of the microbial communities. It becomes important in representing unbiased view of phylogenetic tree and functional diversity of environmental microbial communities. Large-scale sequencing techniques have been applied in many studies such as metagenomics (Delforno et al., 2017; Guo et al., 2017), proteogenomics (Heyer et al., 2017), metatranscriptomic (Hassa et al., 2018; Xia et al., 2018) and whole genome sequencing (Moradigaravand et al., 2018) due to its capability of producing billion of sequence reads at low cost with high speed. The developments of instruments, new sequencing chemistries and bioinformatics have transformed the area of microbial ecology and genomics.

DNA sequencing technology includes Roche/454, Illumina GA/Solexa and SOLiD/Applied Biosystem. These technologies are much faster, accurate, easy-to-operate and less expensive as compared to traditional Sanger's dideoxy sequencing of cloned amplicon and they have been widely used in investigating microbial diversity in various types of wastewater as listed in **Table 2.6**.

**Table 2.6 Various types of wastewater analysed using high-throughput platform to reveal microbial diversity profiles.**

| Type of wastewater | High-throughput platform | Reference |
|---|---|---|
| Municipal wastewater | Illumina MiSeq | Mwaikono et al. (2016) |
| Industrial wastewater | Illumina MiSeq | Selvarajan et al. (2018) |
| Coking wastewater | Illumina MiSeq | Ma et al. (2015b); Shi et al. (2015) |
| Olive oil wastewater | Illumina MiSeq | Maza-Márquez et al. (2017) |
| Industrial and municipal wastewater | Pyrosequencing Roche 454GS-FLX Titanium | Shu et al. (2015) |
| Wastewater from food industry | Pyrosequencing Roche 454GS-FLX | Kim et al. (2014) |
| | Illumina MiSeq | Liu et al. (2016) |
| Domestic and industrial wastewater | Pyrosequencing Roche 454GS-FLX | Ibarbalz et al. (2013) |
| Coal mine | Illumina MiSeq | Ma et al. (2015a) |
| Swine wastewater | Illumina MiSeq | Ye et al. (2016) |
| Starch wastewater | Illumina MiSeq | Antwi et al. (2017) |
| | Pyrosequencing Roche 454 GS-FLX Titanium | Lu et al. (2018) |
| Brewery wastewater | Pyrosequencing Roche 454 GS-FLX titanium | Miran et al. (2015) |
| Wastewater from pulp and paper industry | Pyrosequencing Roche 454 GS-FLX-Titanium | Gupta et al. (2017) |
| Industrial dye wastewater | Illumina MiSeq | Tan et al. (2017) |

The NGS technologies share the same principle of massive paralleling sequencing, but the sequencing chemistry, read length, running time, throughput per run and reads per run are slightly different between one another (Bella et al., 2013). As compared to other technologies, the Illumina (**Table 2.7**) uses pair-ended overlapping reads which has several advantages including the increase of total fragment length, enhance the sequence quality (Kuczynski et al., 2011; Lazarevic et al., 2009) and also produce a very low error rate as compared to other bench-top sequencers (Bella et al., 2013).

**Table 2.7 The properties and applications of different Illumina platforms for an amplicon sequencing** (Adapted from Liu et al. (2012)).

| | MiSeq | HiSeq2000 | HiSeq2500 |
|---|---|---|---|
| Read length | 50, 150, 250 or 300 bp; single read or paired end | 50 or 100 bp; single read or paired end | 50, 100 or 150 bp; single read or paired end |
| Run time | 6 hours – 3 days | 3 – 12 days | 3-12 days in standard mode, 1 – 5 days in rapid mode |
| Data generated per lane | 1 – 25 million fragments sequenced in parallel; 0.5 – 15 Gbases data output | 100 – 200 million fragments sequenced in parallel; 7.5 – 35 Gbases data output | 100 – 150 million fragments sequenced in parallel; 7.5 – 35 Gbases data output |
| Applications | Prokaryotic ChIPseq, small RNAseq, small genome resequencing and targeted capture | RNA-Seq, large genome resequencing and targeted sequencing | *De novo* genome sequencing, large genome resequencing and targeted sequencing; where quick data turn around |

### 2.4.3 Statistical Analysis, Data Representation and Visualisation of High-throughput Data

While modern molecular technologies have allowed for greater and more accurate interrogation of microbial diversity in wastewater treatment systems, the resulting datasets are often not amenable to direct sample comparison. To address this issue, a number of statistical description and comparison methods are used to reduce the complexity of the original dataset and allow for comparison of diversity between samples. The majority of these methods were developed by classical biological ecologists studying relationship of macrobiotic species such as plants or birds in defined environmental plots, however there is support for their application to microbial datasets (Hughes et al., 2001).

#### 2.4.3.1 Diversity and Diversity Indices

Diversity is an important indicator of stability of an ecosystem. The concept and definition of alpha, beta and gamma diversity were proposed earlier by Whittaker (1972). Alpha diversity refers to the richness and evenness within a community, while beta diversity compares the richness and evenness among different communities. As for gamma diversity, it is an overall diversity of a defined region and represented as geographical diversity (Hunter and Gibbs, 2006). These diversity terms and evaluation methods were initially developed for and applied to ecosystems at macro scale, such as forest and fishery. When applied to microbial ecology, alpha and beta diversities

are commonly accepted to describe the diversity in individual samples and among different samples.

Several methods have been developed to evaluate alpha diversity of a community. The simplest one is species richness (S), which is the observed number of species in a given community. It is easy and straightforward when applied to macro ecosystems (such as forest) because individual species, often a small number, can be morphologically identified and counted. However, species richness cannot be determined because no robust and reliable species concept is available for microbes and it is often difficult to ascertain if all the 'species' in a community have been accounted for.

A practical taxonomic unit equivalent to species of microorganisms is operational taxonomic unit (OTU), which is typically defined based on a sequence similarity of 16S rRNA genes. However, true OTU richness is still difficult to determine because not all the OTUs present in a sample can be identified. In most studies, thus, 'true' OTU richness is estimated using rarefaction (Hurlbert, 1971; Sanders, 1968), Chao1 (Chao, 1984) or abundance-based coverage estimator (ACE) (Chao and Lee, 1992). Different models are used in each method and the estimates often differ considerably. Another challenge associated with determining OTU richness stems from huge difference is the number of sequences (ranging from 1 to hundred thousand of sequences) representing each OTUs. Given the high error rates of sequencing data from high-throughput technologies, OTUs represented by a small number of sequences are discarded as artificial ones. However, the cut-off values used in all the studies were arbitrary.

The Shannon diversity index (H') and Simpson diversity index (D) are commonly used to determine the alpha diversity of the associated microbes. Both indices take into account the richness and evenness of the species (OTUs) detected. Shannon diversity index (H') is first proposed by Claude Shannon (Shannon, 1948) and is commonly applied to ecological data. The Shannon index provides a measure of both richness and evenness in a population such that a community with perfect evenness would have a value of H' equal to the natural log of the number of observed species ($H_{max}$). The Shannon equitability measure ($E_H$) is the ratio of H' to $H_{max}$ and provides an additional measure of the evenness of a sample, with a value of 1 indicating a sample has complete evenness. Meanwhile, Simpson diversity index is in a scale from 0 to 1, with a value of 0 representing a completely homogenous community, while a value of 1 representing a completely heterogeneous community (Simpson, 1949).

Meanwhile, beta diversity is the comparison of diversity between samples. Instead of a single value like alpha diversity index, beta diversity is calculated as the distance (or dissimilarity) between a pair of samples. Thus, if more than two samples are compared, a distance (or dissimilarity) matrix will be generated to represent the beta diversity. Many methods have been developed to generate distance (or dissimilarity) matrix, such as Bray-Curtis, Ochiai and Unifrac. The first two methods are non-phylogenetic, with the Bray-Curtis method taking into account the abundance of species or OTUs, while the Ochiai method only comparing the presence or absence of species or OTUs. On another note, Unifrac is a method to generate distance matrix based on phylogenetic trees constructed from the species present in the communities to be

compared. It is specifically developed for 16S rRNA sequences (Lozupone et al., 2007) and widely used in analysis of beta diversity of microbiomes.

#### 2.4.3.2 Multivariate Analysis

While the determination and comparison of the alpha and beta diversity in and between different environments can provide beneficial insight into the diversity and distribution of microbes in an environment, it is unable to explain how microbial communities react to and influence various environmental parameters. Multivariate analysis of large ecological datasets allows reduction of data complexity, so that major patterns and correlation between populations and environmental factors can be established.

In environmental microbiology, principal coordinate analysis (PCO) has been increasingly used in visualising beta diversity and revealing the relationship between bacterial community composition and source of pollutants (Ibekwe et al., 2012) or geological distributions (Cao et al., 2011; Hong et al., 2011). Canonical correspondence analysis (CCA) is another ordination method which can help to explain the variations of community structure patterns in wastewater by revealing the potential correlations between microbial communities or specific groups of microbes with environmental variables. Although multivariate analysis is a powerful tool to elucidate the correlation between microbial community and the environmental factors, extra caution should be taken when interpreting the results and over-interpretation should be avoided because the synthetic variables, axes or clusters derived do not necessarily always have biological meanings (James and McCulloch, 1990).

## 2.5 Bioindicator for Environmental Monitoring

The environmental quality of water, soil and air is increasingly degraded due to the presence of various types of pollution. Therefore, we need to raise the prevention of pollution by monitoring environmental quality. Biological methods were commonly used to monitor the environmental quality by assessing the presence of several species, such as plants, insects, fish, bacteria and viruses as environmental indicators.

Bioindicators are organisms or a species or a group of species, whose function and/or population status can be used to monitor the status of health of the environment in which they live. Changes in the population status, behaviour and physiology of such organisms could be used to predict the occurrence of an environmental problem within a given ecosystem. Bioindicators provide an adequate information that would be difficult to obtain and quantify by other means, or at least not as easily or quickly (Hopkin, 1990; Lindenmayer et al., 2000). Species are identified as bioindicators when their abundance and population fluctuation clearly vary in response to any environmental change in a particular habitat (Mouillot et al., 2002).

Indicator species can be categorised into early warning, diagnostic and compliance. Early warning indicators can reveal the first signs of disturbance in the environment before most other species are affected. Diagnostic indicators are those used to investigate observed environmental disturbances. Compliance indicators are those species, which are used to verify maintenance or either restoration goals have been achieved (Noss, 1990). Bioindicators are habitat specific and react immediately in response to environmental changes. Such species and their habitats are closely linked,

hence it is impossible for such species to resist environmental changes (Celli and Maccagnani, 2003).

The reliability of bioindicators with their habitat could be assessed by sampling in different weather conditions or seasons. It is the specific assemblage of organisms and their environment that makes certain organisms' as a preferable bioindicators (McGeoch et al., 2002, Celli and Maccagnani, 2003). Species that cannot normally live outside forests, grasslands or cultivated lands, those responding to a particular soil management practice and those supported by water logging are examples of bioindicators (Hopkin, 1990; Paoletti, 1999). Therefore, by using bioindicators, it is possible to assess areas of biochemical contamination; impacts monocultures, disposal, industrial and urban settlements and areas neighbouring to power plants.

### 2.5.1 Microbial Indicators for Environmental Monitoring

Microbial indicators have major roles in the agro-ecological management of soil and for application of nutritional improvements. They are considered as efficient ecosystem management tools (Díaz, 2009). Microorganisms present in effluent treatment plants are also known to be responsible in improving the water quality. In addition, different hazardous waste disposal sites could be monitored using target microorganisms. For example, bioluminescent bacteria have been employed to assess the toxicity of heavy metals in aquatic habitats. Bioluminescent bacteria, which emit light as a result of enzymatic mediated chemical reactions involving phosphorus containing molecules, are being used to test water for environmental toxins. When

toxins are present in the water, the cellular metabolism of the bacteria is inhibited or disrupted. This affects the quality or amount of light emitted by the bacteria.

Moreover, microorganisms can also be used as indicators of aquatic or terrestrial ecosystem health. For instance, *Cynobacteria* in the Ethiopian Lake Beseka are dominantly found throughout the dry period and their abundance is associated with excess nutrient loads, particularly phosphate, and the relatively high temperature that favours their growth in that area (Zelalem, 2007). Some microorganisms can even produce stress proteins when exposed to contaminants like cadmium and benzene, hence could be used as an early warning system to detect low levels of pollution.

The monitoring of environmental quality based on bacterial indicator has been widely used to indicate the source of pollution in the water bodies as shown in **Table 2.8**. The criteria of a species to be indicator are: 1) it must be in the polluted water when polluting sources of pathogenic microorganisms were found, 2) not reproduced within the environment, 3) must be found in a larger number than the pathogenic microorganism, 4) must respond to natural environment conditions and water treatment process in the same manner with particular pathogen species, 5) it should be easy to be isolated, identified and counted, 6) must be low cost to allow numerous sampling, and 7) not turn into pathogenic microorganisms (to minimise health risks in the analysis) (Payment and Locas, 2011).

**Table 2.8 Potential bacterial indicators to indicate different types and sources of contamination in the water bodies.**

| Pollution | Site | Bioindicator | Reference |
|---|---|---|---|
| Oil spill | Cíes Island (Atlantic Isle National Park, Galicia, Spain) | *Myxococcales, Desulfarculales* | Acosta-González et al. (2013) |
| Untreated and treated wastewater treatment plant | Beijing-Tianjin region of Haihe River | *Cyanobacteria, Thaumarchaeota, Campylobacteraceae, Methanosaetaceae* | Bai et al. (2014) |
| Perfluorooctanoic acid | Xiaoqing River in Shandong Province of China | *ε-Proteobacteria, Thiobacillus, Sulfurimonas* | Sun et al. (2016) |
| High level nutrient and antibiotics | Hongqi River, Jiangsu Province, China | *Clostridium difficile, Arcobacter butzleri* | Jia et al. (2017) |
| Bisphenol A (BPA) and 4-nonylphenol (NP) | Qinhuai River | *Comamonadaceae, Pseudomonadaceae, Alcaligenaceae, Bacillaceae, Sphingomonadaceae, Burkholderiaceae, Rhizobiaceae* | Cai et al. (2017) |
| Uranium | Cauvery River | *Photobacterium, Halomonas, Idiomarina, Marinobacter, Aeromonas, Loktanella, Gluconobacter, Desulfovibrio* | Suriya et al. (2017) |
| POME final discharge | River water (approximately 3 km from palm oil mill), Malaysia | *Chromatiaceae*, *Alcaligenaceae* | Sharuddin et al. (2017) |

Other than that, the bioindicators are potentially useful tools in assessing sustainable forest management (Paoletti, 1999; Kotwal et al., 2008). Bioindicators are slightly different from key indicators, even though both are useful in deducing information about the environment. Bioindicators tell us information about the environment through their population or particular response to changes in the ecosystem, while key indicator species are those species which are essential to the ecosystem and if such species are affected, the whole ecosystem will perish. There are evidences that the richness of certain taxonomic groups is an indication of overall species richness in the area and by measuring the relative abundance of the indicator species, it is possible to assess the overall biodiversity (Hess et al., 2006). In the most rapidly changing habitat types, indicator species could be monitored to determine whether population trends match variations in landscape composition and patterns. Hence, indicator species can be used as a conservation tool to identify biodiversity hot spots, if spatial patterns of species richness agree across taxa (Betts et al., 2003; Hess et al., 2006, Dalmir and King, 2007; Dung and Webb, 2007).

### 2.5.2 Advantages and Disadvantages of Bioindicators

Bioindicators are important in environmental monitoring because of various reasons. The effect of pollutants on indicator species is clearly recognised and its application is relatively cost effective (Spiegel, 2002). Besides, bioindicator-based studies usually need simple techniques, which can be easily repeated by different individuals from time to time. The method is feasible in different ecosystems and it is also suitable in assessing large areas (Paoletti, 1999). Indicator species are also important in monitoring the environment because they are easier to interpret and less ambiguous than directly sampling and assessing all plant and animal communities found in a given ecosystem (Dale and Beyeler, 2001).

Regardless of their advantages, the application of bioindicators in environmental monitoring has its own limitations. Indicator species should be present at the given locality with sufficient number of individuals, they have to be wide-spread in the locality under investigation and the physiological processes of uptake and retention of toxic substances or environmental contaminants by the bioindicators should be well known (MeGeoch et al., 2002; Mouillot et al., 2002).

The goal of POME treatment is to reduce the polluting power of this wastewater below the effluent discharge standard before being released into the environment, but different biotreatment processes have been employed in the different palm oil mills. In addition, the palm oil waste generated by each palm oil mill was based on the amount of FFB processed (Liew et al., 2017). Theo et al. (2017) in their study even showed

different generations of biomass and biogas depending on the FFB processing capacities of the palm oil mills. Hence, it remains unclear how these different biotreatment processes and capacities could affect the result of the previous study which determined that *Alcaligenaceae* and *Chromatiaceae* were the bioindicators. Since the bacteria are highly sensitive to disturbances and could react differently depending on the properties of pollutants, environmental factors and treatment processes, it is important to assess the practical relevance of the bioindicator for monitoring the river water quality. In this study, the presence of *Alcaligenaceae* and *Chromatiaceae* in the POME final discharge generated by different biotreatment processes of POME was assessed in correlation to the changes of pollutant properties, hence reliable bioindicators could be developed.

## 2.6 Concluding Remarks

The increase generation of POME is an environmental concern due to its highly polluting properties which may cause river water pollution if it is not being properly managed. In order to evaluate the river water quality, the bioindicator assessment has been proposed by several studies which is considered more accurate and reliable as compared to the current physicochemical characterisation of the polluted river water. In addition, as the river water could be contaminated by several types of pollutants, the use of bioindicator would be useful in determining the actual source of contamination in the affected water bodies, in this case due to POME final discharge. Moreover, the limited knowledge about the shift of bacterial populations in relation to biodegradation in the different stages of a full-scale treatment of POME impeded the

monitoring process and the understanding of the severity level of pollution caused by POME. With the emergence of molecular fingerprinting and advanced high-throughput sequencing, the study on the bacterial compositions and functions which could not be conducted previously due to the limitation in conventional methods can now be actively carried out. As bacterial community are highly dynamics and capable of reflecting the consequences of ecosystem stress, the information on the shift of bacterial community may serves as a foundation to identify the potential bioindicators to indicate the contamination caused by POME final discharge, hence could be applied as a sensitive indicator for a rapid assessment of pollution in a particular area.

# CHAPTER 3

# GENERAL MATERIALS AND METHODS

## 3.1 Analyses of Physicochemical Characteristics

The parameters for the characterization of the physicochemical properties were selected based on the parameters required in the calculation of Water Quality Index (WQI) by the Department of Environment (DOE) of Malaysia. The parameters assessed for this study included pH value, temperature, the concentrations of biochemical oxygen demand ($BOD_5$) and chemical oxygen demand (COD).

### 3.1.1 pH and Temperature

The sensitive parameters such as pH and temperature were determined *in situ*. The pH meter was calibrated prior to sampling by immersing the electrode in buffer with a pH value within the range of the samples. Triplicate samples were collected to estimate variability resulting from the sampling and analytical procedures. The samples were agitated to provide homogeneity and the bulb was dipped in the sample prior to the pH and temperature measurement (APHA, 2002).

### 3.1.2 Biochemical Oxygen Demand Concentration

The principle of $BOD_5$ involves measuring the difference of the oxygen concentration in the sample before and after incubation for five days at 20°C. The $BOD_5$ test was conducted according to the procedure in Standard Method APHA 5210-B (APHA, 2002). Dilution water was prepared in a glass container by bubbling the distilled water with clean compressed air for more than 30 mins to allow the water to become saturated with dissolved oxygen (DO) with a value approximately 8 mg/L at room temperature. For every 1 L of bubbling distilled water, 1 mL each of phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride solution was added and the contents were mixed thoroughly. The samples were inserted into three sets of 300 mL BOD bottles and the bottles were filled with distilled water that has been mixed with nutrients. Similarly, a blank was prepared by taking three sets of 300 mL BOD bottles and filled with distilled water. All samples and blank were incubated in BOD Incubator Compact Model 205 (HACH, USA) at 20°C for five days. Prior to incubation, DO content was determined for the blank and the samples using DO meter (YSI 5100, Ohio USA). The formula used to determine the BOD concentration as follows:

Equation 3.1:

$$P = V_s / (V_s + V_{dw})$$

Equation 3.2:

$$BOD = (DO_1 - DO_5) / P$$

Where:

$V_s$ = Volume of sample
$V_{dw}$ = Volume of dilution water
BOD = Biochemical oxygen demand
$DO_1$ = Dissolved oxygen on the first day
$DO_5$ = Dissolved oxygen on the fifth day
P = Dilution factor

### 3.1.3 Chemical Oxygen Demand Concentration

The COD concentration was determined according to the standard method (APHA, 2002). A 2 mL sample was added into the COD digestion reagent vial supplied by HACH Company (HACH, USA). For a blank, 2 mL distilled water was used instead of the sample. Then, the samples and blank were refluxed in COD reactor at 150°C for two hours. Each vial was inverted several times while it is still warm and the vials were placed in a test tube rack to cool down to room temperature prior to measurement using DR 2800$^{TM}$ portable spectrophotometer (HACH, USA).

## 3.2 Genomic DNA Extraction of Palm Oil Mill Effluent Final Discharge

The final discharge samples (1 L) were filtered through a 0.45 µm pore-sized filter membrane (cellulose nitrate membrane filters, 47 mm, Whatman) prior to the DNA extraction process. The DNA extraction of the samples was conducted by filtering the samples with Sterivex™ filter units (Millipore, Germany), and both ends of the filter unit were secured with the inlet and outlet caps. Prior to genomic DNA extraction, Sterivex™ filters (Merck Millipore, German) were eliminated any remaining liquid in the filter housing by syringe.

Total DNA was extracted from the Sterivex™ filters using PowerWater® Sterivex™ DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) as described by Rajala et al. (2015). The inlet cap was removed and 0.9 mL of solution ST1B was added into the filter unit which helped to pull the bacteria from the membrane into solution. A 0.9

mL of ST2 solution which contained a strong lysing reagent was added into the filter unit prior to the incubation process at 90°C for 5 min, followed by vortexing at maximum speed for 5 min. After vortexing, the lysate containing both intact and lysed bacteria was removed from the Sterivex™ filter unit using a sterile syringe. Then, the lysate was added into a tube containing glass beads and vortexed at maximum speed for 5 min which will cause the collision between the beads and the bacterial cells and subsequently causing the cells to break open. The supernatant was then separated from the sample debris and beads before mixing with inhibitor removal solution to remove additional non-DNA organic and inorganic materials. The lysate was then passed through the binding column tube to allow the DNA to selectively bind to silica filter membrane in the binding column basket while contaminants passed through the silica membrane. Upon completing the washing steps, a sterile elution buffer of 50 µL was added to the center of the filter membrane, followed by incubation at room temperature prior to centrifugation at 13,000 *g* for 1 min at room temperature to collect the DNA. The quantity and quality of the extracted DNA were determined using NanoDrop 2000 UV-Vis Spectrophotometer and agarose electrophoresis, respectively (Phan et al., 2016). The extracted DNA was stored at -20°C until further processing steps.

## 3.3 Analysis of Bacterial Community by PCR-Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is one of the most frequently used methods for molecular fingerprinting and has been widely applied in different areas of research. This technique is based on the separation of the same length of the polymerase chain reaction (PCR)-amplified fragments of genes coding for 16S rRNA under denaturing gradient gel condition (Muyzer et al., 1993).

### 3.3.1 Polymerase Chain Reaction Amplification of 16S rRNA Gene

The 16S rRNA gene was amplified using a set of primers, 357F with GC clamp (40 nucleotides GC-rich sequence); 5‘-CCT ACG GGA GGC AGC AG-3‘ and 518R; 5‘-ATT ACC GCG GCT GCT GG-3‘. The PCR procedures were performed according to Zainudin et al. (2014). The PCR reaction mixture and conditions were set as follows: The reaction mixture consisted of 50 µL of total volume containing 1.0 µL of 0.5 ng/µL extracted DNA as a template, 25 µL of Premix *Taq* DNA (Takara Bio. Inc., Japan), 19 µL of sterilized deionized water and 2 µL of each primer (10 µM). The PCR reaction was performed using PCR Thermal Cycler (Takara, Japan) with an initial denaturation temperature at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min. The final extension was performed at 72°C for 10 min to prevent the formation of artificial double bands in DGGE analysis. The PCR products were analysed to confirm the size of 161 bp using agarose gel electrophoresis.

### 3.3.2 PCR Purification

The PCR product of 50 µL was purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instruction. The DNA concentration of each purified PCR product was first measured by using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) prior to preparation for DGGE with a standardised concentration.

### 3.3.3 Denaturing Gradient Gel Electrophoresis

DGGE analysis was performed using DCode System for DGGE (Bio-Rad Laboratories, USA). The DGGE apparatus and glass plate were sterilized using deionized water and absolute ethanol for disinfection process. After assembling the glass plates, a plug of 0% denaturant gradient was prepared and pipetted into the gap between the glass plates. Later, the DGGE gel was prepared with 30% to 65% denaturant gradient as shown in **Appendix A** and allowed to polymerise for 45 mins. The purified PCR product with a total concentration of 600 ng was loaded onto each of the individual lane of 1.5-mm-thick vertical denaturing gel. The DGGE analysis was performed at a constant voltage of 200 V in 1× TAE buffer at 60°C (Rajhi et al., 2016). After running the electrophoresis for 5 hours, the gel was stained with GelRed™ nucleic acid stain (Biotium, USA) for approximately 30 mins (Zhou and Wu, 2012). DGGE profiles were documented with Molecular Imager® Gel Doc™ XR System (Bio-Rad, USA) under UV light.

### 3.3.4 Analysis of PCR-DGGE Gel Images

The DGGE images were converted, normalized and analysed with the software package GelCompar II version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) according to the provider's instructions. To extrapolate the meaningful data from the DGGE analysis gel profiles, cluster analysis was performed using the Pearson correlation coefficient to determine the distance matrices and Unweighted Pair Group Method with Arithmetic mean (UPGMA) to create dendrograms.

### 3.3.5 Sequencing

For sequencing purpose, predominant DGGE bands were excised from DGGE gel followed by amplification and purification before sent to First BASE Laboratories Sdn. Bhd. (Malaysia). To confirm the amplicons with the same gradient and similarity were excised, DGGE was done for at least two times until a single band was obtained. The closest known relatives were determined by the homology searches for the 16S rRNA gene sequence obtained which was performed with Sequence Match provided by the Ribosomal Database Project II (RDPII).

## 3.4 Metagenomic Analysis using Illumina MiSeq Sequencing Platform

Metagenomic studies are commonly performed by analysing the prokaryotic 16S rRNA gene. The amplification of 16S rRNA regions with the generation of multimillion sequence reads by Illumina MiSeq is capable to obtain a complete

coverage of microbial community (Połka et al., 2014). In this study, Illumina MiSeq sequencing platform was applied to analyse the bacterial community composition and diversity in in different POME final discharges produced from different palm oil mills, as well as each stage of full-scale POME treatment systems.

### 3.4.1 16S rRNA Gene Amplification and Illumina MiSeq Sequencing

The extracted DNA samples were amplified with a set of primers targeting the hypervariable V4-V5 region of the 16S rRNA gene. The forward primer was 515F (5′-GTGCCAGCMGCCGCGG-3′) and the reverse primer was 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). The thermos cycling steps were set as follows: an initial denaturation at 94°C for 3 min followed by 94°C for 45 s, 50°C for 60 s and 72°C for 90 s of 35 cycles, and a final extension at 72°C for 10 min. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purified amplicons were determined by using the Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, USA). The amplicons were processed using the Nextera XT DNA Library Preparation Kit according to the Illumina's protocol (Illumina, San Diego, CA). The amplicons were sequenced using the Illumina MiSeq (V2 MiSeq reagent cartridge with 2×250 bp paired-end). The data obtained were demultiplexed and the reads were then classified to different taxonomic levels.

### 3.4.2 Bioinformatics Analysis of High-throughput Sequencing Data

Sequences of bacterial and archaeal 16S rRNA gene amplicons were filtered for quality, trimmed and processed using the Quantitative Insights Into Microbial Ecology (QIIME) v1.3.0 pipeline (Caporaso et al., 2010) with default settings by using the server provided by Malaysia Genome Institute (MGI), Malaysia. The demultiplexed sequences data in fasta.gz format was uploaded into the server and simplified into fastq format prior to pre-process steps to check for the sequence quality. The sequences were then trimmed using Dynamic Trim tool to remove low quality sequence (quality score <20) and any read containing ambiguous bases. Subsequently, the trimmed sequences were assembled using Paired-end Assembler for Illumina sequences (PANDAseq) tool to pair the forward and reverse sequences.

A mapping file which contained the information of each sample necessary for the subsequent data analyses was constructed and validated. In the following task, the assembled sequences underwent quality filtering and were linked to the sample ID stated in the mapping file in the previous step. The high-quality reads were clustered into operational taxonomic units (OTUs) using *de novo* OTU picking pipeline with 97% sequence similarity.

### 3.4.3 Taxonomy and Relative Abundance of Bacteria and Archaea

Taxonomic assignment of OTUs can be performed using a variety of algorithm. The QIIME software supports with algorithm that was used for taxonomic identification of the OTU and phylogenetic alignment. By default, UCLUST consensus taxonomy classifier v1.2.22q was used to classify each representative sequence before querying the Greengenes database v 13.8 using PyNAST program (Caporaso et al., 2010; DeSantis et al., 2006) to search for the closest match to an OTU from which a taxonomic lineage is inferred. By default, QIIME filtered the sequence alignment to remove columns comprised of only gaps and locations known to be excessively variable by using 16S alignment mask. Subsequently, the filtered alignment was used to generate the OTU table which was used to construct the taxonomy plot for analysis of taxonomy and relative abundance of bacteria and archaea.

The resulting biom-files were imported and analysed in Microsoft Excel spreadsheet with pivot tables. The pivot tables were further analysed to create heatmap using the relative abundance of each genus compared to its respective relative abundance in the other samples (Madsen et al., 2015). The colours on the heatmap represent the relative abundance of each taxon compared to the abundance of the same taxon in the remaining samples. For each row, the rule applied identified the highest (dark red) and the lowest (bright red) values and assigned colour gradient for the remaining values.

### 3.4.4 Diversity Analyses

The core diversity analyses including the rarefaction curve, the alpha-diversity indices (i.e., Chao 1 and observed species) and beta-diversity indices (pair-wise sample dissimilarity) were calculated using QIIME software, based on the 0.90- and 0.97-OTU tables and their corresponding phylogenetic tree. The dissimilarity (or distances) between bacterial communities in different samples was computed using both the weighted and unweighted UniFrac metrics. A three-dimensional principal component analyses (3D-PCA) plot was used to visualize the pairwise UniFrac distances among samples.

## 3.5 Statistical Analyses

The analysis of physicochemical characterization were performed in triplicate. The UNIVARIATE procedure of SAS version 9.2 was used to describe the distribution of data. The analysis of variance was applied to evaluate the significance of results, and $p<0.05$ considered to be statistically significant. The statistical analysis was performed using Microsoft Excel spreadsheet. The repeated measurement ANOVA was also conducted to test the significant difference of total cell concentration in different sampling points for different months.

# CHAPTER 4

# BACTERIAL DIVERSITY ASSESSMENT IN PALM OIL MILL EFFLUENT FINAL DISCHARGE TREATED BY DIFFERENT BIOTREATMENT PROCESSES FOR THE DETERMINATION OF BIOINDICATORS

## 4.1 Introduction

Palm oil production is one of the major industries in Malaysia which makes a significant contribution to the national economy. However, concurrent with the high production of crude palm oil, an enormous volume of palm oil mill effluent (POME) is being generated every year (Bala et al., 2015). The most common practice of palm oil mills is discharging the treated and partially treated POME into the nearby river water. This practice has led to unfavourable impacts, not only to the industry, but also to the people and the environment (Rupani et al., 2010). POME is known as a high strength agro-industrial wastewater that contains large amount of organic matter which eventually increases both the biochemical oxygen demand (BOD) and the chemical oxygen demand (COD) (Bala et al., 2015). The anaerobic digestion applied for the treatment of POME could increase the rate of biodegradation (Poh and Chong, 2009), in addition to the conventional POME treatment using the ponding system. However, if the system is not managed efficiently, the discharge of treated or partially treated POME into a nearby river could lead to severe environmental pollution (Rupani et al., 2010).

Currently, the quality of the impacted river water was assessed by using the physicochemical characterisation. However, the current assessment is considered inaccurate as the affected river water may have other anthropogenic sources such as from residential areas and agricultural practices. Therefore, it is crucial to have a reliable indicator for monitoring the specific cause of pollution in the effluent receiving river water. The previous case study was carried out to determine the potential bioindicators that could be used to indicate the contamination of river water by POME final discharge. *Alcaligenaceae* and *Chromatiaceae* which were present in the effluent receiving river water but not in the upstream part were reported to have originated from the POME final discharge, hence were proposed as bioindicators to indicate the river water contamination due to POME (Sharuddin et al., 2017).

## 4.2 Materials and Methods

### 4.2.1 Location of Study Area

The POME final discharges were collected from four different typical palm oil mills in Peninsular Malaysia which had different capacities to process fresh fruit bunch (FFB) and the treatment of POME (**Table 4.1**). These palm oil mills were selected randomly, and their locations are provided in **Figure 4.1**. Each palm oil mill adopted different biotreatment processes of POME (**Figure 4.2**).

**Table 4.1** **Capacity of FFB, anaerobic retention time and total retention time of POME for the treatments A, B, C, and D (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

| Characteristics | A | B | C | D |
|---|---|---|---|---|
| Capacity (tonnes of FFB/hour) | 40 | 30 | 54 | 54 |
| Anaerobic retention time (days) | 40 | 45 | 40 | 45 |
| Total retention time of POME (days) | 61 | 100 | 142 | 82 |


**Figure 4.1** **Locations of four selected typical palm oil mills in Peninsular Malaysia. The GPS coordinates of the palm oil mills are provided as follow: FELDA Pasoh Palm Oil Mill; 3°0'52"N 102°18'12"E, FELDA Trolak Palm Oil Mill; 3°56'16"N 101°21'02"E, FELDA Sungai Tengi Palm Oil Mill; 3°35'07"N 101°24'56"E, FELDA Besout Palm Oil Mill; 3°52'52"N 101°16'37"E** (Source: Google Map).

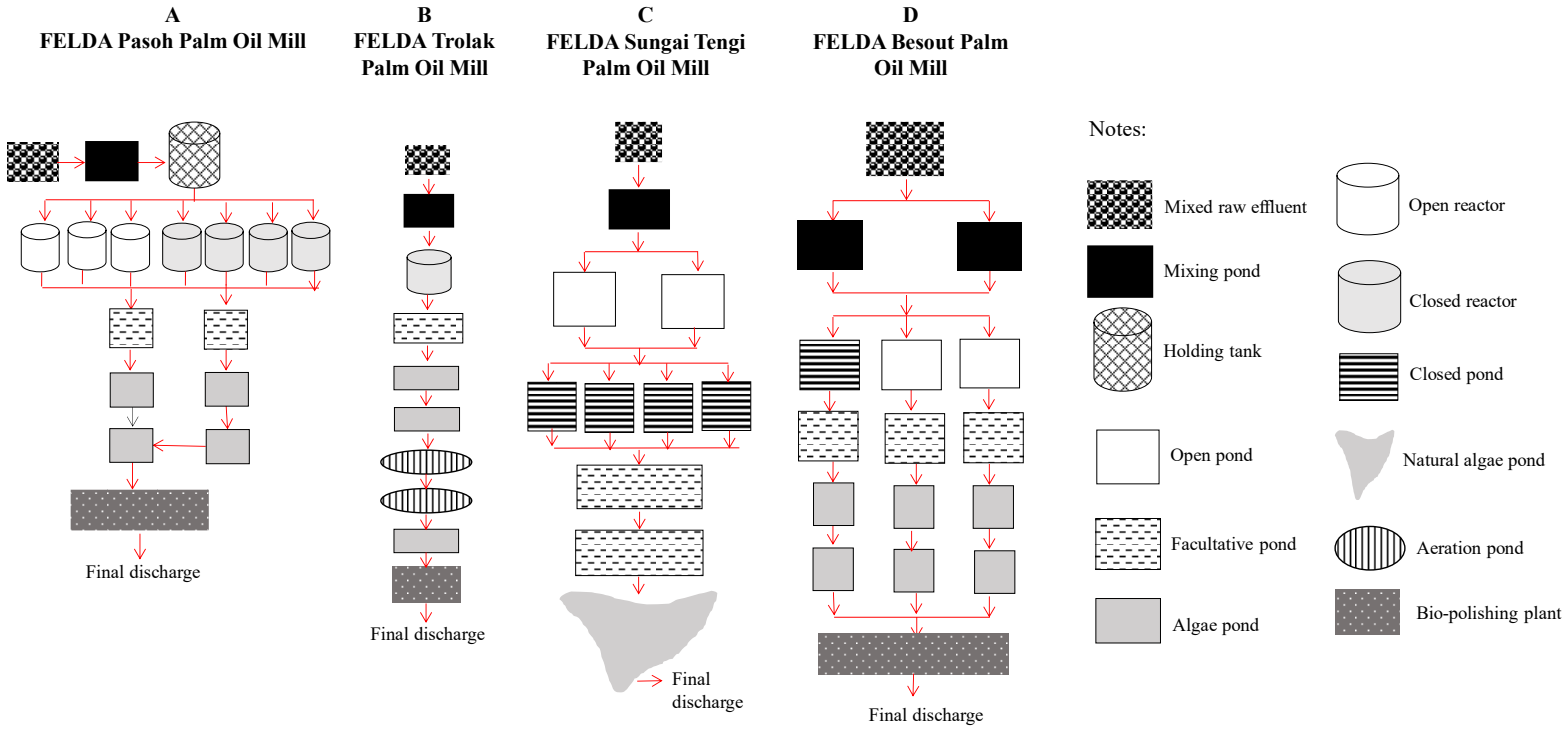



**Figure 4.2** **Process flow schematics of the different stages of full-scale treatment of POME in four typical palm oil mills. Different anaerobic digestion processes were applied either using open and closed reactors (A), closed reactor (B), closed ponds (C) or open and closed ponds (D). The facultative anaerobic (facultative pond) and the aerobic (algae pond) processes were also different with B and C adopting one series of ponding system, while A and D adopted two and three series of ponding system, respectively. All POME treatment systems except C adopted biopolishing as their tertiary treatment.**

### 4.2.2 Sample Collection

The POME final discharge was collected in the sterilized plastic bottle containers from the final release point of POME treatment at the selected palm oil mills. The samples were kept in an ice box during transportation which took approximately 2–4 hours to avoid any biological or chemical reactions in the samples (Standard Method 9060 B). The samples were processed immediately for the physicochemical and nutrient analyses. Meanwhile, for the analysis of the bacterial community, each sample of POME final discharge was passed through the Sterivex™ (Merck Millipore, Germany) membrane filter cartridges (pore size 0.22 μm, diameter 1.7 cm, Millipore, Germany) until the filter clogged (or maximum sample volume of 3 L was filtered). Both ends of the filter unit were secured with the inlet and outlet caps. The volume of filtered water was recorded and Sterivex™ units were immediately kept in sterile zip lock bags and stored at -20°C prior to DNA extraction.

### 4.2.3 Physicochemical Characterisation

The colour analysis was carried out by following platinum-cobalt (PtCo) standard method 8025 and the colour was detected at a wavelength of 455 nm using method 8000). HACH spectrophotometer (DR 2800, Loveland, CO) by placing 10 mL of sample in a specially designed square quartz sample cell (APHA, 2002). The other parameters for the physicochemical characterisation were chosen based on the parameters required in the calculation of Water Quality Index (WQI) by the Department of Environment (DOE) of Malaysia. The parameters measured in this

study were pH, temperature, $BOD_5$ and COD. The pH and temperature values were recorded *in situ* using the portable meter (**Section 3.1.1**). Meanwhile, the concentration of $BOD_5$ was measured according to the procedure in Standard Method APHA 5210-B (APHA, 2002) (**Section 3.1.2**) and the concentration of COD was measured by using reactor digestion method (HACH method 8000) (**Section 3.1.3**).

### 4.2.4 Nutrient Composition Analysis

The sample with the volume of 50 mL was filtered through a 0.45 μm pore-sized filter membrane (cellulose nitrate membrane filters, 47 mm, Whatman) prior to the nutrient analysis. The compositions of nutrients (phosphorus, ammonium, nitrogen, nitrate and potassium) in the POME final discharge were analysed using Inductive Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) at ESPEK Research and Advisory Services, Kajang, Selangor, Malaysia.

### 4.2.5 Genomic DNA Extraction of Palm Oil Mill Effluent Final Discharge

Total genomic DNA was used to analyse bacterial community in the POME final discharge from the selected palm oil mills. The details of the method used were described in **Section 3.2**.

### 4.2.6 Bacterial Community Analysis by PCR-Denaturing Gradient Gel Electrophoresis

PCR-DGGE analysis for bacterial community in the final discharge samples was performed following to the methods as stated in **Section 3.3**.

### 4.2.7 High-throughput Illumina MiSeq Sequencing and Bioinformatic Analysis

A thorough analysis of bacterial community in the different POME final discharge samples using Illumina MiSeq platform was done following the method as described in **Section 3.4**.

### 4.2.8 Principal Coordinate Analysis

Principal coordinate analysis (PCO) (Gower, 1966) was performed on PAST software (Hammer et al., 2001) to correlate the abundance of different bacterial genera to different physicochemical properties of the samples. The PCO was done to graphically illustrate the relationships between bacterial taxa within the phylum *Proteobacteria,* physicochemical characteristics ($BOD_5$ and COD) and nutrients compositions (phosphorus, ammonium, nitrogen, nitrate and potassium) of POME final discharges obtained from four different palm oil mills. Data points were clustered using correlation similarity measure to obtain a PCO scatter plot.

## 4.3 Results and Discussion

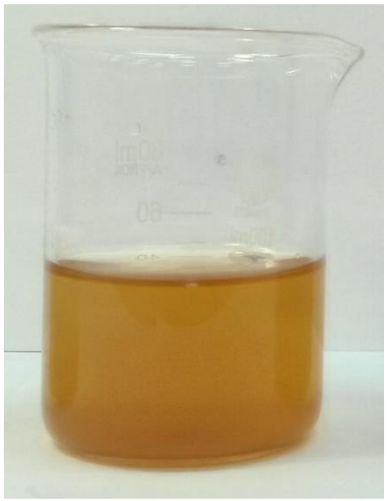
### 4.3.1 Physicochemical Characteristics of Palm Oil Mill Effluent Final Discharge

The assessment of the physicochemical properties of POME final discharge is very crucial to safeguard public health and the environment because large quantities of treated or partially treated POME are being discharged into the water bodies.

#### 4.3.1.1 Colour

The colour of POME final discharge is often objected by the public on the assumption that colour is a sign of pollution. The colour of POME final discharge from each palm oil mill was observed in this study (**Table 4.2**). The highest colour concentration of POME final discharge (2109 PtCO/L) was recorded from FELDA Trolak Palm Oil Mill, followed by FELDA Besout Palm Oil Mill (1304.73 PtCO/L) which is more brownish. Colour in POME final discharge influenced by suspended and dissolved particles. Dissolved organic matter, such as humus, peat or decaying plant matter can produce a yellow or brown colour (Mohammed and Chong, 2014).

**Table 4.2** **The physicochemical characteristics of POME final discharge obtained from four different typical palm oil mills (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

| Characteristics | A | B | C | D |
|---|---|---|---|---|
| |  |  |  |  |
| Colour (PtCo/L) | 584.77 ± 7.85 | 2109.83 ± 0.15 | 650.80 ± 5.80 | 1304.73 ± 4.88 |
| Temperature | 33.23 ± 0.15 | 32. 20 ± 0.30 | 33.17 ± 0.35 | 31. 23 ± 0.31 |
| pH | 8.23 ± 0.02 | 7.81 ± 0.04 | 8.01 ± 0.07 | 8.01 ± 0.02 |

According to Mohammed and Chong (2014), concentrations of naturally dissolved organic acids such as tannins and lignins may also have an effect by giving water a tea colour. Tannins that are yellow to black are the most abundant kind found in POME and can have a great influence on its colour, as well as a musty smell. The brown colouring comes from tannins leaching into run-off water from the manufacturing process of palm oil (Clean Water Team, 2013) .The dark brown colour of POME consisted of many organic compounds such as anthrocyanin and carotene pigment that was extracted from fresh fruit bunches in the sterilization process (Mohammed et al., 2014). Moreover, it included polyphenol compounds, lignin, tannin, polyalcohol, humic acid, melanoidin and other organic matters which are recalcitrant to the conventional treatments (Mohammed, 2013; Zainal et al., 2017). The change of colour of POME is caused by decomposition of lignocellulosic materials (Tan et al., 2014) where it has to decrease to 500 ADMI (American Dye Manufacturers Institutes) for compliance (Bello and Abdul Raman, 2017). Besides aesthetic problem, colour also affects sunlight penetration and limits potential wastewater reuse.

#### 4.3.1.2 pH and Temperature

The temperature and pH value of the samples were measured in this study (Table 4.2) as one of the efforts to determine the properties of the POME final discharges. Temperature is one of the important assessments of water quality because it influences the types of aquatic life present in the water bodies and regulates the maximum dissolved oxygen concentration of the water. While the pH value is a measure of the acid strength in the water. Generally, both properties affected most physical, biological and chemical characteristics of the wastewater (Ibrahim et al., 2012).

It is shown that the average temperature for the POME final discharges from four different typical palm oil mills were comparable and within the range of ambient temperature. Meanwhile, the average pH value recorded for all samples ranging from 7.81 ± 0.04 to 8.23 ± 0.02 indicated that the POME final discharges had the properties of an alkaline pH. In the anaerobic digestion process during the treatment of POME, the acidic bacteria convert the organic components of the raw POME into volatile fatty acids which contribute to the low pH of POME (Rupani et al., 2010). However, as the volatile fatty acids were used as a substrate in an anaerobic phase, the pH value of POME at the later stage of treatment was increased.

As the POME final discharge is introduced into the nearby river water, it is important to compare its physicochemical properties with the standard provided by Malaysia Environmental Quality Report (EQR, 2006). In this study, the pH and temperature recorded in the POME final discharge showed acceptable values whereby the standard temperature should be in the range of ± 2ºC from the normal ambient temperature, while pH value should be ranging from 6.5 to 8.5.

#### 4.3.1.3 Biochemical and Chemical Oxygen Demand

**Figure 4.3** shows the average concentrations of $BOD_5$ and COD for all the POME final discharge. The Department of Environment (DOE) of Malaysia has set that the standard five-day BOD as one of the basic and most important parameters measured to indicate the strength of pollutants in terms of oxygen required to stabilize domestic and industrial wastes (EQR, 2006). As being mentioned in the previous studies, many

mills are unable to achieve the discharge limits set by the DOE of Malaysia even after the treatment using aerobic ponding system (Ahmad and Hameed, 2009; Bhatia et al., 2007). The discharged effluents from the palm oil mills often do not fulfil the regulatory discharge limits which may be also due to several other factors such as the varieties in different batches of FFB, processing techniques, age or type of fruits and also the climate (Wu et al., 2010). The introduction of the POME final discharge with significant concentration of $BOD_5$ was reported to change the microbial ecology in the POME-receiving river water (Sharuddin et al., 2018).

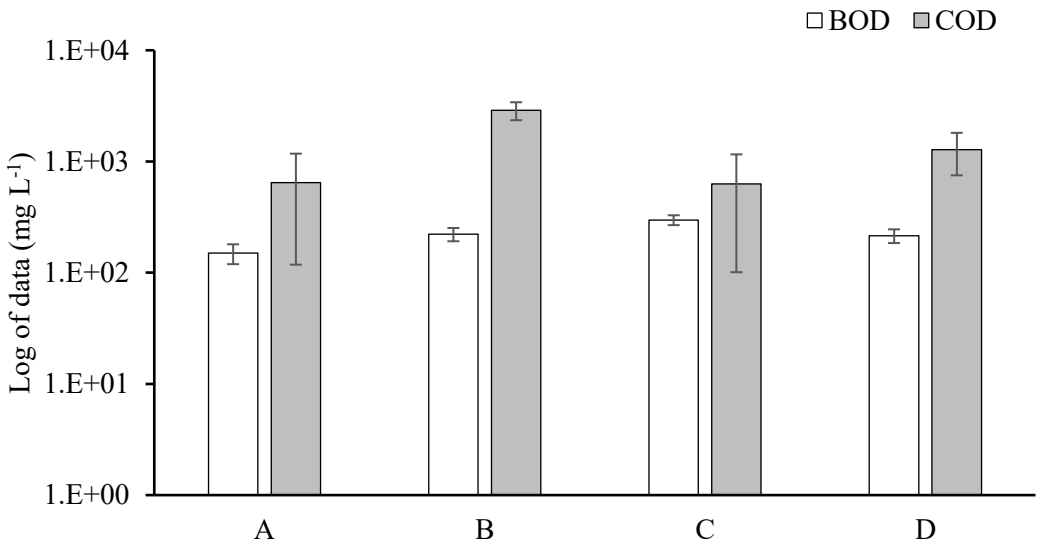



**Figure 4.3** **The concentrations of $BOD_5$ and COD of the POME final discharges obtained from four different palm oil mills with y-axis on a log scale (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill). One-way ANOVA, t-test was performed and demonstrated a significant difference of the concentrations of $BOD_5$ and COD between different POME final discharges at a confidence level of 0.05.**

The COD concentrations for all the POME final discharges were also recorded as shown in **Figure 4.3**. COD concentrations are typically higher than $BOD_5$ and the ratio between them will vary depending on the characteristics of the wastewater. Higher concentrations of COD were recorded in sample B and D, mainly due to the management of POME in the respective palm oil mills including desludging. The desludging process was carried out annually in sample A and C, hence recorded lower COD concentrations. High concentration of COD is commonly associated with POME (Khemkhao et al., 2012) which may indicate the presence of recalcitrant chemicals that could not be degraded biologically. The bioaccumulation of these compounds may give rise to environmental problems (Kanu and Achie, 2011).The ratio of BOD/COD has been commonly used as an indicator for biodegradation capacity (Zaher and Hammam, 2014).

#### 4.3.1.4 Nutrient Composition

In this study, nutrient contents which were nitrogen, nitrate, ammonium, phosphorus and potassium present in the POME final discharge obtained from four different palm oil mills were analyzed as shown in **Table 4.3**. Generally, the raw POME contains high concentrations of organic nitrogen and phosphorus (Hadiyanto et al. 2013) which may cause severe pollution to the aquatic environments if it is being directly discharge into the water bodies. Inappropriate handling and discharging of the improperly treated POME to waterways can also contribute to eutrophication or disruption to the industrial water reuse applications. In this study, the overall nutrient composition was recorded below 2% which indicate that nutrient removal was occurred during the treatment of POME. Even though the partially treated POME (anaerobic sludge) was

reported to contain high amounts of nutrients (Liew et al. 2015; Baharuddin et al. 2009), the nitrogen, nitrate, ammonium, phosphorus and potassium were reported in low percentage in the POME final discharge (**Table 4.3**), perhaps due to efficient biotreatment processes in reducing the polluting strengths of POME. There are many factors affecting the nutrient removal performance of the POME treatment system such as the capacity of treatment facility, pollutant loading, treatment mode, age of mill, characteristics of POME, etc. (Loan et al. 2015).

**Table 4.3 Percentage composition of nutrients in the different POME final discharges obtained from four different typical palm oil mills (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

| Nutrient Composition (%) | A | B | C | D |
|---|---|---|---|---|
| Nitrogen | 1.89 ± 0.02 | 0.84 ± 0.03 | 1.05 ± 0.02 | 0.27 ± 0.04 |
| Nitrate | 2.04 ± 0.05 | 1.01 ± 0.02 | 1.16 ± 0.01 | 0.27 ± 0.02 |
| Ammonium | 1.26 ± 0.02 | 0.42 ± 0.03 | 0.63 ± 0.04 | 0.25 ± 0.01 |
| Phosphorus | 0.15 ± 0.03 | 0.15 ± 0.02 | 0.16 ± 0.03 | 0.05 ± 0.02 |
| Potassium | 0.12 ± 0.04 | 0.14 ± 0.02 | 0.04 ± 0.01 | 0.22 ± 0.02 |

### 4.3.2 PCR-DGGE Analysis of Bacterial Community Structure

The structural patterns of the bacterial community provide important clues about the underlying mechanisms that structured the ecological communities in the difference POME final discharges. However, the dependency on the conventional methods may limit the identification of the representative samples to be further analysed by deep

sequencing, especially when it involves a large number of samples. Thus, in this study, the molecular analysis of bacterial community structure was conducted using the PCR-denaturing gradient gel electrophoresis (DGGE) method to unravel the changes in community structure and composition in the POME final discharges. Over the last decade, a more extensive analysis of bacterial communities using methods based on direct PCR amplification and analysis of ribosomal RNA genes have been developed (Boon et al., 2002). The analysis of the amplified fragments of 16S rRNA genes by DGGE has been successfully used to evaluate the bacterial diversity in environmental samples (An et al., 2016; Araya et al., 2003; Zhao et al., 2014).

#### 4.3.2.1 DGGE Band Sequencing Analysis

Prior to DGGE, the amplification of the extracted DNA was done by using a pair of 16S rRNA universal primer, producing DNA fragment with 161 bp as shown in **Figure 4.4**. The DGGE analysis of four different POME final discharges was done (**Figure 4.5**) for a rapid detection of bacterial community changes, as well as to analyse the distribution and composition of community structures. In general, the sequencing analysis of the dominant bands in these samples revealed the presence of at least seven different bacterial taxa. The phylogenetic affiliation of the bacterial community represented by dominant bands in DGGE profiles (**Table 4.4**) revealed that the bacterial communities were mainly composed of organisms affiliated with *Bacteroidetes* (2/7) and *Proteobacteria* (3/7).

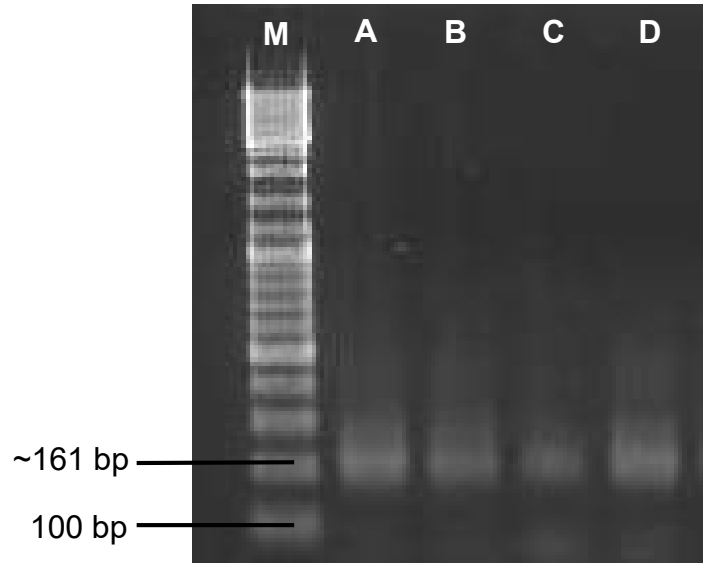



**Figure 4.4** **Representative of 16S rRNA region amplified by PCR separated on 1.6% agarose gel (M: 100 bp DNA Ladder marker, A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

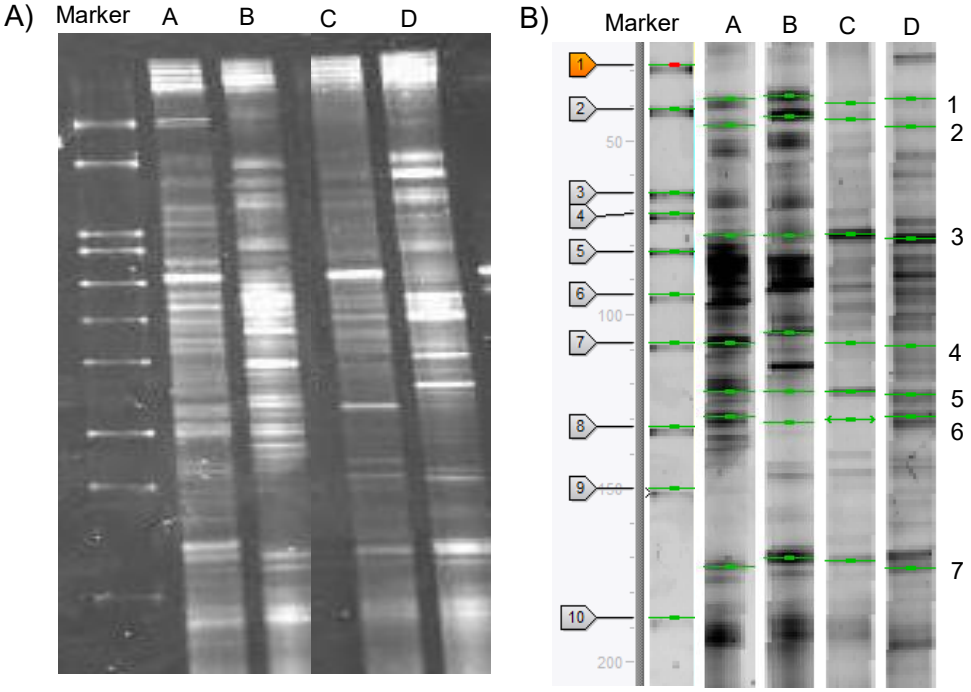



**Figure 4.5** **DGGE fingerprint profile (A) and normalized DGGE image (B) of POME final discharges obtained from four different palm oil mills (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

**Table 4.4 Phylogenetic sequence affiliation and similarity to the closest relative of amplified 16S rRNA gene sequences excised from DGGE gels. (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

| Band ID | S_ab score | Closest relative | Phylum | Family |
|---|---|---|---|---|
| 1 | 0.930 | *Macellibacteroides* | *Bacteroidetes* | *Porphyromonadaceae* |
| 2 | 0.918 | *Povalibacter* | *Proteobacteria* | *Sinobacteraceae* |
| 3 | 1.000 | *Polynucleobacter* | *Proteobacteria* | *Burkholderiaceae* |
| 4 | 0.904 | *Prevotella* | *Bacteroidetes* | *Prevotellaceae* |
| 5 | 0.924 | *Unclassified* | *Acetothermia* | - |
| 6 | 0.953 | *Unclassified* | *Proteobacteria* | - |
| 7 | 0.939 | *Ilumatobacter* | *Actinobacteria* | *Acidimicrobiaceae* |

Sequences belonging to the *Proteobacteria* have previously been retrieved from other types of wastewater (Remmas et al., 2017). Many of them were related to the organisms involved in the pollutant degradation, which suggest the importance of these communities for wastewater treatment. The results were in accordance with the bacterial community studies in the treated wastewater from urban and industrial effluents (Moura et al., 2009) and wastewater treatment systems (Adrados et al., 2014). *Proteobacteria* was also found as the dominant phylum in the different municipal wastewater treatment plants (Hu et al., 2012), constructed wetland treatment systems (Zhong et al., 2014) and domestic sewage wastewater treatment reactor (Xia et al., 2010).

*Proteobacteria* play a central role in the biological processes involved in the removal of organic matter and nitrogen in the eco-ditch systems, but the distributions of subdivision may differ depending on the ambient environment, its salinity, and whether they are facing aerobic or anaerobic conditions (Ye and Zhang, 2013). The

sequences related to *Povalibacter*, a member of *Sinobacteraceae* were reported to possess the ability to metabolize polycyclic aromatic hydrocarbons (PAHs) (Nogi et al., 2014). While, *Polynucleobacter* from the family of *Burkholderiaceae* is known as one of the most typical freshwater clusters (Andersson et al., 2008; Araya et al., 2003; Cottrell et al., 2005).

Meanwhile, a group of chemoheterotrophic bacteria from *Bacteroidetes* phylum was also detected that known by its ability to degrade complex organic matter. *Prevotella* and *Macellibacteroides*, members of *Bacteroidetes* were reported as being a fermentative bacteria that capabale to turn the organic matters into volatile fatty acids, which were used as electronic donors for biological desulphurization (Salminen and Rintala, 2002). Acording to Zhang et al. (2017), in the actual operation of dyeing wastewater treatment processes, the bioactivity and quantity of fermentative bacteria can be related to the performance of organic pollutant degradation. Therefore, it is hypothesised that the *Prevotella* and *Macellibacteroides* found in the POME final discharge played an important role in the degradation of organic pollutant.

The presence of *Actinobacteria* was also detected as a dominant band in the POME final discharge (**Figure 4.5**), generally recognized as organotrophic bacteria that able to decompose recalcitrant and poorly accessible substrates at later stages of microbial succession. In addition, they are possibly involved in the synthesis and decomposition of humic substances (Bagatini et al., 2014; Zakharova et al., 2013). For instance, the genus *Ilumatobacter*, a member of *Actinobacteria* has been recently found in abundant during degradation processes of freshwater diatoms (Zakharova et al., 2013) and at the stationary growth phase of freshwater-cultured phytoplankton (Bagatini et al., 2014).

This study demonstrates that PCR-DGGE provides a reproducible assessment of microbial community structure but could result in a biased quantification of species richness and relative species abundance. This method is known to only provide the monitoring of dominant bacterial population. Several studies have postulated that each member of rich bacterial populations could not be detected by PCR-DGGE and appear as a weak band when their concentration is lower than $10^3$ CFU/mL (Lucena-Padrós et al., 2015). The high number of the weak bands is also due to the high number of bacterial diversity which resulted in a smear bands (Boon et al., 2002).

Nevertheless, despite such limitations, PCR-DGGE is best adapted for generating community snapshots in the monitoring and identifying bacterial community shifts in response to seasonal changes, bioremediation applications or environmental perturbations. Specifically, PCR-DGGE can be applied to screen large number of samples to identify a subset of interest for further examination using the high throughput sequencing.

### 4.3.3 High-throughput Sequencing Analysis

The current determination of physicochemical properties in assessing the level of pollution is considered inaccurate as the affected river water may have other anthropogenic sources such as from residential areas and agricultural practices. Therefore, it is crucial to have a reliable indicator for assessing the specific cause of pollution in the effluent receiving river water. A study conducted by Shade et al. (2012) showed that the compositions of microorganisms and their functions were most sensitive to disturbances.

Therefore, the use of bacteria as an indicator of the presence of pollutants is now widely regarded as a potential approach in determining the actual cause of contamination in water bodies. The use of a microbial source tracking method in identifying fecal contamination was suggested as a complement to the traditionally used bacterial indicators and environmental variables (Zhang et al., 2014). Furthermore, an assessment of the bacteriological water quality and the monitoring of microbial contamination could also serve as biomonitoring standards and hence would be beneficial in environmental planning and management (Baghel et al., 2005).

In the previous study by Sharuddin et al. (2017), *Alcaligenaceae* and *Chromatiaceae* which were originated from the POME treatment were present in the downstream part of the river. Even though the distance between the final release point of POME to the river water was about 2 km, these bioindicators were still reported to be present in the downstream part of the river. Interestingly, these bioindicators were not detected in the upstream part of the river that was unpolluted due to POME final discharge.

Therefore, this study was undertaken to confirm the reliability of the proposed potential bioindicators, *Alcaligenaceae* and *Chromatiaceae* to specifically indicate contamination in river water by POME final discharge. The compositions of the bacterial communities in the POME final discharge obtained from four different palm oil mills which used different biotreatments of POME were elucidated using a high-throughput MiSeq.

#### 4.3.3.1 Taxonomic Relative Abundance of Bacterial Community

The MiSeq analysis of the 16S rRNA marker gene amplicons from the four samples produced a total of 299,466 high quality reads and 261,273 observed OTUs. FastQC reports generated by the quality check tool in QIIME pipeline revealed that these libraries represented the majority of 16S rRNA sequences present in each sample, with values ranging from 90% to 99%. A detailed summary of the analyzed sequences is presented in **Table 4.5**.

**Table 4.5 Number of raw reads, high-quality reads, OTU and alpha-diversity indices measured as Shannon-Weaver index (H') and Evenness (E') in the POME final discharge obtained from different palm oil mills (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

| | A | B | C | D |
|---|---|---|---|---|
| Number of raw reads a | 132,259 | 66,563 | 64,275 | 87,752 |
| Number of high-quality reads b | 118,100 | 55,093 | 56,467 | 69,806 |
| Number of OTU c | 98,613 | 37,787 | 56,364 | 68,509 |
| Shannon-Weaver index (H') [d] | 2.252 | 2.097 | 1.331 | 1.788 |
| Evenness (E') [e] | 0.144 | 0.145 | 0.059 | 0.096 |

[a] Image data output from the sequencing machine was transformed by base calling into sequence data and stored in fastq format
[b] Poor quality sequences were discarded (*i.e.*, sequences of <200 bp with an average quality score of <25 and ambiguous characters)
[c] Clustered according to the similarity to one another at 97% threshold
[d] Calculated as $H = \sum(pi * \ln pi)$, where *pi* is the abundance of each species
[e] Calculated as $E = H/\ln S$, where S is the total number of species

The alpha-diversity analyses conducted using H' and E' showed different richness and evenness of bacterial communities in all the samples. As discussed in a study of Silva et al. (2016), microbial communities were stable during the longer sludge retention time (SRT) of 60 days, while the community was much more in dynamic composition during shorter SRT of 20 days. However, SRT was not the only factor affecting the composition and diversity of bacterial community in the POME but may also due to the different biotreatment processes. The results obtained in this study were supported by Vuono et al. (2015) who similarly stated that the system performance and the microbial community of the activated sludge were different based on the operating conditions.

In this study, the bacterial community compositions were compared among the four different POME final discharges at phyla level (**Figure 4.4**). Taxonomically, the major phylum observed in all samples belonged to *Proteobacteria*, ranging from 22.09% to 67.37%, in accordance with the results of PCR-DGGE mentioned earlier whereby *Proteobacteria* was detected as the dominant community in all the samples (**Table 4.4**). Becerra-Castro et al. (2016) also documented *Proteobacteria* as the most frequently encountered taxa in the secondary effluent from urban wastewater treatment plants comprising up to 50% of the total sequence on average. *Proteobacteria* was also reported as being the most predominant phylum to be found in wastewater, such as from coking wastewater treatment plants in the steel industry (Ma et al., 2015), aerobic biofilter treating textile effluent (Köchling et al., 2017), the constructed wetland for municipal wastewater treatment (Chen et al., 2015) and many others.

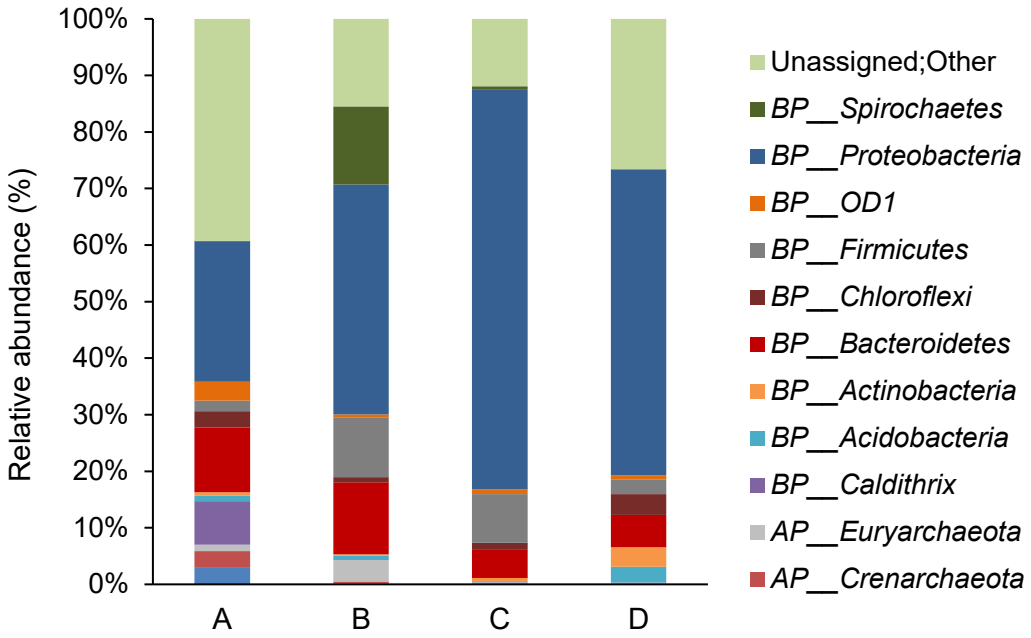



**Figure 4.6: Relative abundance of the bacterial phyla (>2%) in the POME final discharge obtained from four different palm oil mills. (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill). BP: bacterial phylum, AP: archaeal phylum.**

On the other hand, the *Bacteroidetes* was detected as the second most abundant phylum in all the samples ranging from 4.84% to 11.54% which may attribute to its ability in degrading the recalcitrant dissolved organic matters (Zhang et al., 2015), hence enabling them to inhabit in a wide range of environments. These results are in accordance with the bacterial community analysis using PCR-DGGE as described earlier in **Section 4.3.2** where the *Proteobacteria* was found as a dominant community, followed by *Bacteroidetes*.

#### 4.3.3.2 Assessment of Reliable Bioindicators

In order to have a thorough evaluation on the bacterial community structure in different POME final discharges, a heatmap displaying 22 order level of *Proteobacteria* with an average relative abundance >0.7% was constructed (**Figure 4.7**). In the previous study, *Alcaligenaceae* and *Chromatiaceae* were proposed as potential bioindicators for the assessment of polluted river water due to POME final discharge. These bioindicators were found to be present in the downstream (polluted due to POME final discharge) but not in the upstream (unpolluted due to POME final discharge) where they were suggested to be originated from the treatment of POME. This was in accordance with the study conducted by Guo et al. (2016) which selected the potential indicator bacteria based on the increment of that particular community in the river water exposed to paper mill effluent. Interestingly, *Alcaligenaceae* and *Chromatiaceae* from the class *beta-* and *gamma-Proteobacteria*, respectively were also being detected in all the four final discharges despite them having undergone different processes of POME treatment. Hence, it was determined that *Chromatiaceae* and *Alcaligenaceae* as reliable bioindicators to indicate the contamination by POME final discharge.

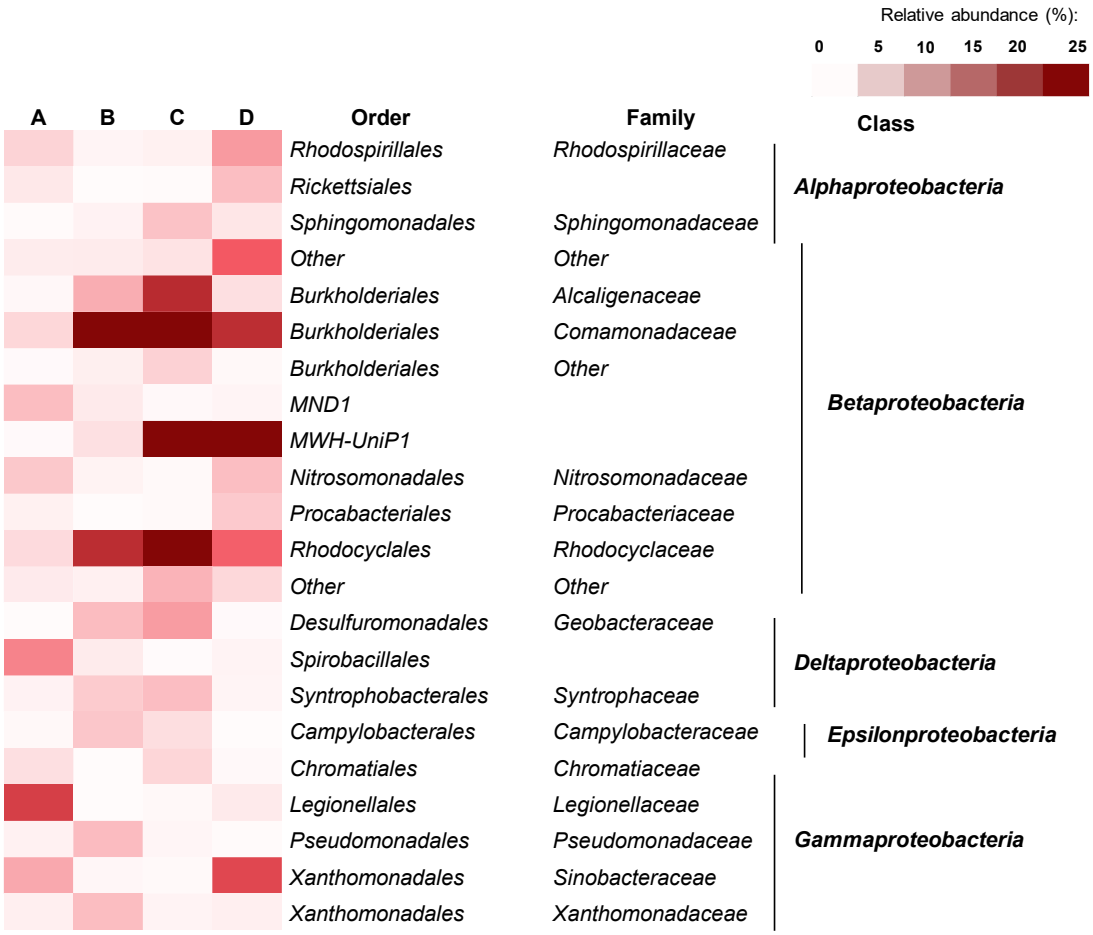



**Figure 4.7** **Heatmap of the relative abundance of 22 bacterial orders from the total *Proteobacteria* in the POME final discharges obtained from different palm oil mills (A: FELDA Pasoh Palm Oil Mill, B: FELDA Trolak Palm Oil Mill, C: FELDA Sungai Tengi Palm Oil Mill, D: FELDA Besout Palm Oil Mill).**

### 4.3.4 Principle Coordinate Analysis to Correlate Bacterial Community Composition with the Pollutant Properties

The PCO was done to correlate the *Proteobacteria* community, physicochemical characteristics and nutrients compositions of the POME final discharge (**Figure 4.8**). The *Alcaligenaceae* and *Chromatiaceae* were shown to be positively, strongly correlated with the concentration of $BOD_5$. The relative abundance of *Alcaligenaceae* (4.81%) and *Chromatiaceae* (0.71%) were shown to be highest in FELDA Sungai Tengi Palm Oil Mill (C) (**Figure 4.7**) which were correlated with the highest concentration of $BOD_5$ in this palm oil mill (298 ± 31.83 mg/L), as compared to the other mills. Hudson et al. (2008) in their study also found the strongest correlation between the bacterial community and the amount of biodegradable organic matter present in the effluent which was measured as $BOD_5$. However, a weak correlation between these bioindicators and the nutrients present in the POME final discharge was observed that might be due to their different biodegradation abilities.

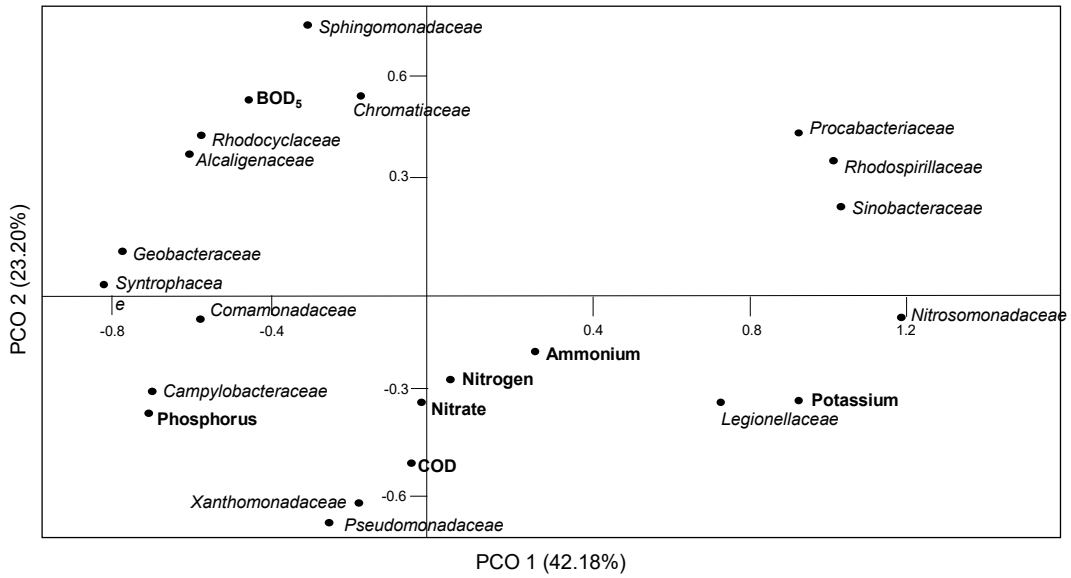



**Figure 4.8 PCO ordinations of phylum *Proteobacteria* based on relative abundance of OTU, physicochemical properties ($BOD_5$ and COD) and nutrients compositions (phosphorus, ammonium, nitrogen, nitrate and potassium) of POME final discharges obtained from four different palm oil mills. Variance explained in each axis is given in parentheses.**

*Alcaligenaceae* was known to have potential in degrading phenolic (Rehfuss and Urban, 2005) and aromatic compounds (Pérez-Pantoja et al., 2012) that might have resulted from lignocellulosic degradation during the treatment of POME. Sharip et al. (2016) in their study also found phenolic and aromatic compounds which resulted from the lignocellulosic degradation of oil palm mesocarp fiber (OPMF) condensates by using superheated steam. However, *Chromatiaceae* was reported as a non-responsive community on lignocellulose with the ability to fix its own carbon (Darjany et al., 2014). No previous study had been conducted to ascertain whether *Chromatiaceae* was able to absorb and digest plant-derived carbon or whether it could compete for the substrates derived from lignocellulosic degradation.

## 4.4 Concluding Remarks

The information on the shift of bacterial community is of practical relevance in designing the bioindicator for monitoring the river water quality. The detection of the proposed bioindicators, *Alcaligenaceae* and *Chromatiaceae* in all the four POME final discharges, despite them having different properties and been generated from different processes of POME treatment, showed the potential of using these bacterial indicators to indicate river water contamination caused by this effluent. The bioindicator can be used to model a more sensitive and reliable pollution assessment method to complement the current conventional monitoring using the physicochemical properties.

# CHAPTER 5

# SHIFT OF BACTERIAL AND METHANOGENIC ARCHAEAL POPULATIONS IN THE DIFFERENT STAGES DURING THE TREATMENT OF PALM OIL MILL EFFLUENT

## 5.1 Introduction

The pond and anaerobic treatment systems in the mills were using undefined microbial populations (McHugh et al., 2003) which were responsible for the biodegradation of organic compounds and contaminants. However, the unknown mechanism for the degradative pathways might lead to the digestion problems, causing a failure of the treatment system. In addition, the POME influences microbial ecology where the low nucleic acid (LNA) bacteria cells were shown to be shifted to high nucleic acid (HNA) bacterial cells in the affected river water due to POME final discharge (Sharuddin et al., 2018). Therefore, the issues of POME treatment cannot be figured out without understanding the biodegradative pathways throughout the treatment of POME.

The focus of current studies has largely been on the microbial community structure of POME in various bioreactor configurations (Rana et al., 2017). However, less attention was given to the bacterial and archaeal consortia involved in the biodegradation in the different stages of a full-scale treatment of POME. Insights into the bacterial and archaeal communities and key players catalysing a complex series of biochemical reactions to reduce the polluting power of POME in the treatment system are still unclear. Therefore, the substrates causing an inefficient treatment of POME are not ascertained. Moreover, the current traditional monitoring approaches evaluated by

determining the BOD and COD removal efficiency could not be directly used to correlate between microbial biomass and any particulate organic matters present in the treatment system. Hence, the assessment of microbial community that is responsible for the biodegradation in the different stages of POME treatment is important to understand the performance of the treatment system.

The determination on the composition of microbial community during the wastewater treatment process using molecular approach, such as PCR-DGGE is commonly adopted in many previous studies (Abdullah et al., 2013; Gray et al., 2002; Sánchez et al., 2011; Venkata Mohan et al., 2010). The changes in the microbial community structure can be visualized through the PCR-DGGE fingerprint patterns. However, the PCR-DGGE could result in low resolution of DNA patterns and it is also difficult to identify the major DNA bands. In addition, the DNA sequences information is limited up to the genus level due to short sequences (< 200 bp). Despite its limitations, PCR-DGGE still remains a useful method for monitoring shifts in microbial community structure over time, especially to be used as a preliminary approach prior to in-depth analysis of next generation sequencing.

The recent development of high-throughput sequencing technology such as Illumina MiSeq has been helpful to analyse even low-abundance microorganisms, and therefore can elucidate the composition of the microbial community more completely and accurately. In this study, bacterial and archaeal diversity and composition at the different stages of POME treatment were assessed by using PCR-DGGE, followed by MiSeq sequencing, allowing insights into details of the distribution of these populations and their roles in the biodegradation processes. A comprehensive

assessment of the bacterial and methanogenic archaeal communities in the different stages would be beneficial for understanding the biodegradation mechanisms involved in the full-scale treatment of POME.

## 5.2 Materials and Methods

### 5.2.1 Samples Collection

POME samples were collected monthly from January until December 2015 at FLEDA Pasoh 4 Palm Oil Mill which situated at Simpang Pertang, Negeri Sembilan, Malaysia (3º0’52” N, 102º18’12” E). Eight samples were collected from the different stages comprising of anaerobic, facultative anaerobic and aerobic processes, including the mixed raw effluent (MRE), mixing pond, holding tank and final discharge in a typical POME treatment system as shown in **Figure 5.1**. The 2 L sample from each sampling point was collected in a pre-cleaned plastic container. The samples were collected from the collection pit at the end of each stage, except for MRE which was taken from the fresh raw POME.

### 5.2.2 Physicochemical Characterisation of POME

The physicochemical characterisation of selected parameters was conducted for all samples for one whole year period from January 2015 until December 2015.

#### 5.2.2.1 pH and Temperature

The pH and temperature values were recorded *in situ* using the portable meter according to the method as described in **Section 3.1.1**.

#### 5.2.2.2 Biochemical Oxygen Demand Concentration

The concentration of $BOD_5$ was measured for all samples according to the procedure in Standard Method APHA 5210-B (APHA, 2002) (**Section 3.1.2**).

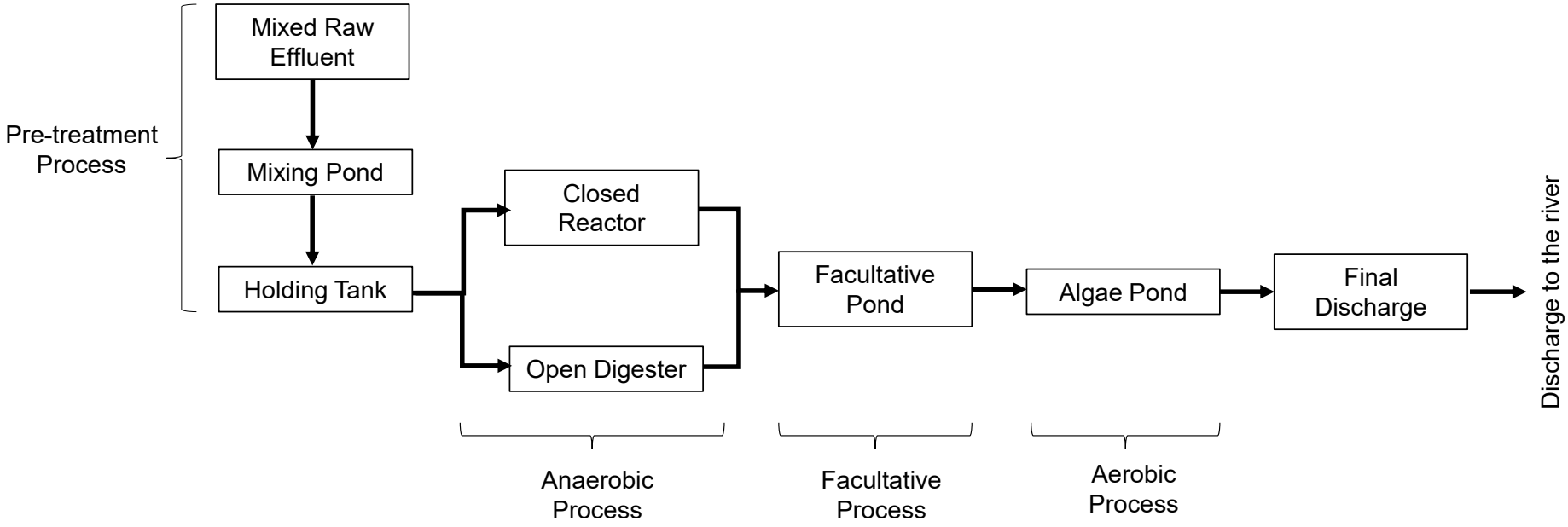



**Figure 5.1** **Sampling points of the different stages of palm oil mill effluent (POME) treatment at FELDA Pasoh Palm Oil Mill, Negeri Sembilan, Malaysia.**

#### 5.2.2.3 Chemical Oxygen Demand Concentration

The concentration of COD for all samples were measured by using reactor digestion method (HACH method 8000) (**Section 3.1.3**).

#### 5.2.2.4 Determination of Total Organic Carbon

The total organic carbon (TOC) of water samples was determined using a TOC-VCSH Analyzer (Shimadzu, Japan) by a differential method according to TOC Analyzer Manual (Annual Book of ASTM Standard, Standard D 7573-09). This technique is regarded as the most applicable method for measurement of organic carbon in the range of 0.3 mg/L to 1000 mg/L. The diluted water samples were injected into a quartz bed and heated at 680°C. The aqueous samples were then converted into a gaseous phase and forced through a layer of catalyst to ensure all compounds containing carbon were converted into $CO_2$ which was then measured by the non-dispersive infrared detector. For TOC analysis which consisted of all organic carbon regardless of form, a portion of the sample was injected to determine the total carbon (TC) which is the sum of all organic and inorganic carbon species. A portion of the sample was then acidified and purged to remove the inorganic carbon (IC) which consisted of $CO_3^{2-}$, $HCO_3^-$, $H_2CO_3$ and dissolved $CO_2$. The purged IC was measured as a total of IC. The TOC, TC and IC were calculated based on the following equations:

Equation 5.1:

$$TC\ (mg/L) = IC\ (mg/L) + TOC\ (mg/L)$$

Equation 5.2:

$$TOC\ (mg/L) = TC\ (mg/L) - IC\ (mg/L)$$

### 5.2.3 Compounds Characterisation

An understanding of the chemical composition of wastewater is important since this allows an understanding of reactions and interactions with the organic and inorganic compounds. The characterization of the chemical compounds of various types of wastewater (Huang et al., 2010; Sophonsiri and Morgenroth, 2004; Talib et al., 2016) is important to allow an understanding of reactions and interactions between the organic and inorganic compounds (Shon et al., 2006). Fundamental information on specific characteristics of organic matter is crucial to ensure the POME treatment process is in the optimized condition.

#### 5.2.3.1 Organic Acids Determination

The sample was centrifuged at 10,000 *g* for 15 min to remove the biomass. The organic acids were identified according to the analytical methods of NREL/TP-510-42623.43 (Sluiter et al., 2012) by high-performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) detector (Shimadzu, Japan). A 20 µL of sample was pre-filtered using 0.2 µm nylon membrane before it was injected into the HPLC system. The organic acids were separated on an Aminex HPX-87H column, 300 mm × 7.8 mm (Bio-Rad, California, USA) using 0.08 M sulphuric acid as a mobile phase at a flow rate of 0.6 mL/min and an oven temperature of 50°C. Detection was carried out using UV at 210 nm, while the peak of each acid was identified by referring the retention time obtained to that of standard compounds using a standard curve **(Appendix B)**. All chemicals used in the preparation of standard solutions for HPLC analyses were of analytical grade.

#### 5.2.3.2 Gas Chromatography-Mass Spectrometry Analysis

Compositions of other lignocellulosic degradation products in the samples were determined by using Gas Chromatography-Mass Spectrometry (GCMS) (Shimadzu, Japan) following the method of Chokwe et al. (2012), with a detection limit of peak area >2%. Liquid–liquid extraction pretreatment was carried out prior to GCMS analysis using $CH_2Cl_2$ (chromatogram pure grade, Fisher Corporation, USA). A 20 mL sample was freezed dried prior to dissolve in 2 mL methanol. The sample was then filtered through a 0.22 μm nylon membrane before being analysed. GCMS was equipped with a DB-5 column (Agilent, USA) of 30 m × 0.25 mm internal diameter, 0.25 μm film thickness. The conditions were set as follows: initial oven temperature was held at 70°C for 2 min and ramped at 20°C/min to 230°C, then increased to 270°C. Helium was used as a carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 250°C. A 1 μL sample was injected neat with a split ratio of 1:10. The mass spectra were recorded over the 50–650 amu range at one scan per second with ionization energy of 70 eV and ion source temperature at 230°C. The compositions of samples were qualitatively identified through comparison of sample's mass spectrum with library and literature.

### 5.2.4 Genomic DNA Extraction of POME Sludge and Final Discharge

For the purpose of bacterial community analyses, each sample except final discharge was dispensed into a sterile Falcon tube and centrifuged at 14,000 *g* for 10 min at 4°C. After decanting the supernatant, the pellet was stored at −20°C prior to further analysis.

Total genomic DNA was extracted from sludge samples using the Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., USA) following the manufacturer's instructions. Briefly, approximately 2 g of sludge sample was added to the Power Bead and lysis buffer-containing tubes provided in the kit, followed by the addition of Solutions C1 and C5. The DNA was eluted from the column using the C6 elution buffer. The DNA concentration and quality were then determined using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis, respectively. The extracted genomic DNA samples were stored at −20°C prior to further analysis. Meanwhile, the total genomic DNA extraction for final discharge samples was done according to the method as described in **Section 3.2**.

### 5.2.5 Bacterial Community Analysis by PCR-Denaturing Gradient Gel Electrophoresis

Bacterial community analysis in POME and final discharge samples were performed following the methods as described in **Section 3.3**.

### 5.2.6 Illumina MiSeq Sequencing and Bioinformatic Analysis

For the metagenomics analysis of bacterial and archaeal community, extracted genomic DNA was proceeded to high-throughput MiSeq sequencing and bioinformatics procedure. The details of the methods used were described in **Section 3.4**.

## 5.3 Results and Discussion

### 5.3.1 Physicochemical Characteristics of Palm Oil Mill Effluent

The assessment of the physicochemical properties of POME in each stage of treatment is one of the important aspects in understanding the influence of treatment conditions on the changes of bacterial community structure. Hence, in this study, the physicochemical characterization of selected parameters was conducted for all samples which included the pre-treatment, anaerobic, facultative, aerobic and final discharge stages of POME treatment for one-year period from January until December 2015.

#### 5.3.1.1 pH and Temperature

The pH value is a measure of the acid strength in the water, while the water temperature is defined as a measure of heat content of the water mass which both generally affected most physical, biological and chemical characteristics of a river water (Ibrahim et al., 2012). The pH values and temperature of the samples were recorded (**Figure 5.2**) as one of the efforts to determine the influence of pH and temperature towards POME biodegradation throughout the treatment.

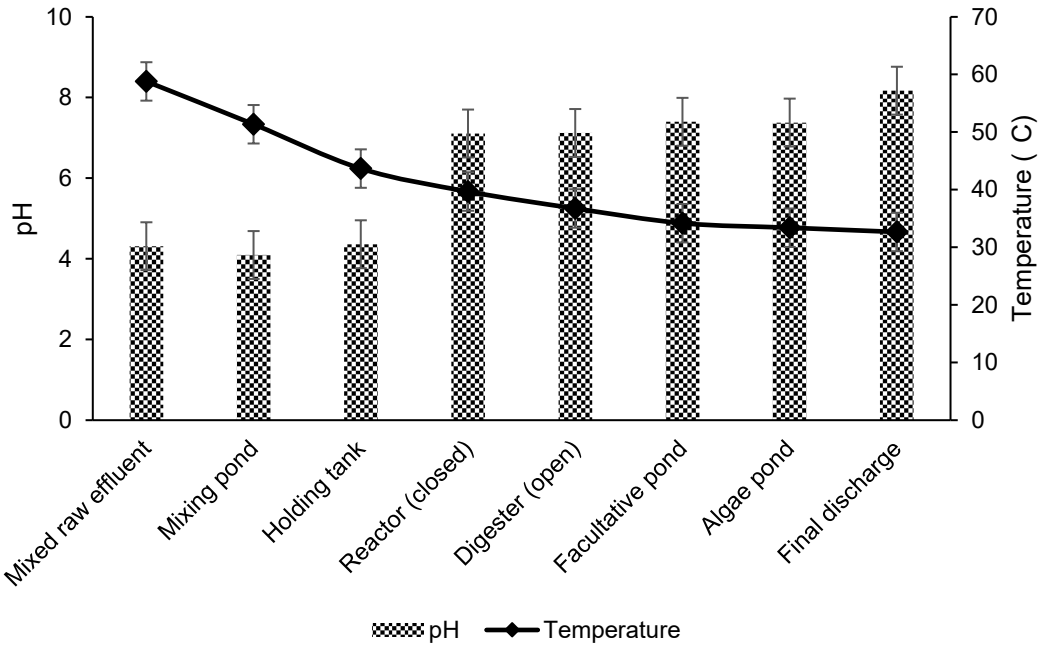



**Figure 5.2 The profiles of pH and temperature in the different stages of POME treatment.**

POME has a substantially high temperature during the initial generation of the fresh raw POME itself and during the first stage of effluent treatment in the ponds (Bala et al., 2014a). In this study, fresh raw POME (MRE) recorded a slightly low temperature of 59°C (**Figure 5.2**) as compared to the other studies which reported the temperature range of 80 to 90°C (Alhaji et al., 2016; Bello and Abdul-Raman, 2017). The POME was then cooled down in the first pond to 60 – 63°C (Yoochatchaval et al., 2011). Generally, in palm oil processing, hot steam is used in most of the stages which include the sterilization, digestion and oil extraction processes, hence producing a fresh raw POME with a very high temperature. Following that, the holding tank and ponding system are function to cool down the high temperature of POME as the effluent went through the treatment stages. The temperature was reducing steadily throughout the treatment stages (Igwe and Onyegbado, 2007) where the final discharge recorded the

temperature of 33°C. It is shown that the average temperature for final discharge was comparable and within the range of ambient temperature as reported in the other studies (Saeed et al., 2016; Zainal et al., 2017).

Meanwhile, the samples from pre-treatment processes of POME (MRE, mixing pond and holding tank) recorded an acidic pH at 4.0 - 4.3, mainly due to the generation of organic and free fatty acids resulted from the partial degradation of palm fruits (Salihu and Alam, 2012). pH values then continued to rise slowly until pH 7.1 during anaerobic treatment (reactor and digester tank). This might be due to the degradation of organic acids, as well as the consumption of $H^+$ during methanogenic digestion (Feng et al., 2015). Methanogenesis is strongly affected by pH, as such, methanogenic activity will decrease when pH in the digester deviates from the optimum value. Gerardi (2006) reported that optimum pH for most microbial growth in biological treatment unit is between 6.8 and 7.2, while pH lower than 4 and higher than 9.5 are not tolerable.

Meanwhile, the pH was increased from 7.38 ± 0.23 to 8.17 ± 0.54 from facultative pond to final discharge. Facultative and algae ponds are necessary to further reduce the organic content in the wastewater before it is discharged into rivers. The facultative ponds rely on bacteria to break down the organic matters into simple end products of methane, carbon dioxide, hydrogen sulphide and water (Azmi and Yunos, 2014). Meanwhile, the algae ponds rely on algae to consume carbon dioxide during photosynthesis, and this consumption is responsible for an increase in pH before discharge into the river (Gerardi and Lytle, 2015).

Nevertheless, as stated by Malaysia Environmental Quality Act 1974 (DOE, 1977), the final discharge showed acceptable pH and temperature values whereby the standard temperature should be below 45°C, while pH value should be ranging from pH 5.0 to 9.0.

#### 5.3.1.2 Correlation between Biochemical Oxygen Demand, Chemical Oxygen Demand and Biogas Production

Biochemical oxygen demand ($BOD_5$) is one of the basic and most important water quality parameters because it provides an index to assess and indicate the strength of pollutants in terms of oxygen required to stabilise domestic and industrial wastes (Malaysia Environmental Quality Report, 2006). In this study, the concentration of $BOD_5$ was measured to indicate the functionality of POME treatment system. **Figure 5.3** shows the average changes of $BOD_5$ concentration throughout POME treatment for all sampling points whereby the MRE showed the highest average of $BOD_5$ value with 15,068 mg/L due to high organic contents in fresh raw POME (Bala et al., 2014b).

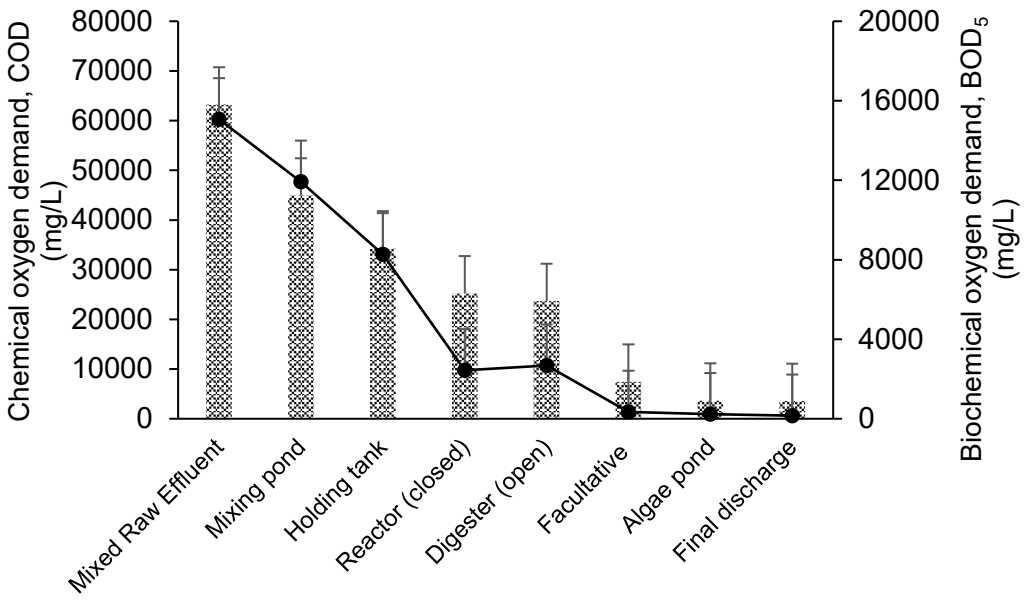



**Figure 5.3 The changes of $BOD_5$ (●) and COD (▒) throughout the different stages of POME treatment. The error bars represent standard deviations of experiments performed in twelve months.**

Fresh raw POME contains biodegradable constituents with a BOD/COD ratio of 0.5 and this implies that POME can be easily treated using biological means (Tchobanoglous et al., 2003). A large reduction of $BOD_5$ concentration was detected in the closed reactor (83.8%) and open digester (82.3%), hence indicated the satisfactory performance of anaerobic processes. According to Satyawali and Balakrishnan (2008), more than 80% of BOD can be removed from an anaerobic treatment with the potential of energy recovery in the form of biogas.

In order to complement the anaerobic treatment in reducing the $BOD_5$ concentration effectively until it meets the standard discharge limit for river water quality by the Department of Environment (DOE) of Malaysia, aerobic processes have been introduced as a part of the POME treatment. In this study, the average $BOD_5$ concentration in the final discharge was appeared to be slightly higher (155 mg/L)

from the standard discharge limit (100 mg/L). It might be due to the high processing FFB season in the year that produced a high load of incoming POME into the treatment plant (Liew et al., 2014).

The generation of POME is also commonly associated with high level of COD concentration (Khemkhao et al., 2012). A high COD concentration may indicate the presence of recalcitrant chemicals which could not be degraded biologically, hence, the bioaccumulation of these compounds may give rise to environmental problems (Kanu and Achie, 2011). The COD concentrations for all sampling points throughout POME treatment stages were recorded as shown in **Figure 5.3** where it showed a similar reducing trend as $BOD_5$ data. Anaerobic treatment is regarded as one of the most effective ways to reduce COD reduction in high organic waste including POME. The COD removal was recorded with 60.1% reduction in the closed reactor and 62.5% reduction in the open digester. According to Wilkie et al. (2000), more than 50% of COD can be converted to biogas.

The biogas production was also measured in the studied palm oil mill from the commercial scale of closed reactors (1800 $m^3$ each; four units) with a total biogas production of 1,178,620 $m^3$/year (**Figure 5.4**), hence indicates the anaerobic treatment is well functioned. Theoretically, the generation of methane can be estimated based on COD removal. In this study, the average COD removal in one unit of the closed reactor was 38,008 mg/L (60.1%). Therefore, methane was estimated to be produced in the closed reactor as stated in **Table 5.1**, following the calculations by Battacharya et al. (2005).

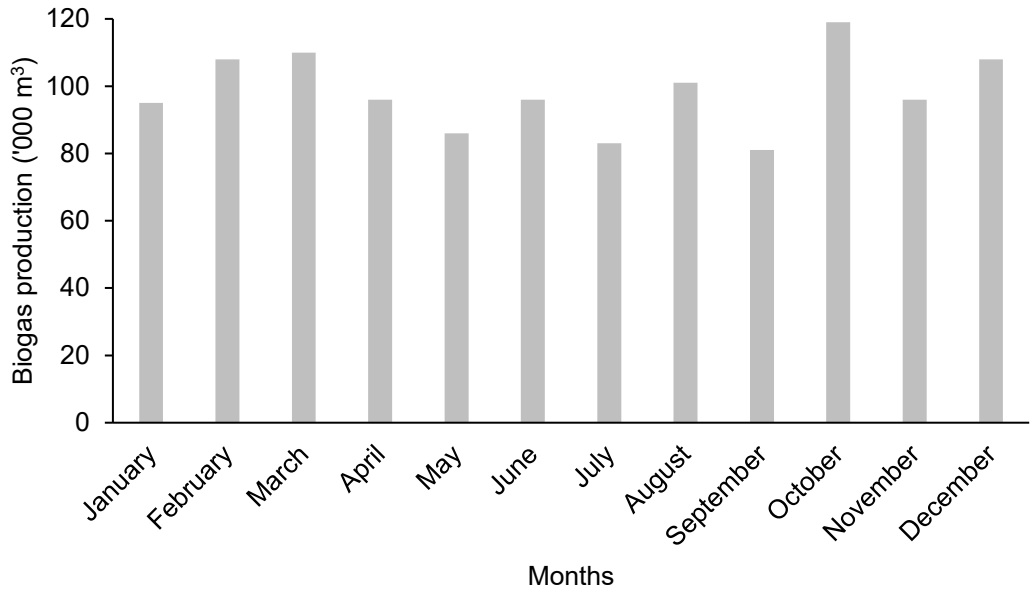



**Figure 5.4** **Monthly production of biogas in commercial scale reactors (four units) with the capacity of 1800 m$^3$ each in the studied palm oil mill in 2015** (Source: Data provided by FELDA Pasoh Palm Oil Mill).

**Table 5.1** **Estimated methane production from POME based on 60.1% COD removal in one unit of the anaerobic (closed) reactor with the capacity of 1800 m$^3$.**

| Parameter | Unit | Closed reactor |
|---|---|---|
| POME generated [a] | m$^3$ | 1800 |
| COD concentration in raw POME | mg/L | 63,233 |
| COD in tank | mg/L | 25,226 |
| COD converted | tonnes | 68.41 |
| $CH_4$ produced [b] | tonnes | 17.10 |

[a] Capacity of tank
[b] Theoretical methane conversion factor is 0.25 kg $CH_4$ per kg COD

Following the anaerobic process, the average COD concentration was reduced until it reached 3,571 mg/L of COD for final discharge sample. These data reflected the feasibility of aerobic process where it is implemented as the secondary treatment to further treat the POME before it is being released into nearby river. The result was in accordance with Vijayaraghavan et al. (2007) who showed a remarkable removal of COD in POME by using aerobic treatment.

#### 5.3.1.3 Total Organic Carbon

The TOC analysis is one of the measurement methods for identifying the amount of organic matter in wastewater and also plays an important role in assessing the efficiency of wastewater treatment system (Poh et al., 2010). Generally, it is a sum of dissolved organic carbon and particulate organic carbon. The average TOC concentration recorded in this study for each sampling point is shown in **Figure 5.5**. Based on the results obtained, the TOC concentration was reduced from MRE to the final discharge point. The reduction of TOC concentration was shown to be in accordance with the $BOD_5$ and COD data which supports the correlation between these parameters as stated by Dubber and Gray (2010). This finding also indicated that the implementation of POME treatment in the mill is successful in reducing the TOC content of the POME, in correlation with the reduction of $BOD_5$ and COD concentrations.

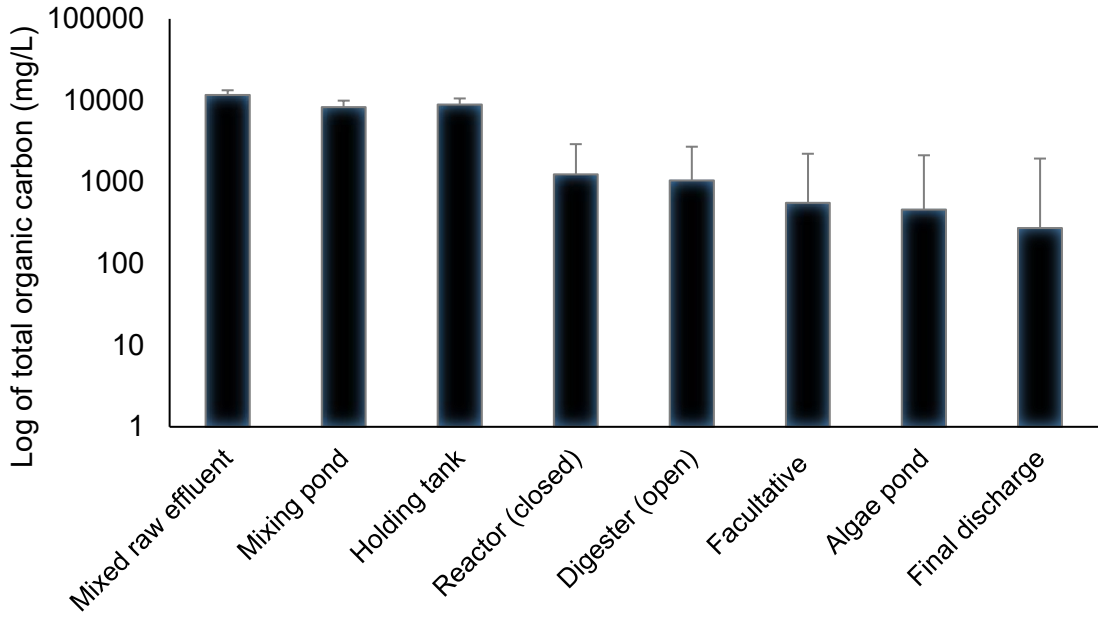



**Figure 5.5 The changes of TOC concentration throughout the different stages of POME treatment. The error bars represent standard deviations of experiments performed in twelve months.**

### 5.3.2 PCR-DGGE Analysis of Bacterial Community Structure

Biological treatment of POME is relying upon the activity of complex microbial communities, hence they must be functionally stable in order to continuously and steadily remove organic pollutants in POME. However, knowledge regarding the microbial community structures in the POME treatment and their correlation to the environmental variables are still lacking. The PCR-DGGE of 16S rRNA genes is a common molecular tool used in assessing the microbial community in the effluent treatment plant (Bafana et al., 2015). This method has also been successfully used to investigate the bacterial diversity in municipal wastewater (Miura et al., 2007). In this study, a PCR-DGGE method was used as a preliminary and confirmatory step prior to in-depth sequencing analysis using Illumina MiSeq.

In general, the coupling of PCR amplification of taxonomical targets with DGGE enable the separation of DNA fragments of identical length but of different sequence which allows the evaluation of the dominant bacterial community diversity in the samples (Lucena-Padrós et al., 2015). Prior to DGGE, the amplification of the extracted DNA was done using a pair of 16S rRNA universal primer, producing DNA fragment with 161 base pair as shown in **Figure 5.6**. The DGGE analyses were then carried out for the samples from all treatment stages of POME for a period of one year for rapid detection of bacterial community changes, as well as to analyse the distribution and composition of bacterial community (**Figure 5.7**).

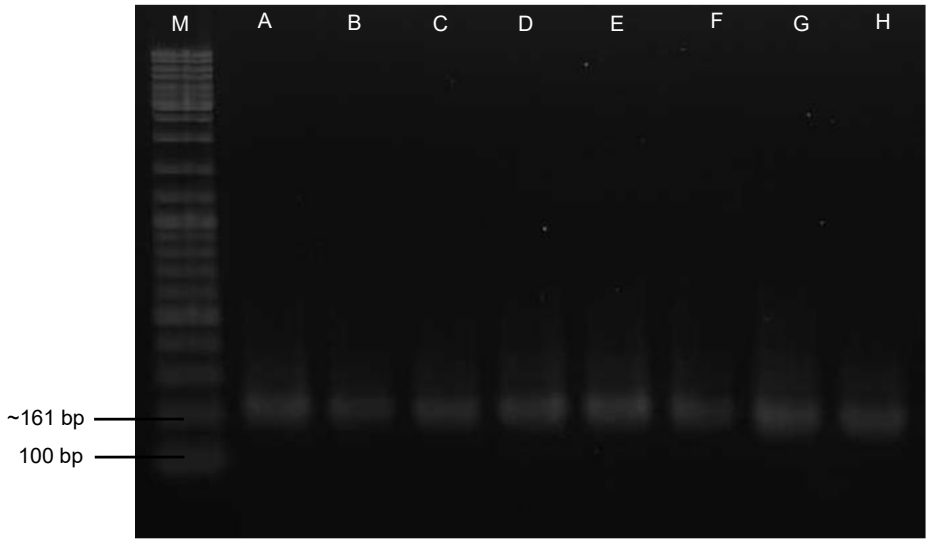
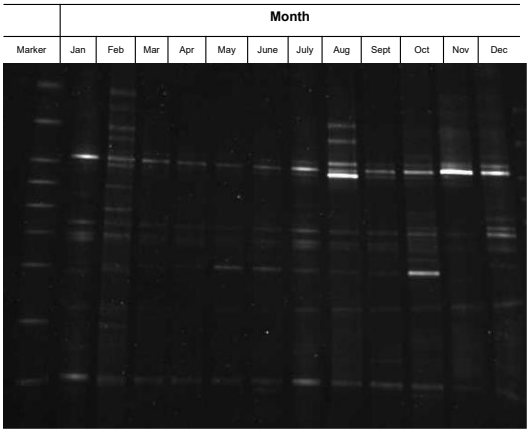
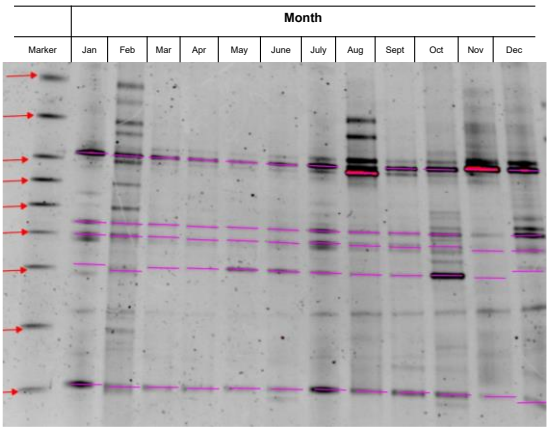



**Figure 5.6** **Representative of 16S rRNA regions amplified by PCR separated on 1.6% agarose gel electrophoresis with the approximate size of 161 bp. (A: MRE, B: Mixing pond, C: Holding tank, D: Reactor (closed), E: Digester (open), F: Facultative, G: Algae, H: Final discharge). M: 100 bp DNA ladder as a molecular marker.**

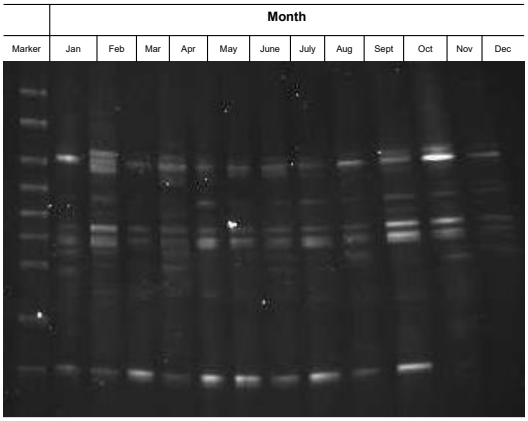
**A (i)**



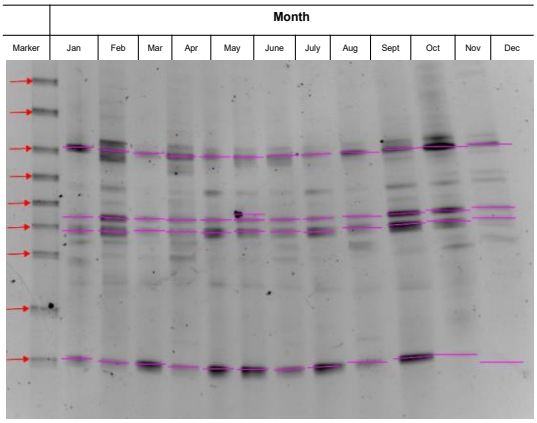

**A(ii)**



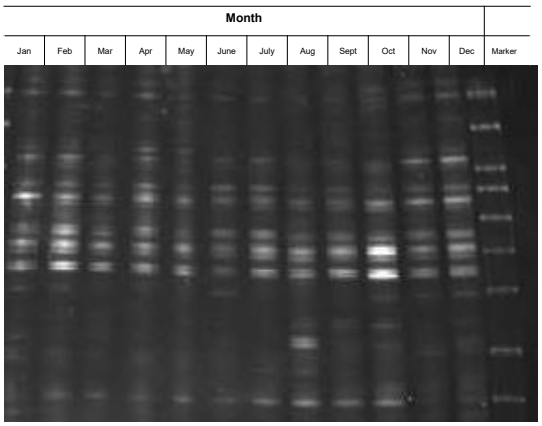

**B (i)**



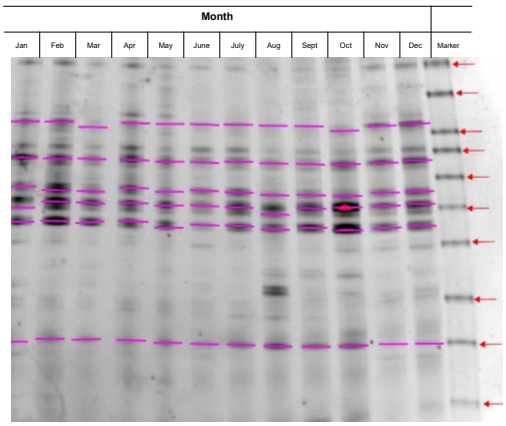

**B (ii)**



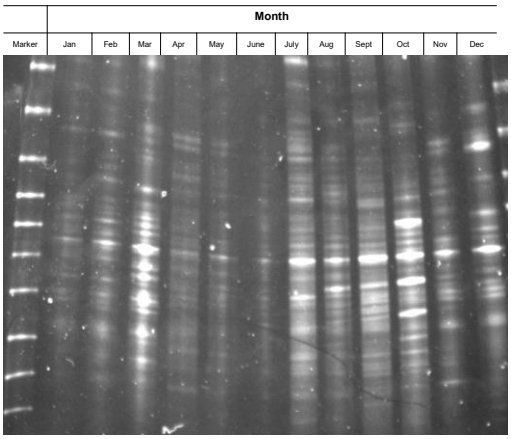

**C (i)**



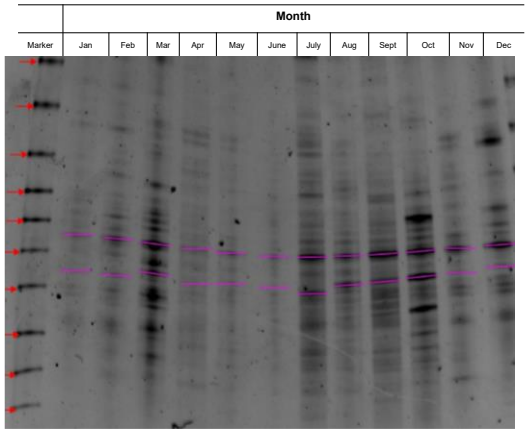

**C (ii)**



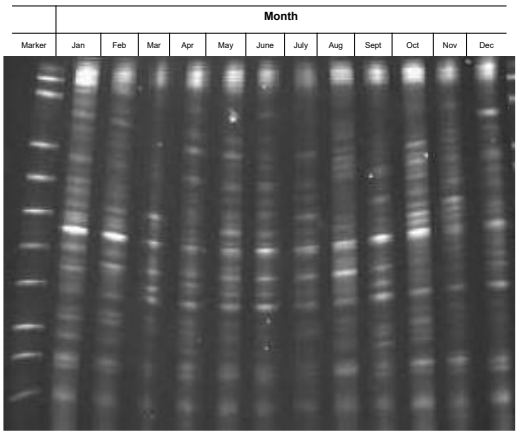
**D (i)**



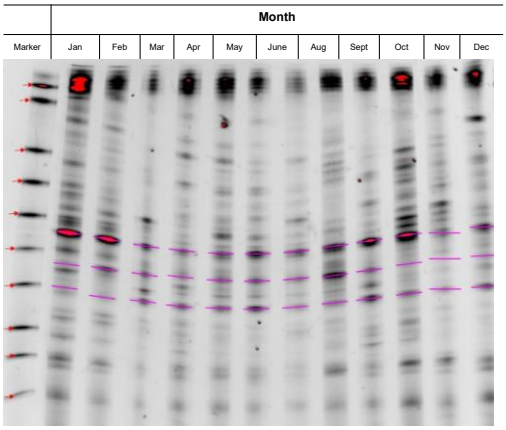

**D (ii)**



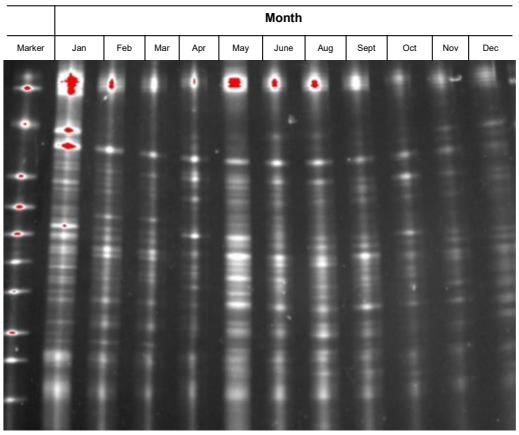

**E (i)**



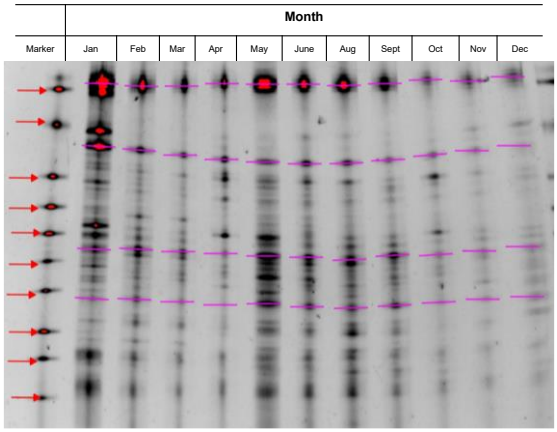

**E (ii)**



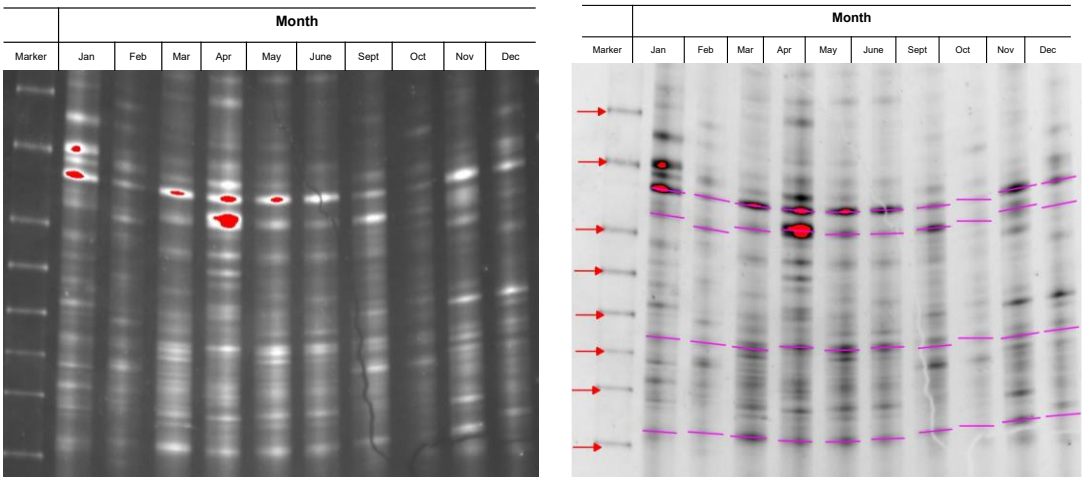

**F (i)**




**F (ii)**



**G (i)** **G (ii)**




**H (i)** **H (ii)**

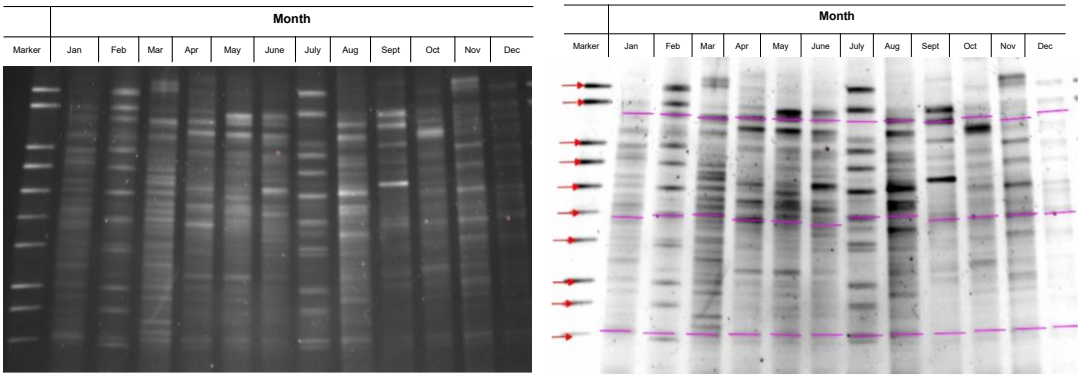



**Figure 5.7** **DGGE fingerprint profiles (i) and normalized-DGGE images (ii) respectively for A: MRE, B: mixing pond, C: holding tank, D: reactor (closed), E: digester (open), F: facultative, G: algae, H: final discharge for one-year monitoring period.**

The sequencing analysis of the dominant bands in all the POME samples revealed the presence of at least 30 different bacterial species with most of these species belonging to the *Firmicutes* phylum (**Table 5.2**). The diverse bacterial population along POME treatment is due to the highly complex nature of POME. The presence of palm fibers in POME composed of cellulose, hemicelluloses and lignin can be biodegraded by hydrolysis reactions. The temperature of 51 – 59°C recorded in the MRE, mixing pond and holding tank (**Figure 5.2**) is beneficial for the hydrolysis of the fiber (Khemkhao et al., 2015). A higher temperature not only increases thermal degradation, but also, based on PCR-DGGE analysis (**Figure 5.7**), enriches a number of working hydrolytic, acidogenic and acetogenic bacteria (**Table 5.2**), for instance members of *Clostridiales* family such as *Thermoanaerobacterales*, *Thermoanaerobacterium thermosaccharolyticum* and *Caldicellulosiruptor*. It has been reported that the well-known anaerobic cellulolytic bacteria require high temperatures for their growth and also for cellulose degradation (Pérez et al., 2002).

**Table 5.2** **Phylogenetic sequence affiliation and similarity to the closest relative of amplified 16S rRNA gene sequences excised from DGGE gels for A: MRE, B: mixing pond, C: holding tank, D: reactor (closed), E: digester (open), F: facultative, G: algae, H: final discharge.**

| Band ID | S_ab score | Closest relative | Phylum | Family |
|---|---|---|---|---|
| **A: Mixed raw effluent (MRE)** | | | | |
| 1 | 0.941 | *Thermoanaerobacterium thermosaccharolyticum* | *Firmicutes* | *Syntrophomonadaceae* |
| 2 | 0.958 | *Caldanaerobius* sp. *CTT12* | *Firmicutes* | *Thermoanaero-bacteraceae* |
| 3 | 0.987 | *Brachybacterium* sp. *K473* | *Actinobacteria* | *Dermabacteraceae* |
| 4 | 0872 | *Lactobacillus mucosae* | *Firmicutes* | *Lactobacillaceae* |
| 5 | 0.85 | *Thermoanaerobacterales bacterium* | *Firmicutes* | *Clostridiales* |
| **B: Mixing pond** | | | | |
| 1 | 1.00 | *Lactobacillus delbrueckii* subsp. *lactis* | *Firmicutes* | *Bacilli* |
| 2 | 1.00 | *Thermoanaerobacterium thermosaccharolyticum* | *Firmicutes* | *Clostridiales* |
| 3 | 1.00 | *Caldanaerobius* sp. | *Firmicutes* | *Thermoanaero-bacteraceae* |
| 4 | 0.916 | *Caldicellulosiruptor* | *Firmicutes* | *Clostridiales* |
| **C: Holding tank** | | | | |
| 1 | 0.917 | *Lactobacillus delbrueckii subsp. delbrueckii* | *Firmicutes* | *Lactobacillaceae* |
| 2 | 0.939 | *Prevotella* sp. | *Bacteroidetes* | *Prevotellaceae* |
| 3 | 1.00 | *Thermoanaerobacterium thermosaccharolyticum* | *Firmicutes* | *Clostridiales* |
| 4 | 0.658 | *Thermoanaerobacterales bacterium* | *Firmicutes* | *Clostridiales* |
| 5 | 0.921 | Uncultured *Clostridium sp.* | *Firmicutes* | *Ruminococcaceae* |
| 6 | 0.846 | *Caldanaerobius* sp. | *Firmicutes* | *Thermoanaero-bacteraceae* |
| **D: Reactor (closed)** | | | | |
| 1 | 0.586 | Uncultured bacterium | *Bacteroidetes* | *Bacteroidaceae* |
| 2 | 0.948 | uncultured bacterium | *Firmicutes* | *Syntrophomonadaceae* |
| **E: Digester (open)** | | | | |
| 1 | 0.988 | Uncultured bacterium | *Bacteroidetes* | *Prolixibacteraceae* |

| | | | | |
|---|---|---|---|---|
| 2 | 0.994 | Uncultured bacterium | *Bacteroidetes* | Unclassified |
| 3 | 0.976 | Uncultured bacterium | Unclassified bacteria | |
| **F: Facultative** | | | | |
| 1 | 0.902 | *Chloroflexi* bacterium | *Chloroflexi* | *Anaerolineaceae* |
| 2 | 1.000 | *Bacillus lentus* | *Firmicutes* | *Bacillaceae* |
| 3 | 0.374 | *Polaromonas* sp. | *Proteobacteria* | *Comamonadaceae* |
| 4 | 0.336 | *Gallibacterium anatis* | *Proteobacteria* | *Pasteurellaceae* |
| **G: Algae** | | | | |
| 1 | 0.915 | *Arcobacter butzleri* | *Proteobacteria* | *Campylobacteraceae* |
| 2 | 0.645 | *Thermotogaceae* bacterium | *Thermotogae* | *Kosmotogaceae* |
| 3 | 1.000 | Uncultured bacterium | *Synergistetes* | *Synergistaceae* |
| **H: Final discharge** | | | | |
| 1 | 0.966 | *Pseudomonas* sp. | *Proteobacteria* | *Pseudomonadaceae* |
| 2 | 0.918 | *Povalibacter* | *Proteobacteria* | *Sinobacteraceae* |
| 3 | 1.000 | *Polynucleobacter* | *Proteobacteria* | *Burkholderiaceae* |

It is generally known that the fermentative bacteria are required to metabolise the complex organic materials of POME into fermentation products (Jong et al., 2011). The fermentative bacteria for instance *Firmicutes* that present in the early stages of POME treatment (MRE, mixing pond and holding tank) may play a role in fermenting the complex compositions (lignocellulosic) of POME into simple organic compounds. The results were in accordance with the bacterial community studies in simultaneous treatment of raw POME and biodegradation of palm fiber in high-rate continuous strirred tank reactor (CSTR) conducted by Khemkhao et al. (2015) and Nitipan et al. (2014) whereby *Firmicutes* was found as a dominant phylum.

In addition, DGGE fingerprinting also revealed the presence of *Lactobacillaceae*, *Prevotellaceae*, *Ruminococcaceae* and *Thermoanaerobacteraceae* (**Table 5.2 (C)**) dominantly in the holding tank. According to Schmidt et al. (2015), *Ruminococcaceae* function as main anaerobic degraders of cellulose-derived carbon, while

*Thermoanaerobacteraceae* was reported to be consistently detected in cellulose-containing wastewater (Liu et al., 2003).

On the other hand, the DGGE fingerprints of the final discharge sample revealed a distinct shift of bacterial community due to aerobic condition and biodegradation processes throughout POME treatment with the appearance of dominant bands corresponding to sequences affiliated to the *Pseudomonadaceae*, *Sinobacteraceae* and *Burkholderiaceae* families (**Table 5.2 (H)**). *Pseudomonas* sp., a member of *Pseudomonadaceae* family was reported to be able to assist plants in phytoremediation for removing pollutants from POME final discharge (Hamzah et al., 2016). Elmi et al. (2015) also suggested that the presence of *Pseudomonas* sp. was able to reduce the COD value to 58%. Besides, the ability of *Pseudomonas* sp. to reduce COD concentration was also being reported by Sonune and Garode (2015) where 60% of COD reduction from municipal wastewater was achieved. Meanwhile, *Sinobacteraceae* prefers to utilize aliphatic, aromatic hydrocarbon compounds and small organic acids for degradation (Gutierrez et al., 2012). According to Zhang et al. (2018), *Sinobacteraceae* is one of key players in the biodegradation of hazardous organic pollutants in the textile-dyeing industrial wastewater system.

However, it should be noted that there are some limitations in the PCR-DGGE approach, for instance low abundance microorganisms may not clearly appear in distinct bands in DGGE gel, hence the analysis gives incomplete distribution and composition of species in a particular sample. According to Lucena-Padrós et al. (2015), PCR-DGGE method only provides the monitoring of dominant bacterial population whereby each member of bacterial populations could not be detected by

PCR-DGGE and might only appear as a weak band when their concentration is lower than $10^3$ CFU/mL. Due to the high number of bacterial diversity in a particular sample, it may results in smear bands cause by the high number of the weak bands (Boon et al., 2002). Hence, a high-throughput sequencing using Illumina MiSeq was applied in this study to thoroughly investigate the compositions of bacterial and archaeal communities that are responsible in the biodegradation of POME throughout the treatment, as discussed in the next section.

### 5.3.3 Next Generation Sequencing (Illumina MiSeq) Analysis

The functional stability of POME treatment system primarily relies on the dominant microbial activities and their interactions within highly diverse communities. An anaerobic digestion of POME uses unknown microbial populations (McHugh et al., 2003) which were responsible for the biodegradation of organic compounds to reduce the polluting power of wastes and wastewater (David and Japareng, 2015). With a limited knowledge of microbial community that is responsible for the biodegradation particularly in the full-scale treatment of POME, this study offers a novel insight into the composition and diversity of the bacterial and methanogenic archaeal populations during POME treatment.

Recently, many studies have investigated the dynamics and diversity of microbial communities from different wastewater treatment systems by using high-throughput sequencing, which enables us to detect massive microbial populations at a high throughput and low cost (Ma et al., 2015; Wang et al., 2012; Wang et al., 2017; Zhang et al., 2015). Illumina MiSeq, in particular, has been successfully used to study

microbial compositions in various industrial systems (Caporaso et al., 2012). A thorough understanding on the microbial community structure of POME has also been carried out in various bioreactor configurations (Rana et al., 2017). However, to our best knowledge, there is very limited studies on the microbial communities that are responsible in the biodegradation of POME in the different stages of a full-scale treatment. Therefore, a thorough in-depth sequencing using Illumina MiSeq was carried out in this study to analyse the compositions and diversity of bacterial and archaeal populations from different stages of POME treatment for one whole year period.

#### 5.3.3.1 Richness and Diversity of the Bacterial Community

The normalised DGGE images (**Figure 5.7(ii)**) provide the important clues on the bacterial community structure and composition in the POME treatment system for a year period. Since the dominant bacterial populations in each stage showed almost a similar pattern throughout a year, only samples from January, June and December were selected to be further analysed using Illumina MiSeq, mainly due to seasonal difference of temperatures between rainy and dry season in these three months in the studied sites.

In total, 1,134,163 effective sequences with an average length of 253 bp were obtained from high throughput sequencing and reads for each sample ranged from 82,687 to 200,631 (**Figure 5.8**). Sequences with ≥ 97% similarity were grouped into OTUs. The sequence number of each sample was normalized and 38,069 – 148,262 OTUs were generated using the QIIME platform. An average of 89,509 ± 36,055 OTUs was

identified in all samples. These data have been deposited into the NCBI database under the accession number SRP108921. A total of 240,733 OTUs were assigned and the sequencing coverage rate was exceeded with values ranging from 90% to 99% for each sample, indicating that exhaustive information on the microbial community was obtained through MiSeq.

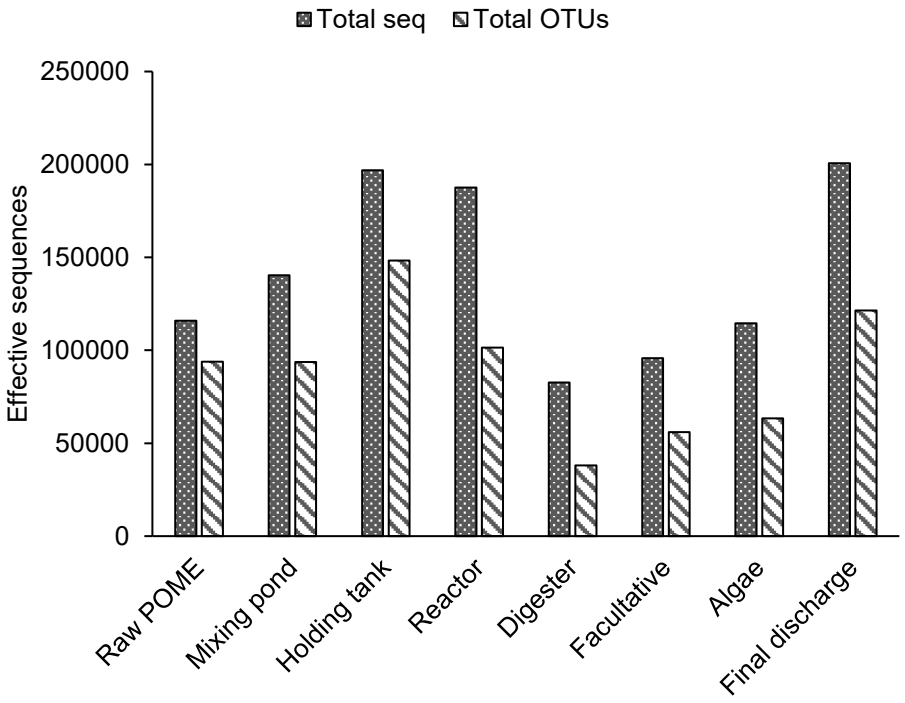



**Figure 5.8 Summary of analysed sequences in the different stages of POME treatment.**

In this study, the Shannon diversity index (H′) and Evenness index (E') were used to measure the species richness and evenness of distribution of species within a community, where higher values of indices can be regarded as higher genetic diversity at a site. The richness and diversity of the bacterial and archaeal communities in the different stages of POME treatment are shown in **Table 5.3**. In the OTU-based analyses, the alpha diversity, including OTU richness, H' and E' indices were

significantly increased from initial to the later stages of POME treatment. It was suggested that the bacteria that played important roles in the biodegradation at the early phases might become excessively dominant, hence lowering the H', as reported by Sharuddin et al. (2017). In addition, the high concentration of organic matter that was readily available to most bacteria could contribute to the increment of the bacterial population (Jang et al., 2014), hence reducing the H' and E' in the early phases of POME treatment. Cardinali-Rezende et al. (2016) in their study also reported on the shift of bacterial community during the treatment of municipal solid waste with the increased diversity over time based on the biodegradative ability of microorganisms during anaerobic digestion.

**Table 5.3 Microbial alpha-diversity characteristics using Shannon-Weaver index (H') and Evenness (E') for POME samples from each stage of treatment.**

| POME treatment stages | | Alpha-diversity analyses | |
|---|---|---|---|
| | | **Shannon-Weaver index (H')** | **Evenness (E')** |
| Mixed raw effluent | | 0.869 | 0.050 |
| Mixing pond | | 0.800 | 0.077 |
| Holding tank | | 1.119 | 0.073 |
| Closed reactor | Anaerobic | 1.908 | 0.120 |
| Open digester | Anaerobic | 1.725 | 0.115 |
| Facultative anaerobic pond | | 1.933 | 0.111 |
| Aerobic (algae) pond | | 1.929 | 0.109 |
| Final discharge | | 2.272 | 0.145 |

#### 5.3.3.2 Shift of Bacterial Community Involved in the Initial Hydrolysis

The analysed OTUs were assigned to 38 phyla, 92 classes, 155 orders, 236 families and 694 genera. The bacterial community at the family level is shown to be shifted throughout the treatment of POME (**Figure 5.9**). At the family level, a total of 370 families was obtained and the major sequences were classified into 21 families with ≥2% on average.

MRE was suggested to contain microorganisms derived from palm fruit and lignocellulosic materials and contain various organic compounds generated in the process of palm oil production (Ahmad et al., 2011a; Hassan et al., 2005). *Lactobacillaceae* of the phylum *Firmicutes* showed the highest abundance in the MRE (35.9%) where it was suggested to contribute to the higher lactic acid production (3.127 ± 1 g/L) (**Figure 5.10**). A high proportion of *Lactobacillaceae* detected in the MRE might have originated from the remaining fiber since fresh raw POME contained a high concentration of suspended solids which mainly consisted of debris from the palm fruit mesocarp generated during the crude palm oil extraction process (Lam and Lee, 2011). According to Baharuddin et al. (2009), lactic acid bacteria were found as the prominent community in the oil palm empty fruit bunch (OPEFB) due to the remaining oil content.

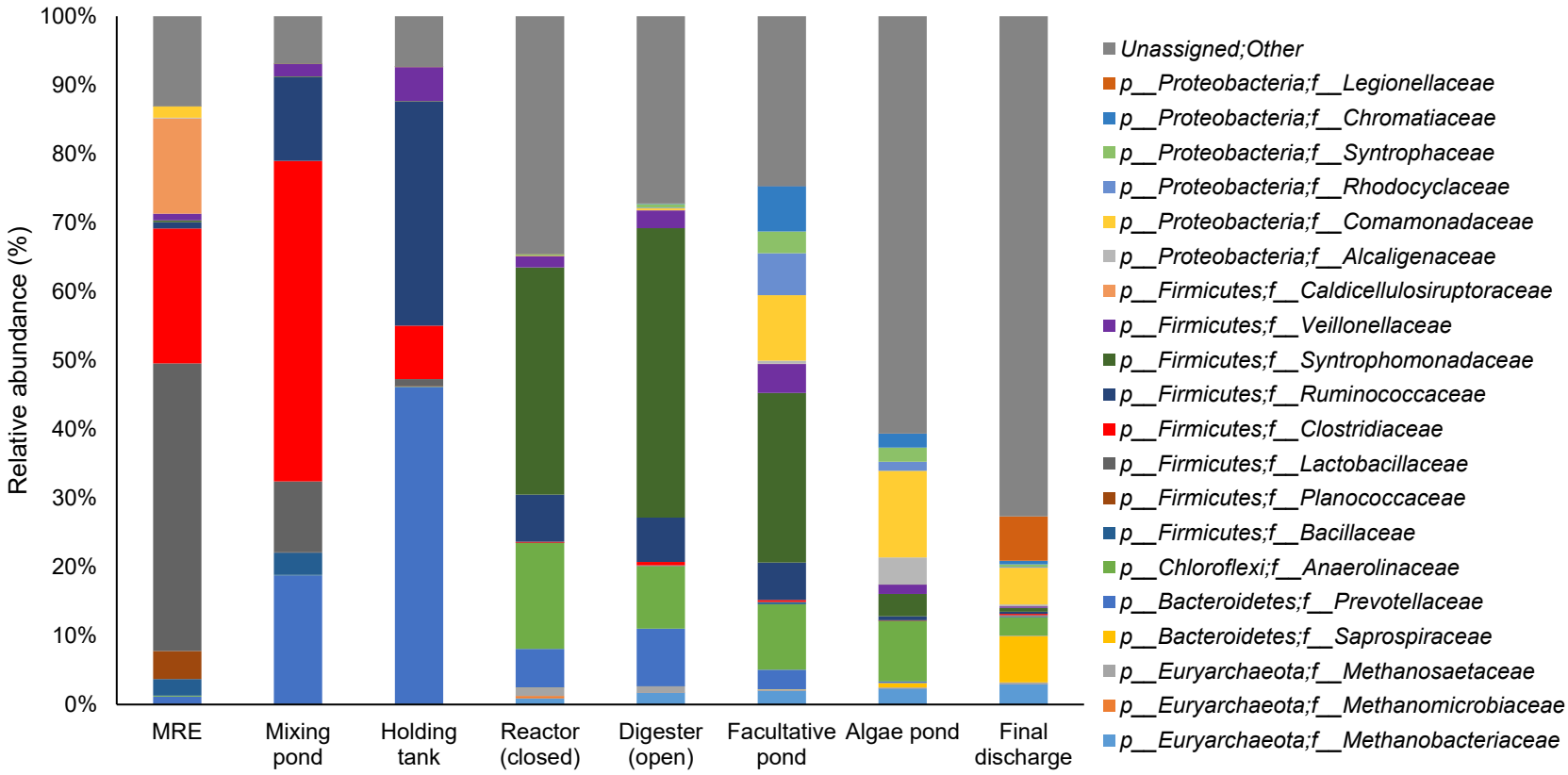



**Figure 5.9** **Relative abundance of the bacterial and archaeal communities categorized at the taxonomic family level in the different stages of a full-scale treatment of POME that were summarized from three sampling months (January, June and December 2015). *p*: phylum, *f*: family.**

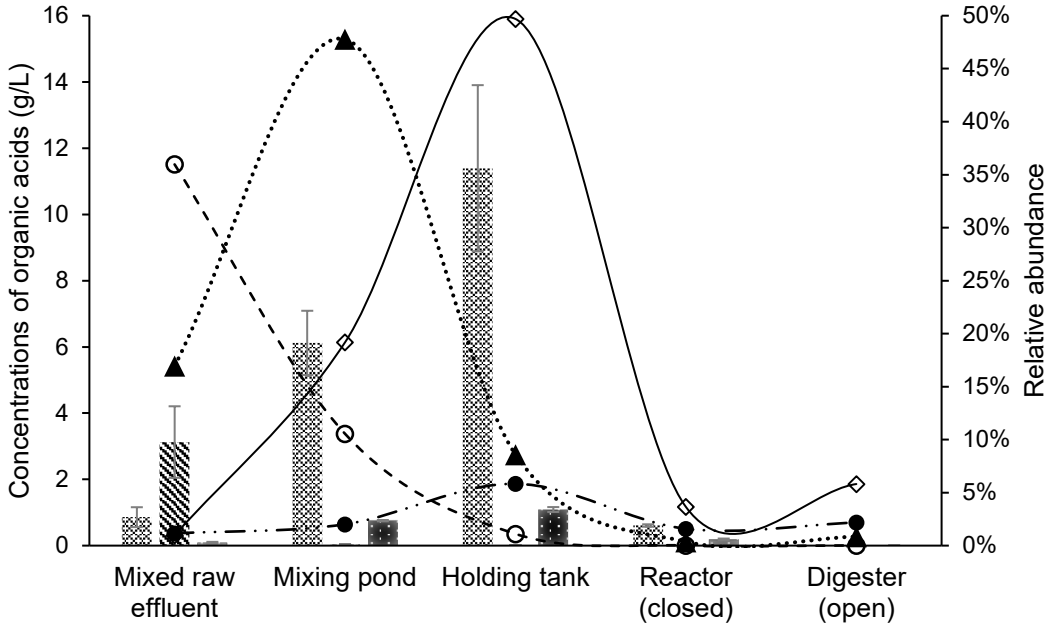



**Figure 5.10** **Relative abundance of *Lactobacillaceae* (○), *Veillonellaceae* (●), *Prevotellaceae* (◊) and *Clostridiaceae* (▲) in relation to the concentrations of acetic (▨), lactic (▧) and propionic (▩) acids detected in the MRE, mixing pond, holding tank and closed reactor. No acids were detected in the open digester and the subsequent stages of POME treatment. The error bars represent standard deviations of experiments performed in triplicate.**

This study demonstrated the production of lactic acid by *Lactobacillaceae* as the earliest intermediate in the MRE, but as the reaction progressed, lactic acid showed a reduction while propionic and acetic acids were produced. The conversion of lactic acid to propionic acid, acetic acid and $CO_2$ was previously reported to be catalysed by *Veillonellaceae* family (Cibis et al., 2016). This bacterium was found to be increased in the mixing pond (2.0%) and holding tank (5.83%) (**Figure 5.9**) in accordance with the increased concentrations of propionic acid and acetic acid (**Figure 5.10**). However, a large increment of acetic acid in the mixing pond to 6.121 ± 1 g/L was suggested to be produced by *Clostridiaceae* (47.8%) through the fermentation of cellulose and glucose. A recent study by Mustapha et al. (2018) suggested that a high population of

*Clostridia* in the waste sewage sludge might be involved in the hydrolysis and acidogenesis. Meanwhile, further increment of acetic acid concentration in the holding tank (11.393 ± 3 g/L) was suggested to be contributed by the *Prevotellaceae*, a member of *Bacteroidetes* which was increased to 19.2% in the mixing pond and to 49.7% in the holding tank. *Prevotellaceae* was reported as being a saccharolytic fermentative anaerobe involved in acidogenesis (Lee et al., 2015) and able to produce volatile fatty acids (VFAs) including acetic acid as metabolic end products (Shah and Gharbia, 1992).

On the other hand, a high proportion of the *Caldicellulosiruptoraceae* detected in the MRE (11.9%), followed by *Ruminococcaceae* in the mixing pond (6.1%) and holding tank (17.3%) (**Figure 5.9**) were suggested to play a role in the initial cellulose hydrolysis. According to Rainey et al. (1994), *Caldicellulosiruptor* sp. could utilize cellulose, cellobiose, xylan and xylose by the actions of hydrolytic enzymes. Meanwhile, *Ruminococcaceae* was reported to contain a number of cellulolytic and amylolytic species which appear exclusively in biomass-derived culture of an anaerobic digestion (Weiß et al., 2016).

#### 5.3.3.3 Biodegradation of Lignocellulosic Materials

The potential of lignocellulosic degradation was also observed during the treatment of POME. Alcohols detected in the early stages of POME treatment (**Table 5.4**) were suggested as the main degradation products of cellulose and hemicellulose, as being discussed by Yamaguchi et al. (2016). For instance, a dominance of *Lactobacillaceae* in the MRE and mixing pond was suggested to be involved in the production of

1,3−propanediol and 2,3−butanediol. The bioconversion of glucose into 2,3−butanediol by *Lactobacillus* was previously reported by Celińska and Grajek (2009). It was also reported as being a 1,3−propanediol producer from the co-fermentation of glycerol and glucose at the expense of ethanol and lactate (El-Ziney et al., 1998). The presence of glycerol in the POME has been described in a study by Jin and Viidanoja (2017). A higher population of *Clostridiaceae* in the MRE and holding tank was also suggested to be involved in the production of 1,3−propanediol. The utilization of lignocellulosic hydrolysates as co-substrates together with glycerol by *Clostridia* was reported able to enhance the production of 1,3−propanediol (Xin et al., 2016). The disappearance of these compounds in the subsequent stages could be due to the major reduction of *Lactobacillaceae* and *Clostridiaceae*.

**Table 5.4 Presence of alcohols and phenolics as main degradation compounds from lignocellulosic materials in the different stages during the full-scale treatment of POME.**

| Name of Compounds | MRE[a] | MP[b] | HT[c] | CR[d] | OD[e] | FP[f] | AP[g] | FD[h] |
|---|---|---|---|---|---|---|---|---|
| *Alcohols* | | | | | | | | |
| 1,3-Propanediol | √ | √ | √ | √ | √ | - | - | - |
| 2,3-Butanediol | √ | √ | - | - | - | - | - | - |
| Benzyl alcohol | √ | - | - | - | - | - | - | - |
| Phenylethyl alcohol | √ | - | - | - | - | - | - | - |
| 3-Hexanol, 4-methyl | √ | √ | - | - | - | - | - | - |
| Resorcinol | √ | - | - | - | - | - | - | - |
| 3-Pyridinol | √ | - | - | - | - | - | - | - |
| Triethylene glycol | √ | - | - | - | - | - | - | - |
| *Phenolics* | | | | | | | | |
| 1,2-Benzenediol | √ | √ | √ | - | - | - | - | - |
| Phenol, 3,4,5-trimethoxy- | √ | - | - | - | - | - | - | - |
| Phenol, 2-methoxy- | √ | - | - | - | - | - | - | - |
| 2-Methoxy-4-vinylphenol | √ | - | - | - | - | - | - | - |
| Phenol, 2,6-dimethoxy- | √ | - | - | - | - | - | - | - |
| 3-Pyridinol | √ | - | - | - | - | - | - | - |
| Phenol, 4-(2-propenyl)- | √ | - | - | - | - | - | - | - |
| Tyrosol | √ | - | - | - | - | - | - | - |
| Homovanillic acid | √ | - | - | - | - | - | - | - |
| Coumaran | √ | - | - | - | - | - | - | - |

Notes: [a] mixed raw effluent, [b] mixing pond, [c] holding tank, [d] closed reactor, [e] open digester, [f] facultative pond, [g] algae pond, [h] final discharge

In addition, the phenolic compounds were detected as the major degradation products in the MRE (**Table 5.4**) which was suggested due to the presence of lignin in the lignocellulosic materials. Sharip et al. (2016) in their study on the characterization of oil palm mesocarp fiber superheated steam condensate also found phenolics as the major compounds which were suggested to be contributed by the lignin degradation. A high temperature and acidic pH of raw POME was suggested to assist in the degradation of lignin (Ibrahim et al., 2013). According to Álvarez et al. (2005), the treatment of lignocellulosic material (sawdust) with acid could modify its chemical composition. Moreover, a study by Zakaria et al. (2015) suggested that the hydrothermal pretreatment of oil palm biomass could partially remove the hemicellulose and modify the lignin structure, resulting in the changes of the cellulose-hemicellulose-lignin matrix.

On the other hand, the presence of *Comamonadaceae* in the MRE (1.3%) was suggested to play a key role in the recycling of plant-derived carbon of aromatic compounds including phenols, as suggested by Pérez-Pantoja et al. (2012). Moreover, *Alcaligenaceae* which was detected in the MRE (0.05%) also known to have potential in the degradation of phenolics (Rehfuss and Urban, 2005) and aromatic (Pérez-Pantoja et al., 2012) compounds. The *Comamonadaceae* and *Alcaligenaceae* were also found to be emerged in the facultative pond, algae pond and final discharge which are hypothesised to function in the degradation of certain compounds resulted from ultraviolet (UV) irradiation. Pereira et al. (2007) in their study stated that the selected group of compounds present in the aquatic environment can be degraded by UV photolysis and UV/$H_2O_2$ oxidation. As discussed in Chapter 4, the *Alcaligenaceae* was also being detected in the different POME final discharges treated by different

biotreatment processes, hence was proposed as one of the reliable bioindicators to indicate the river water contamination due to POME final discharge (Mohd-Nor et al., 2018). The results presented in this study indicated that the phenols and alcohols present in the MRE were degraded throughout the POME treatment. These compounds were mostly detected in the MRE and mixing pond but not detected in the final discharge, hence indicated the successful degradation of lignocellulosic materials in the POME treatment system.

#### 5.3.3.4 Shift of Bacterial and Archaeal Communities Involved in the Methanogenesis

A very large shift of bacterial community was recorded as the anaerobic treatments in the closed reactor and open digester started (**Figure 5.9**). The shift of methanogenic populations in the closed reactor and open digester was suggested to influence the generation of methane, as mentioned in the study of Chong et al. (2014). The concentration of VFAs including acetic acid was known to be correlated with the population of methanogens in an anaerobic system. In this study, the increased aceticlastic methanogen *Methanosaetaceae* in the closed reactor (0.8%) and open digester (0.6%) was suggested to play a role in degrading acetate for the generation of methane, which was supported by the large reduction of acetic acid in the reactor (**Figure 5.10**).

A large increment of *Syntrophomonas* in the closed reactor (21.5%) and open digester (29.2%) could also function as acetate-oxidizing bacteria, provided the $H_2CO_2$ produced were subsequently utilized by hydrogenotrophic methanogen (Hattori,

2008). In addition, the co-culture of hydrogenotrophic methanogen *Methanobacteriaceae* (0.6–1.3%) with *Syntrophomonas* in the closed reactor and open digester could be involved in β-oxidation of fatty acids. In a previous work by Hatamoto et al. (2007), the co-culture of hydrogenotrophic methanogen *Methanospirillum hungatei* with *Syntrophomonas palmitatica* sp. nov. isolated from granular sludge in a mesophilic upflow anaerobic sludge blanket reactor for POME treatment was able to oxidise straight-chain saturated fatty acids including palmitate with carbon chain lengths of C4–C18. Long chain fatty acids, especially palmitate was previously reported to be present in the POME (Fang et al., 2011). It was reported that this co-culture played a role in β-oxidation of fatty acids whereby the fatty acids with ad odd number of carbon atoms were converted into propionate and methane, while acetate and methane were produced from the fatty acids with an even number of carbon atoms. The presence of hydrogenotrophic methanogen and syntrophic bacteria is also important to obtain maximum COD removal and optimum biogas production (Amani et al., 2012). However, the *Syntrophomonas* detected in the facultative pond (15.0%) might be carried over from the upstream anaerobic process.

The results of this study indicated that the bacterial and methanogenic archaeal communities were shifted due to biodegradation in the different stages of the full-scale treatment of POME. The biodegradation by dominant bacteria from the hydrolysis to methanogenesis obtained from this study is illustrated in **Figure 5.11**. The findings of this research provide insights on the importance of the bacterial and methanogenic archaeal communities and their balanced populations in catalysing the biodegradation of a variety of compounds throughout the POME treatment.

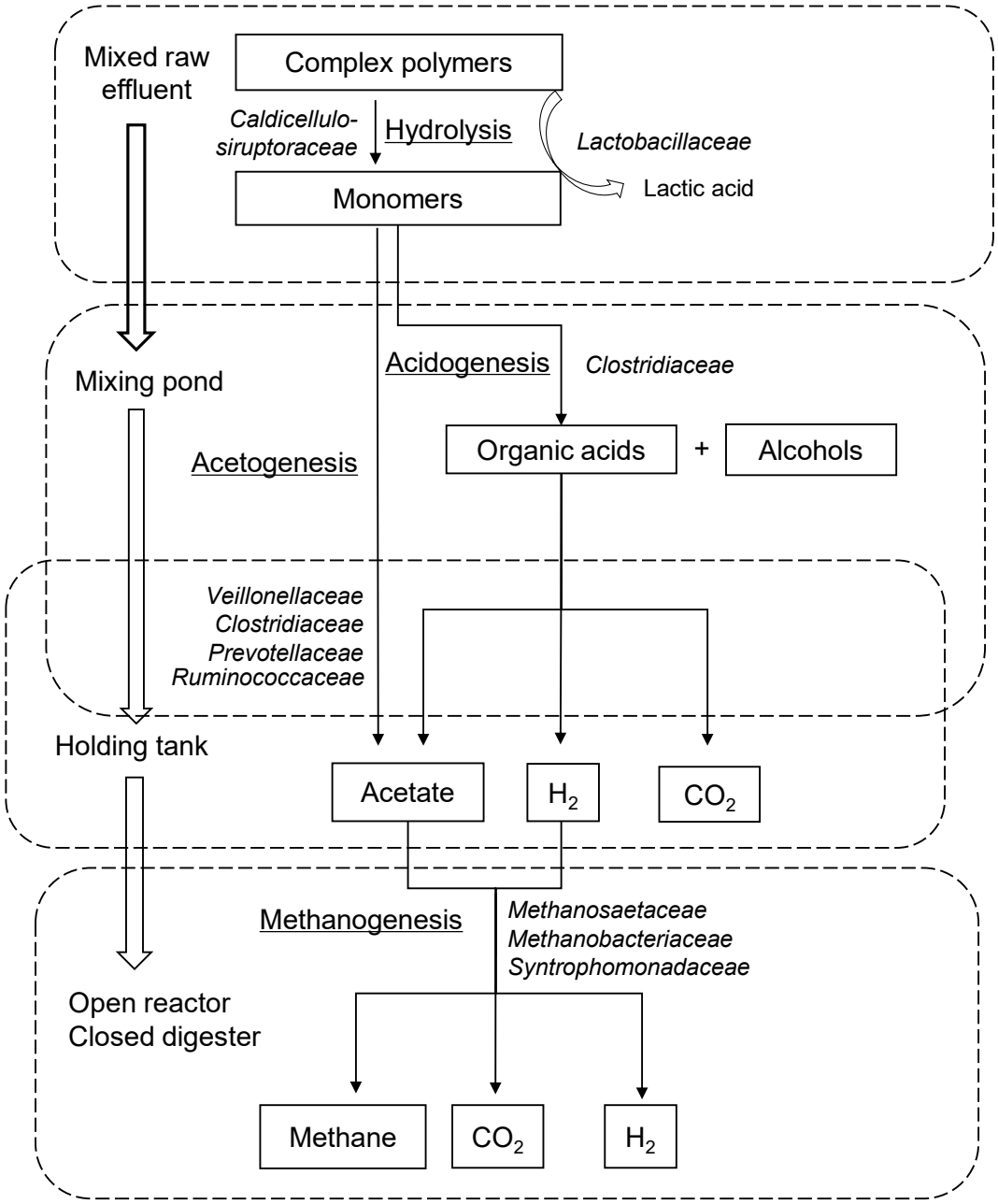



**Figure 5.11 Schematic flow process diagram of biodegradation in the different stages during the treatment of POME catalysed by dominant bacteria and archaea populations for the generation of methane as end-product.**

## 5.4 Concluding Remarks

The presence of dominant bacterial and methanogenic archaeal populations in the different stages during the treatment of POME were mainly contributed to the biodegradation of POME. The presence of *Caldicellulosiroptoraceae* and *Lactobacillaceae* in the raw POME initiated the hydrolysis step in degrading complex lignocellulosic compounds. Products from hydrolysis were converted into organic acids through acidogenesis and acetogenesis processes by *Clostridaceae*, *Veillonellaceae*, *Prevotellacea*e and *Ruminococcacea*e. Meanwhile, *Methanosaetaceae*, *Methanobacteriaceae* and *Syntrophomodaceae* which were previously known to be involved in the methanogenesis process were predominantly detected during the anaerobic stage and contributed to the generation of methane. The changes in physiochemical properties, particularly in pH, $BOD_5$, COD and TOC values throughout the treatment of POME were mainly due to the actions of these microbial communities which were supported by the continuous supply of substrate from biodegradation products. The findings of this research provide insights on the importance of the bacterial and methanogenic archaeal communities and their balanced populations in catalysing the biodegradation of a variety of compounds throughout the treatment of POME. The information obtained is an important first step in our understanding of the mechanisms involved in the different treatment stages of POME.

# CHAPTER 6

# CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

## 6.1 Conclusions

Based on molecular-based methods of partial 16S rRNA gene identification through PCR-DGGE and Illumina MiSeq sequencing, the changes of the bacterial community in the different final discharges and throughout the treatment of POME were determined. The information on the shift of bacterial community is of practical relevance in designing the bioindicator for monitoring the river water quality. The detection of the proposed bioindicators, *Alcaligenaceae* and *Chromatiaceae*, in all the four POME final discharges, despite them having different properties and been generated from different processes of POME treatment, showed the potential of using these bacterial indicators to detect the river water contamination caused by this effluent.

In addition, the shift of bacterial and methanogenic archaeal community structure in the different stages of POME treatment was mainly related to the biodegradation potential of microorganisms in degrading the pollutants. *Caldicellulosiruptoraceae* and *Lactobacillaceae* played an important role in the hydrolysis stage which contributed to the generation of lactic acid. Meanwhile, *Clotridiaceae*, *Vellonellaceae*, *Prevotellaceae* and *Ruminococcaceae* dominated the acidogenesis and acetogenesis processes which contributed to the higher production of acetic acid. The aceticlastic methanogen, *Methanosaetaceae* was involved in the acetic acid degradation during anaerobic process for methane generation, meanwhile hydrogenotrophic methanogen,

*Methanobacteriaceae* might be involved in fatty acids and acetic acid degradations by syntrophic cooperation with *Syntrophomonas*. Moreover, the detection of phenols and alcohols in the early phases but not in the final discharge indicated the successful degradation of lignocellulosic materials.

## 6.2 Recommendations for Future Studies

Concurrent with the massive efforts in transforming palm oil industry in Malaysia into a more sustainable industry, a thorough waste management system to determine the impact of POME towards the bacterial community must be established. Hence, for the future studies, it is recommended to quantify the genes involved in the biodegradation specifically caused by *Alcaligenaceae* and *Chromatiaceae* as part of the development of bioindicators to the indicate river water contamination due to POME final discharge.

While sequencing of 16S rRNA amplicon using Illumina MiSeq can provide detailed information on the microbial community involved in the biodegradation of POME, they are however unable to describe the metabolic function of the system. The application of recent advance in the fields of meta-transcriptomics can be used further in the future to understand the microbial functions in the biodegradation of POME. Meanwhile, the advance in Illumina sequencing technology could provide outstanding levels of transcriptional libraries. These metagenomes collection could serve as a platform for mapping of the recorded reads, allowing the tracking of changes in microbial activity. On top of that, quantitative-PCR (qPCR) technique can be used to specifically identify and quantify the target microorganism and gene involved in the biodegradation of POME.

## REFERENCES


Abdullah, N., Yuzir, A., Curtis, T.P., Yahya, A., Ujang, Z., 2013. Characterization of aerobic granular sludge treating high strength agro-based wastewater at different volumetric loadings. Bioresour. Technol. 127, 181–187. https://doi.org/10.1016/j.biortech.2012.09.047

Abdullah, R., 2003. Short-Term and Long-Term Projection of Malaysian Palm Oil Production. Oil palm Ind. Econ. J. 3, 32–36.

Acosta-González, A., Rosselló-Móra, R., Marqués, S., 2013. Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: Aromatic biodegradation potential after the Prestige oil spill. Environ. Microbiol. 15(1), 77–92. https://doi.org/10.1111/j.1462-2920.2012.02782.x

Adrados, B., Sánchez, O., Arias, C.A., Becares, E., Garrido, L., Mas, J., Brix, H., Morató, J., 2014. Microbial communities from different types of natural wastewater treatment systems: Vertical and horizontal flow constructed wetlands and biofilters. Water Res. 55, 304–312. https://doi.org/10.1016/j.watres.2014.02.011

Ahmad, A., Ghufran, R., Wahid, Z.A., 2011a. Role of calcium oxide in sludge granulation and methanogenesis for the treatment of palm oil mill effluent using UASB reactor. J. Hazard. Mater. 198, 40–48. https://doi.org/10.1016/j.jhazmat.2011.10.008

Ahmad, A., Ghufran, R., Wahid, Z.A., 2011b. Bioenergy from anaerobic degradation of lipids in palm oil mill effluent. Rev. Environ. Sci. Biotechnol. 10, 353–376. https://doi.org/10.1007/s11157-011-9253-8

Ahmad, A.A., Hameed, B.H., 2009. Reduction of COD and color of dyeing effluent from a cotton textile mill by adsorption onto bamboo-based activated carbon. J. Hazard. Mater. 1538e1543.

Ahmad, A.L., Sumathi, S., Hameed, B.H., 2006. Coagulation of residue oil and suspended solid in palm oil mill effluent by chitosan, alum and PAC. Chem. Eng. J. 118, 99–105. https://doi.org/10.1016/j.cej.2006.02.001

Ahmad, A.L., Sumathi, S., Hameed, B.H., 2005. Residual oil and suspended solid removal using natural adsorbents chitosan, bentonite and activated carbon: A comparative study. Chem. Eng. J. 108, 179–185. https://doi.org/10.1016/j.cej.2005.01.016

Ahmed, Y., Yaakob, Z., Akhtar, P., Sopian, K., 2015. Production of biogas and performance evaluation of existing treatment processes in palm oil mill effluent (POME). Renew. Sustain. Energy Rev. 42, 1260–1278. https://doi.org/10.1016/j.rser.2014.10.073

Alhaji, M.H., Sanaullah, K., Lim, S.F., Khan, A., Hipolito, C.N., Abdullah, M.O., Bhawani, S.A., Jamil, T., 2016. Photocatalytic treatment technology for palm oil mill effluent (POME) - A review. Process Saf. Environ. Prot. 102, 673–686. https://doi.org/10.1016/j.psep.2016.05.020

Ali, M., Yee, L., Lai, P., Ariffin, H., Rahim, A., Shirai, Y., Sudesh, K., 2012. Sustainable production of polyhydroxyalkanoates from renewable oil-palm biomass. Biomass and Bioenergy 50, 1–9. https://doi.org/10.1016/j.biombioe.2012.10.014

Álvarez, P., Santamaría, R., Blanco, C., Granda, M., 2005. Thermal degradation of lignocellulosic materials treated with several acids. J. Anal. Appl. Pyrolysis 74, 336–343. https://doi.org/10.1016/j.jaap.2004.11.030

Amani, T., Nosrati, M., Mousavi, S.M., 2012. Response surface methodology analysis of anaerobic syntrophic degradation of volatile fatty acids in an upflow anaerobic sludge bed reactor inoculated with enriched cultures. Biotechnol. Bioprocess Eng. 17(1), 133-144. https://doi.org/10.1007/s12257-011-0248-7

Ambuchi, J.J., Liu, J., Wang, H., Shan, L., Zhou, X., Mohammed, M.O.A., Feng, Y., 2016. Microbial community structural analysis of an expanded granular sludge bed (EGSB) reactor for beet sugar industrial wastewater (BSIW) treatment. Appl. Microbiol. Biotechnol. 100, 4651–4661. https://doi.org/10.1007/s00253-015-7245-2

An, X., Baker, P., Li, H., Su, J., Yu, C., Cai, C., 2016. The patterns of bacterial community and relationships between sulfate-reducing bacteria and hydrochemistry in sulfate-polluted groundwater of Baogang rare earth tailings. Environ. Sci. Pollut. Res. 23, 21766–21779. https://doi.org/10.1007/s11356-016-7381-y

Anderson, I., Ulrich, L.E., Lupa, B., Susanti, D., Porat, I., Hooper, S.D., Lykidis, A., Sieprawska-Lupa, M., Dharmarajan, L., Goltsman, E., Lapidus, A., Saunders, E., Han, C., Land, M., Lucas, S., Mukhopadhyay, B., Whitman, W.B., Woese, C., Bristow, J., Kyrpides, N., 2009. Genomic characterization of methanomicrobiales reveals three classes of methanogens. PLoS One 4, e5797. https://doi.org/10.1371/journal.pone.0005797

Andersson, S., Kuttuva Rajarao, G., Land, C.J., Dalhammar, G., 2008. Biofilm formation and interactions of bacterial strains found in wastewater treatment systems. FEMS Microbiol. Lett. 283, 83–90. https://doi.org/10.1111/j.1574-6968.2008.01149.x

Angenent, L.T., Karim, K., Al-Dahhan, M.H., Wrenn, B.A., Domíguez-Espinosa, R., 2004. Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol. 22, 477–485. https://doi.org/http://dx.doi.org/10.1016/j.tibtech.2004.07.001

Antwi, P., Li, J., Boadi, P.O., Meng, J., Koblah Quashie, F., Wang, X., Ren, N., Buelna, G., 2017. Efficiency of an upflow anaerobic sludge blanket reactor treating potato starch processing wastewater and related process kinetics, functional microbial community and sludge morphology. Bioresour. Technol. 239, 105–116. https://doi.org/10.1016/j.biortech.2017.04.124

APHA, 2002. Stand. Methods Exam. Water Wastewater, 20th ed. Am. Public Heal. Assoc., Washington DC USA.

Araya, R., Tani, K., Takagi, T., Yamaguchi, N., Nasu, M., 2003. Bacterial activity and community composition in stream water and biofilm from an urban river determined by fuorescent in situ hybridization and DGGE analysis. FEMS Microbiol. Ecol. 43, 111–119. https://doi.org/10.1111/j.1574-6941.2003.tb01050.x.

Awalludin, M.F., Sulaiman, O., Hashim, R., Nadhari, W.N.A.W., 2015. An overview of the oil palm industry in Malaysia and its waste utilization through thermochemical conversion, specifically via liquefaction. Renew. Sustain. Energy Rev. 50, 1469–1484. https://doi.org/10.1016/j.rser.2015.05.085

Azmi, N.S., Yunos, K.F.M., 2014. Wastewater Treatment of Palm Oil Mill Effluent (POME) by Ultrafiltration Membrane Separation Technique Coupled with Adsorption Treatment as Pre-treatment. Agric. Agric. Sci. Procedia 2, 257–264. https://doi.org/10.1016/j.aaspro.2014.11.037

Bafana, A., Kumar, G., Kashyap, S.M., Kanade, G.S., Shinde, V.M., 2015. Dynamics

of effluent treatment plant during commissioning of activated sludge process unit. Environ. Sci. Pollut. Res. 22, 3538–3546. https://doi.org/10.1007/s11356-014-3597-x

Bagatini, I.L., Eiler, A., Bertilsson, S., Klaveness, D., Tessarolli, L.P., Vieira, A.A.H., 2014. Host-specificity and dynamics in bacterial communities associated with bloom-forming freshwater phytoplankton. PLoS One 9, e85950. https://doi.org/10.1371/journal.pone.0085950

Baharuddin, A.S., Wakisaka, M., Shirai, Y., Abd-Aziz, S., Abdul Rahman, N.A., Hassan, M.A., 2009. Co-composting of empty fruit bunches and partially treated palm oil mill effluents in pilot scale. Int. J. Agric. Res. 4, 69–78. https://doi.org/10.3923/ijar.2009.69.78

Bai, Y., Qi, W., Liang, J., Qu, J., 2014. Using high-throughput sequencing to assess the impacts of treated and untreated wastewater discharge on prokaryotic communities in an urban river. Appl. Microbiol. Biotechnol. 98, 1841–1851. https://doi.org/10.1007/s00253-013-5116-2

Bala, J.D., Lalung, J., Ismail, N., 2015. Studies on the reduction of organic load from palm oil mill effluent (POME) by bacterial strains. Int. J. Recycl. Org. Waste Agric. 4, 1–10. https://doi.org/10.1007/s40093-014-0079-6

Bala, J.D., Lalung, J., Ismail, N., 2014a. Biodegradation of palm oil mill effluent (POME) by bacterial. Int. J. Sci. Res. Publ. 4, 2250–3153.

Bala, J.D., Lalung, J., Ismail, N., 2014b. Palm oil mill effluent (POME) treatment "'Microbial communities in an anaerobic digester'": A review. Int. J. Sci. Res. Publ. 4, 1–24.

Bapteste, É., Brochier, C., Boucher, Y., 2005. Higher-level classification of the Archaea: Evolution of methanogenesis and methanogens. Archaea 1, 353–363. https://doi.org/10.1155/2005/859728

Becerra-Castro, C., Macedo, G., Silva, A.M.T., Manaia, C.M., Nunes, O.C., 2016. Proteobacteria become predominant during regrowth after water disinfection. Sci. Total Environ. 573, 313–323. https://doi.org/10.1016/j.scitotenv.2016.08.054

Bella, J.M., Bao, Y., Gloor, G.B., Burton, J.P., Reid, G., 2013. High throughput sequencing methods and analysis for microbiome research. J. Microbiol. Methods 95, 401–414. https://doi.org/10.1016/j.mimet.2013.08.011

Bello, M.M., Abdul Raman, A.A., 2017. Trend and current practices of palm oil mill effluent polishing: Application of advanced oxidation processes and their future perspectives. J. Environ. Manage. 198, 170–182. https://doi.org/10.1016/j.jenvman.2017.04.050

Bello, M.M., Nourouzi, M.M., Abdullah, L.C., Choong, T.S.Y., Koay, Y.S., Keshani, S., 2013. POME is treated for removal of color from biologically treated POME in fixed bed column: Applying wavelet neural network (WNN). J. Hazard. Mater. 262, 106–113. https://doi.org/10.1016/j.jhazmat.2013.06.053

Bertin, L., Colao, M.C., Ruzzi, M., Marchetti, L., Fava, F., 2006. Performances and microbial features of an aerobic packed-bed biofilm reactor developed to post-treat an olive mill effluent from an anaerobic GAC reactor. Microb. Cell Fact. 5, 1–11. https://doi.org/10.1186/1475-2859-5-16

Bhatia, S., Othman, Z., Ahmad, A.L., 2007. Coagulation-flocculation process for POME treatment using Moringa oleifera seeds extract: Optimization studies. Chem. Eng. J. 133, 205–212. https://doi.org/10.1016/j.cej.2007.01.034

Bing-Ru, L., Guo-Mei, J., Jian, C., Gang, W., 2006. A review of methods for studying microbial diversity in soils. Pedosphere 16, 18–24.

https://doi.org/10.1016/S1002-0160(06)60021-0
Bitton, B., 2005. Wastewater microbiology. Wiley, Hoboken,New Jersey, pp. 345–369.
Boon, N., De Windt, W., Verstraete, W., Top, E.M., 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol. Ecol. 39, 101–112. https://doi.org/10.1016/S0168-6496(01)00198-2
Braga, P.A.C., Tata, A., Gonçalves Dos Santos, V., Barreiro, J.R., Schwab, N.V., Veiga Dos Santos, M., Eberlin, M.N., Ferreira, C.R., 2013. Bacterial identification: From the agar plate to the mass spectrometer. RSC Adv. 3, 994–1008. https://doi.org/10.1039/c2ra22063f
Cai, W., Li, Y., Wang, P., Niu, L., Zhang, W., Wang, C., 2017. Erratum to: Effect of the pollution level on the functional bacterial groups aiming at degrading bisphenol A and nonylphenol in natural biofilms of an urban river. Environ. Sci. Pollut. Res. 23, 15727–15738. https://doi.org/10.1007/s11356-017-8422-x
Cao, H., Hong, Y., Li, M., Gu, J.D., 2011. Diversity and abundance of ammonia-oxidizing prokaryotes in sediments from the coastal Pearl River estuary to the South China Sea. Antonie Van Leeuwenhoek 100, 545. https://doi.org/10.1007/s10482-011-9610-1
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. and Huttley, G.A., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 10.1038/nm, 335–336. https://doi.org/10.1038/nmeth.f.303.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M. and Gormley, N., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621. https://doi.org/10.1038/ismej.2012.8
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G. a, Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C. a, Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W. a, Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat. Publ. Gr. 7, 335–336. https://doi.org/10.1038/nmeth0510-335
Cardinali-Rezende, J., Rojas-Ojeda, P., Nascimento, A.M.A., Sanz, J.L., 2016. Proteolytic bacterial dominance in a full-scale municipal solid waste anaerobic reactor assessed by 454 pyrosequencing technology. Chemosphere 146, 519–525. https://doi.org/10.1016/j.chemosphere.2015.12.003
Celińska, E., Grajek, W., 2009. Biotechnological production of 2,3-butanediol-Current state and prospects. Biotechnol. Adv. 27, 715–725. https://doi.org/10.1016/j.biotechadv.2009.05.002
Chao, A., Lee, S.M., 1992. Estimating the number of classes via sample coverage. J. Am. Stat. Assoc. 87, 210–217. https://doi.org/10.1080/01621459.1992.10475194
Chen, Y., Wen, Y., Tang, Z., Huang, J., Zhou, Q., Vymazal, J., 2015. Effects of plant biomass on bacterial community structure in constructed wetlands used for tertiary wastewater treatment. Ecol. Eng. 84, 38–45. https://doi.org/10.1016/j.ecoleng.2015.07.013

Chin, K.K., Lee, S.W., Mohammad, H.H., 1996. A study of palm oil mill effluent treatment using a pond system. Water Sci. Technol. 34, 119–123. https://doi.org/10.1016/S0273-1223(96)00828-1

Chokwe, T.B., Okonkwo, J.O., Sibali, L.L., Ncube, E.J., 2012. Optimization and simultaneous determination of alkyl phenol ethoxylates and brominated flame retardants in water after SPE and heptafluorobutyric anhydride derivatization followed by GC/MS. Chromatographia 75, 1165–1176. https://doi.org/10.1007/s10337-012-2293-6

Chong, L., Zhuping, Z., Tongjun, G., Yongming, L., Hongmin, D., 2014. Changes in methane emission, rumen fermentation, and methanogenic community in response to silage and dry cornstalk diets. J. Basic Microbiol. 54, 521–530. https://doi.org/10.1002/jobm.201200678

Chong, M.L., Rahim, R.A., Shirai, Y., Hassan, M.A., 2009. Biohydrogen production by Clostridium butyricum EB6 from palm oil mill effluent. Int. J. Hydrogen Energy 34, 764–771. https://doi.org/10.1016/j.ijhydene.2008.10.095

Choong, Y.Y., Chou, K.W., Norli, I., 2018. Strategies for improving biogas production of palm oil mill effluent (POME) anaerobic digestion: A critical review. Renew. Sustain. Energy Rev. 82, 2993–3006. https://doi.org/10.1016/j.rser.2017.10.036

Choorit, W., Wisarnwan, P., 2007. Effect of temperature on the anaerobic digestion of palm oil mill effluent. Electron. J. Biotechnol. 10, 376–385. https://doi.org/10.2225/vol10-issue3-fulltext-7

Cibis, K.G., Gneipel, A., König, H., 2016. Isolation of acetic, propionic and butyric acid-forming bacteria from biogas plants. J. Biotechnol. 220, 51–63. https://doi.org/10.1016/j.jbiotec.2016.01.008

Clean Water Team, 2013. Colour of Water Fact Sheet, State Water Resources Control Board.

Cottrell, M.T., Waidner, L.A., Yu, L., Kirchman, D.L., 2005. Bacterial diversity of metagenomic and PCR libraries from the Delaware River. Environ. Microbiol. 7, 1883–1895. https://doi.org/10.1111/j.1462-2920.2005.00762.x

David, J., Japareng, B., 2015. Studies on the reduction of organic load from palm oil mill effluent (POME) by bacterial strains 1–10. https://doi.org/10.1007/s40093-014-0079-6

De Souza, F.A., Kowalchuk, G.A., Leeflang, P., Van Veen, J.A., Smit, E., 2004. PCR-Denaturing gradient gel electrophoresis profiling of inter- and intraspecies 18S rRNA gene sequence heterogeneity is an accurate and sensitive method to assess species diversity of arbuscular mycorrhizal fungi of the genus gigaspora. Appl. Environ. Microbiol. 70, 1413–1424. https://doi.org/10.1128/AEM.70.3.1413-1424.2004

Delforno, T.P., Lacerda Júnior, G.V., Noronha, M.F., Sakamoto, I.K., Varesche, M.B.A., Oliveira, V.M., 2017. Microbial diversity of a full-scale UASB reactor applied to poultry slaughterhouse wastewater treatment: integration of 16S rRNA gene amplicon and shotgun metagenomic sequencing. Microbiologyopen 6, e00443. https://doi.org/10.1002/mbo3.443

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Rev. Environ. Sci. Biotechnol. https://doi.org/10.1007/s11157-008-9131-1

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ.

Microbiol. 72, 5069–5072. https://doi.org/10.1128/AEM.03006-05
Ding, L., Zhou, Q., Wang, L., Zhang, Q., 2011. Dynamics of bacterial community structure in a full- scale wastewater treatment plant with anoxic-oxic configuration using 16S rDNA PCR-DGGE fingerprints. African J. Biotechnol. 10, 589–600. https://doi.org/10.5897/AJB10.386
Dubber, D., Gray, N.F., 2010. Replacement of chemical oxygen demand (COD) with total organic carbon (TOC) for monitoring wastewater treatment performance to minimize disposal of toxic analytical waste. J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 45, 1595–1600. https://doi.org/10.1080/10934529.2010.506116
El-Ziney, M.G., Arneborg, N., Uyttendaele, M., Debevere, J., Jakobsen, M., 1998. Characterization of growth and metabolite production of Lactobacillus reuteri during glucose/glycerol cofermentation in batch and continuous cultures. Biotechnol. Lett. 20, 913–916.
Elijah, I.O., Sylvester, C.I., Nimi, J., 2013. Physicochemical and Microbial Screening of Palm Oil Mill Effluents for Amylase Production. Greener J. Biol. Sci. 3, 307–318. https://doi.org/10.15580/GJBS.2013.8.100913894
Elmi, A., 2015. Colour and COD Removal from Palm Oil Mill Effluent (POME) Using Pseudomonas Aeruginosa Strain NCIM 5223 in Microbial Fuel Cell. Int. J. Waste Resour. 05, 3–5. https://doi.org/10.4172/2252-5211.1000181
EQR, 2006. Malaysia Environmental Quality Report. Petaling Jaya, Malaysia.
Fang, C., O-Thong, S., Boe, K., Angelidaki, I., 2011. Comparison of UASB and EGSB reactors performance, for treatment of raw and deoiled palm oil mill effluent (POME). J. Hazard. Mater. 189, 229–234. https://doi.org/10.1016/j.jhazmat.2011.02.025
Felda Group Venture, 2017. FELDA Annual Report 2016. Annu. Rep. Integr. Rep. 2016 May 2017, 363.
Felföldi, T., Székely, A.J., Gorál, R., Barkács, K., Scheirich, G., András, J., Rácz, A., Márialigeti, K., 2010. Polyphasic bacterial community analysis of an aerobic activated sludge removing phenols and thiocyanate from coke plant effluent. Bioresour. Technol. 101, 3406–3414. https://doi.org/10.1016/j.biortech.2009.12.053
Feng, Y., Zhang, Y., Chen, S., Quan, X., 2015. Enhanced production of methane from waste activated sludge by the combination of high-solid anaerobic digestion and microbial electrolysis cell with iron-graphite electrode. Chem. Eng. J. 259, 787–794. https://doi.org/10.1016/j.cej.2014.08.048
Gerardi, M.., 2006. Wastewater Bacteria. Wiley-Interscience, New Jersey, pp. 19–31.
Gerardi, M.H., Lytle, B., 2015. Algae, Alkalinity, and pH, in: The Biology and Troubleshooting of Facultative Lagoons. pp. 105–109.
Gich, F.B., Amer, E., Figueras, J.B., Charles A. Abella, C. a. A., Balaguer, M.D., Poch, M., 2010. Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). Int. Microbiol. 3, 103–106. https://doi.org/10.2436/im.v3i2.9255
Gray, N.D., Miskin, I.P., Kornilova, O., Curtis, T.P., Head, I.M., 2002. Occurrence and activity of archaea in aerated activated sludge wastewater treatment plants. Environ. Microbiol. 4, 158–168. https://doi.org/10.1046/j.1462-2920.2002.00280.x
Guo, J., Ni, B.J., Han, X., Chen, X., Bond, P., Peng, Y., Yuan, Z., 2017. Data on metagenomic profiles of activated sludge from a full-scale wastewater treatment

plant. Data Br. 15, 833–839. https://doi.org/10.1016/j.dib.2017.10.048
Gupta, V., Capalash, N., Gupta, N., Sharma, P., 2017. Bio-prospecting laccases in the bacterial diversity of activated sludge from pulp and paper industry. Indian J. Microbiol. 57, 75–82. https://doi.org/10.1007/s12088-016-0624-2
Gutierrez, T., Green, D.H., Nichols, P.D., Whitman, W.B., Semple, K.T., Aitken, M.D., 2012. Polycyclovorans algicola gen. nov., sp. nov., an aromatic-hydrocarbon- degrading marine bacterium found associated with laboratory cultures of marine phytoplankton. Appl. Environ. Microbiol. AEM-02833. https://doi.org/10.1128/AEM.02833-12
Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST : Paleontological Statistics Software Package for Education and Data Analysis 4, 1–9.
Hamzah, M.F., Yusof, N., Alimon, H., 2016. Microbial assisted phytoremediation of palm oil mill final discharge (POMFD) wastewater. J. Oil Palm Res. 28, 320–330. https://doi.org/10.21894/jopr.2016.2803.08
Hassa, J., Maus, I., Off, S., Pühler, A., Scherer, P., Klocke, M., Schlüter, A., 2018. Metagenome, metatranscriptome, and metaproteome approaches unraveled compositions and functional relationships of microbial communities residing in biogas plants. Appl. Microbiol. Biotechnol. 102, 5045–5063. https://doi.org/10.1007/s00253-018-8976-7
Hassan, M.A., Shirai, Y., Yung-Tse Hung, 2005a. Treatment of Palm Oil Wastewaters, in: Lawrence, K.W., Hung, Y.-T., H.Lo, H., Yapijakis, C. (Eds.), Waste Treatment in the Food Processing Industry. Taylor & Francis, pp. 101–107.
Hassan, M.A., Yacob, S., Shirai, Y., Hung, Y., 2005b. Treatment of Palm Oil Wastewaters. Waste Treat. Food Process. Ind. 101–117. https://doi.org/10.1201/9781420037128.ch4
Hatamoto, M., Imachi, H., Fukayo, S., Ohashi, A., Harada, H., 2007. Syntrophomonas palmitatica sp. nov., an anaerobic syntrophic, long-chain fatty-acid-oxidizing bacterium isolated from methanogenic sludge. Int. J. Syst. Evol. Microbiol. 57, 2137–2142. https://doi.org/10.1099/ijs.0.64981-0
Hattori, S., 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. 23, 118–127. https://doi.org/10.1264/jsme2.23.118
Hesham, A.E.-L., Qi, R., Yang, M., 2011. Comparison of bacterial community structures in two systems of a sewage treatment plant using PCR-DGGE analysis. J. Environ. Sci. 23, 2049–2054. https://doi.org/10.1016/S1001-0742(10)60647-X
Heyer, R., Schallert, K., Zoun, R., Becher, B., Saake, G., Benndorf, D., 2017. Challenges and perspectives of metaproteomic data analysis. J. Biotechnol. 261, 24–36. https://doi.org/10.1016/j.jbiotec.2017.06.1201
Hii, K.L., Yeap, S.P., Mashitah, M.D., 2012. Cellulase production from palm oil mill effluent in Malaysia: Economical and technical perspectives. Eng. Life Sci. 12, 7–28. https://doi.org/10.1002/elsc.201000228
Hong, Y.G., Li, M., Cao, H., Gu, J.D., 2011. Residence of habitat-specific anammox bacteria in the deep-sea subsurface sediments of the South China Sea: analyses of marker gene abundance with physical chemical parameters. Microb. Ecol. 62, 36–47. https://doi.org/10.1007/s00248-011-9849-0
Hossain, M.A., Jewaratnam, J., Ganesan, P., 2016. Prospect of hydrogen production from oil palm biomass by thermochemical process – A review. Int. J. Hydrogen Energy 41, 16637–16655. https://doi.org/10.1016/j.ijhydene.2016.07.104
Hu, M., Wang, X., Wen, X., Xia, Y., 2012. Microbial community structures in

different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresour. Technol. 117, 72–79. https://doi.org/10.1016/j.biortech.2012.04.061
Huang, M.H., Li, Y.M., Gu, G.W., 2010. Chemical composition of organic matters in domestic wastewater. Desalination. https://doi.org/10.1016/j.desal.2010.05.037
Hugenholtz, P., Goebel, B.M., Pace, N.R., 1998. Impact of culture-Independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 4765–4774.
Hughes, J.B., Hellmann, J.J., Ricketts, T.H., Bohannan, B.J.M., 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. 67, 4399–4406. https://doi.org/10.1128/AEM.67.10.4399-4406.2001
Hunter, M.L., Gibbs, J.P., 2006. Fundamentals of conservation biology, 3rd ed. Wiley Blackwell, Malden, MA.
Hurlbert, S.H., 1971. The nonconcept of species diversity: a critique and alternative parameters. Ecology 52, 577–586. https://doi.org/10.2307/1934145
Ibarbalz, F.M., Figuerola, E.L.M., Erijman, L., 2013. Industrial activated sludge exhibits unique bacterial community composition at high taxonomic ranks. Water Res. 47, 3854–3864. https://doi.org/10.1016/j.watres.2013.04.010
Ibekwe, A.M., Grieve, C.M., Lyon, S.R., 2003. Characterization of microbial communities and composition in constructed dairy wetland wastewater effluent. Appl. Environ. Microbiol. 69, 5060–5069. https://doi.org/10.1128/AEM.69.9.5060
Ibrahim, A.H., Dahlan, I., Adlan, M.N., Dasti, A.F., 2012. Comparative study on characterization of Malaysian palm oil mill effluent. Res. J. Chem. Sci. 2, 1–5. https://doi.org/10.1016/j.watres.2013.02.032
Ibrahim, N., Jahim, J.M., Lim, S.S., 2013. Initial study of thermophilic hydrogen production from raw palm oil mill effluent (POME) using mixed microflora, in: Developments in Sustainable Chemical and Bioprocess Technology. Springer, pp. 43–49.
Igwe, J.C., Onyegbado, C.C., 2007. A Review of Palm Oil Mill Effluent (POME) Water Treatment. Glob. J. Environ. Res. 1, 54–62.
James, F.C., McCulloch, C.E., 1990. Multivariate analysis in ecology and systematics: Panacea or Pandora's Box? Annu. Rev. Ecol. Syst. 21, 129–166. https://doi.org/10.1146/annurev.es.21.110190.001021
Jang, H.M., Kim, J.H., Ha, J.H., Park, J.M., 2014. Bacterial and methanogenic archaeal communities during the single-stage anaerobic digestion of high-strength food wastewater. Bioresour. Technol. 165, 174–182. https://doi.org/10.1016/j.biortech.2014.02.028
Jia, S., Zhang, X.X., Miao, Y., Zhao, Y., Ye, L., Li, B., Zhang, T., 2017. Fate of antibiotic resistance genes and their associations with bacterial community in livestock breeding wastewater and its receiving river water. Water Res. 124, 259–268. https://doi.org/10.1016/j.watres.2017.07.061
Jin, C., Viidanoja, J., 2017. Determination of glycerol in oils and fats using liquid chromatography chloride attachment electrospray ionization mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1041–1042, 94–97. https://doi.org/10.1016/j.jchromb.2016.11.041
Jong, B.C., Liew, P.W.Y., Juri, M.L., Kim, B.H., Mohd. Dzomir, A.Z., Leo, K.W., Awang, M.R., 2011. Performance and microbial diversity of palm oil mill effluent

microbial fuel cell. Lett. Appl. Microbiol. 53, 660–667. https://doi.org/10.1111/j.1472-765X.2011.03159.x
Julaidi, R., 2014. Regulatory requirements for biogas plants, effluent discharge and flue gas emissions for palm oil mill. Semin. Palm Oil Mill, Refinery, Environ. Qual.
Kanu, I., Achie, O.K., 2011. Industrial effluents and their impact on water quality of receiving rivers in Nigeria. J. Applieal Technol. Environ. Sanit. 1, 75–86.
Kapley, A., Prasad, S., Purohit, H.J., 2007. Changes in microbial diversity in fed-batch reactor operation with wastewater containing nitroaromatic residues. Bioresour. Technol. 98, 2479–2484. https://doi.org/10.1016/j.biortech.2006.09.012
Khemkhao, M., Nuntakumjorn, B., Techkarnjanaruk, S., Phalakornkule, C., 2012. UASB performance and microbial adaptation during a transition from mesophilic to thermophilic treatment of palm oil mill effluent. J. Environ. Manage. 103, 74–82. https://doi.org/10.1016/j.jenvman.2012.03.004
Khemkhao, M., Techkarnjanaruk, S., Phalakornkule, C., 2015. Simultaneous treatment of raw palm oil mill effluent and biodegradation of palm fiber in a high-rate CSTR. Bioresour. Technol. 177, 17–27. https://doi.org/10.1016/j.biortech.2014.11.052
Kim, D.H., Jang, S., Yun, Y.M., Lee, M.K., Moon, C., Kang, W.S., Kwak, S.S., Kim, M.S., 2014. Effect of acid-pretreatment on hydrogen fermentation of food waste: Microbial community analysis by next generation sequencing. Int. J. Hydrogen Energy 39, 16302–16309. https://doi.org/10.1016/j.ijhydene.2014.08.004
Kim, M., Gomec, C.Y., Ahn, Y., Speece, R.E., 2003. Hydrolysis and acidogenesis of particulate organic material in mesophilic and thermophilic anaerobic digestion. Environ. Technol. (United Kingdom) 24, 1183–1190. https://doi.org/10.1080/09593330309385659
Kim, S.H., Choi, S.M., Ju, H.J., Jung, J.Y., 2013. Mesophilic co-digestion of palm oil mill effluent and empty fruit bunches. Environ. Technol. (United Kingdom) 34, 2163–2170. https://doi.org/10.1080/09593330.2013.826253
Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., Trevors, J.T., 2004. Methods of studying soil microbial diversity. J. Microbiol. Methods 58, 169–188. https://doi.org/10.1016/j.mimet.2004.04.006
Köchling, T., Ferraz, A.D.N., Florencio, L., Kato, M.T., Gavazza, S., 2017. 454-Pyrosequencing analysis of highly adapted azo dye-degrading microbial communities in a two-stage anaerobic–aerobic bioreactor treating textile effluent. Environ. Technol. (United Kingdom) 38, 687–693. https://doi.org/10.1080/09593330.2016.1208681
Kuczynski, J., Lauber, C.L., Walters, W.A., Parfrey, L.W., Clemente, J.C., Gevers, D., Knight, R., 2011. Experimental and analytical tools for studying the human microbiome. Nat. Rev. Genet. 13, 47. https://doi.org/10.1038/nrg3129
Lam, M.K., Lee, K.T., 2011. Renewable and sustainable bioenergies production from palm oil mill effluent (POME): Win-win strategies toward better environmental protection. Biotechnol. Adv. 29, 124–141. https://doi.org/10.1016/j.biotechadv.2010.10.001
Lapara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J., 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. 49. Environ. Sci. Technol. 45, 9543–9549. https://doi.org/10.1021/es202775r
LaPara, T.M., Nakatsu, C.H., Pantea, L.M., Alleman, J.E., 2002. Stability of the

bacterial communities supported by a seven-stage biological process treating pharmaceutical wastewater as revealed by PCR-DGGE. Water Res. 36, 638–646. https://doi.org/10.1016/S0043-1354(01)00277-9

Latif, A., Suhaimi, A.A., Romzay, R.A., 2014. Application of elaeis guineensis leaves in palm oil mill effluent treatment. Indian J. Sci. Technol. 7, 254–261.. https://doi.org/10.1007/s13246-018-0622-2

Laurance, W.F., Koh, L.P., Butler, R., Sodhi, N.S., Bradshaw, C.J.A., Neidel, J.D., Consunji, H., Vega, J.M., 2010. Improving the performance of the roundtable on sustainable palm oil for nature conservation. Conserv. Biol. 24, 377–381. https://doi.org/10.1111/j.1523-1739.2010.01448.x

Lazarevic, V., Whiteson, K., Huse, S., Hernandez, D., Farinelli, L., Østerås, M., Schrenzel, J., François, P., 2009. Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. J. Microbiol. Methods 79, 266–271. https://doi.org/10.1016/j.mimet.2009.09.012

Leclerc, M., Delgènes, J.P., Godon, J.J., 2004. Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16S rDNA sequencing. Environ. Microbiol. 6, 809–819. https://doi.org/10.1111/j.1462-2920.2004.00616.x

Lee, J.Z., Logan, A., Terry, S., Spear, J.R., 2015. Microbial response to single-cell protein production and brewery wastewater treatment. Microb. Biotechnol. 8, 65–76. https://doi.org/10.1111/1751-7915.12128

Li, A.J., Yang, S.F., Li, X.Y., Gu, J.D., 2008. Microbial population dynamics during aerobic sludge granulation at different organic loading rates. Water Res. 42, 3552–3560. https://doi.org/10.1016/j.watres.2008.05.005

Li, H., Shen, T.T., Wang, X.L., Lin, K.F., Liu, Y. Di, Lu, S.G., Gu, J.D., Wang, P., Lu, Q., Du, X.M., 2013. Biodegradation of perchloroethylene and chlorophenol co-contamination and toxic effect on activated sludge performance. Bioresour. Technol. 137, 286–293. https://doi.org/10.1016/j.biortech.2013.02.050

Liew, L.L., Loh, S.K., Kassim, M.A., Muda, K., 2015. Efficiency of nutrients removal from palm oil mill effluent treatment systems efficiency of nutrients removal from palm oil mill effluent treatment systems. J. Palm Oil Res. 27, 433–443.

Liew, W.L., Kassim, M.A., Muda, K., Loh, S.K., Affam, A.C., 2014. Conventional methods and emerging wastewater polishing technologies for palm oil mill effluent treatment: A review. J. Environ. Manage. 149, 222–235. https://doi.org/10.1016/j.jenvman.2014.10.016

Liu, H., Zhang, T., Fang, H.H.P., 2003. Thermophilic H2production from a cellulose-containing wastewater. Biotechnol. Lett. 25, 365–369. https://doi.org/10.1023/A:1022341113774

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., Law, M., 2012. Comparison of next-generation sequencing systems. J. Biomed. Biotechnol. 2012, 11. https://doi.org/10.1155/2012/251364

Liu, S.P., Yu, J.X., Wei, X.L., Ji, Z.W., Zhou, Z.L., Meng, X.Y., Mao, J., 2016. Sequencing-based screening of functional microorganism to decrease the formation of biogenic amines in Chinese rice wine. Food Control 64, 98–104. https://doi.org/10.1016/j.foodcont.2015.12.013

Lozupone, C.A., Hamady, M., Kelley, S.T., Knight, R., 2007. Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. Appl. Environ. Microbiol. 73, 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lu, X., Ni, J., Zhen, G., Kubota, K., Li, Y.Y., 2018. Response of morphology and microbial community structure of granules to influent COD/SO42 – ratios in an upflow anaerobic sludge blanket (UASB) reactor treating starch wastewater. Bioresour. Technol. 256, 456–465. https://doi.org/10.1016/j.biortech.2018.02.055

Lucena-Padrós, H., Jiménez, E., Maldonado-Barragán, A., Rodríguez, J.M., Ruiz-Barba, J.L., 2015. PCR-DGGE assessment of the bacterial diversity in Spanish-style green table-olive fermentations. Int. J. Food Microbiol. 205, 47–53. https://doi.org/10.1016/j.ijfoodmicro.2015.03.033

Ma, Q., Qu, Y.-Y., Zhang, X.-W., Shen, W.-L., Liu, Z.-Y., Wang, J.-W., Zhang, Z.-J., Zhou, J.-T., 2015a. Identification of the microbial community composition and structure of coal-mine wastewater treatment plants. Microbiol. Res. 175, 1–5. https://doi.org/10.1016/j.micres.2014.12.013

Ma, Q., Qu, Y., Shen, W., Zhang, Z., Wang, J., Liu, Z., Li, D., Li, H., Zhou, J., 2015b. Bacterial community compositions of coking wastewater treatment plants in steel industry revealed by Illumina high-throughput sequencing. Bioresour. Technol. 179, 436–443. https://doi.org/10.1016/j.biortech.2014.12.041

Madaki, Y.S., Seng, L., 2013. Pollution Control: How feasible is Zero Discharge Concepts in Malaysia Palm Oil Mills. Am. J. Eng. Res. 2, 239–252.

Madsen, A.M., Zervas, A., Tendal, K., Nielsen, J.L., 2015. Microbial diversity in bioaerosol samples causing ODTS compared to reference bioaerosol samples as measured using Illumina sequencing and MALDI-TOF. Environ. Res. 140, 255–267. https://doi.org/10.1016/j.envres.2015.03.027

Malakahmad, A., Lahin, F.A., Yee, W., 2014. Biodegradation of high-strength palm oil mill effluent (POME) through anaerobes partitioning in an integrated baffled reactor inoculated with anaerobic pond sludge. Water. Air. Soil Pollut. 225. https://doi.org/10.1007/s11270-014-1883-3

Mamimin, C., Thongdumyu, P., Hniman, A., Prasertsan, P., Imai, T., O-Thong, S., 2012. Simultaneous thermophilic hydrogen production and phenol removal from palm oil mill effluent by Thermoanaerobacterium-rich sludge. Int. J. Hydrogen Energy 37, 15598–15606. https://doi.org/10.1016/j.ijhydene.2012.04.062

Maza-Márquez, P., González-Martínez, A., Rodelas, B., González-López, J., 2017. Full-scale photobioreactor for biotreatment of olive washing water: Structure and diversity of the microalgae-bacteria consortium. Bioresour. Technol. 238, 389–398. https://doi.org/10.1016/j.biortech.2017.04.048

McHugh, S., O'Reilly, C., Mahony, T., 2003. Anaerobic granular sludge bioreactor technology. Rev. … 225–245. https://doi.org/10.1023/B:RESB.0000040465.45300.97

McHugh, S., O'Reilly, C., Mahony, T., Colleran, E., O'Flaherty, V., 2003. Anaerobic granular sludge bioreactor technology. Rev. Environ. Sci. Biotechnol. 2, 225–245. https://doi.org/10.1023/B:RESB.0000040465.45300.97

Metcalf, E., 2003. Wastewater engineering treatment and reuse, in: Constituents in Wastewater. Elsevier Science B.V., Amsterdam, pp. 96–97.

Miran, W., Nawaz, M., Kadam, A., Shin, S., Heo, J., Jang, J., Lee, D.S., 2015. Microbial community structure in a dual chamber microbial fuel cell fed with brewery waste for azo dye degradation and electricity generation. Environ. Sci. Pollut. Res. 22, 13477–13485. https://doi.org/10.1007/s11356-015-4582-8

Miura, Y., Hiraiwa, M.N., Ito, T., Itonaga, T., Watanabe, Y., Okabe, S., 2007. Bacterial community structures in MBRs treating municipal wastewater:

Relationship between community stability and reactor performance. Water Res. 41, 627–637. https://doi.org/10.1016/j.watres.2006.11.005
Mohammed, R.R., 2013. Decolorisation of Biologically Treated Palm Oil Mill Effluent (POME) Using Adsorption Technique. Int. Ref. J. Eng. Sci. 2, 01-11.
Mohammed, R.R., Chong, M.F., 2014. Treatment and decolorization of biologically treated Palm Oil Mill Effluent (POME) using banana peel as novel biosorbent. J. Environ. Manage. 132, 237–249. https://doi.org/10.1016/j.jenvman.2013.11.031
Mohammed, R.R., Ketabachi, M.R., McKay, G., 2014. Combined magnetic field and adsorption process for treatment of biologically treated palm oil mill effluent (POME). Chem. Eng. J. 243, 31–42. https://doi.org/10.1016/j.cej.2013.12.084
Mohd-Nor, D., Ramli, N., Sharuddin, S.S., Hassan, M.A., Mustapha, N.A., Amran, A., Sakai, K., Shirai, Y., Maeda, T., 2018. Alcaligenaceae and Chromatiaceae as reliable bioindicators present in palm oil mill effluent final discharge treated by different biotreatment processes. Ecol. Indic. 95, 468–473. https://doi.org/10.1016/j.ecolind.2018.08.007
Moradigaravand, D., Gouliouris, T., Ludden, C., Reuter, S., Jamrozy, D., Blane, B., Naydenova, P., Judge, K., H. Aliyu, S., F. Hadjirin, N., A. Holmes, M., Török, E., M. Brown, N., Parkhill, J., Peacock, S., 2018. Genomic survey of Clostridium difficile reservoirs in the East of England implicates environmental contamination of wastewater treatment plants by clinical lineages. Microb. Genomics 4. https://doi.org/10.1099/mgen.0.000162
Moura, A., Tacão, M., Henriques, I., Dias, J., Ferreira, P., Correia, A., 2009. Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR-DGGE analysis. Microbiol. Res. 164, 560–569. https://doi.org/10.1016/j.micres.2007.06.005
MPOB, 2017. Oil palm planted area 2017, 2–3.
MPOB, 2016. Overview of the Malaysian Oil Palm Industry. Malaysian Palm Oil Board 1–4. https://doi.org/10.1017/CBO9781107415324.004
Mustapha, N.A., Hu, A., Yu, C.P., Sharuddin, S.S., Ramli, N., Shirai, Y., Maeda, T., 2018. Seeking key microorganisms for enhancing methane production in anaerobic digestion of waste sewage sludge. Appl. Microbiol. Biotechnol. 1–12. https://doi.org/10.1007/s00253-018-9003-8
Muyzer, G., 1999. DGGE / TGGE a method for identifying genes from natural ecosystems. Curr. Opin. Microbiol. 2, 317–322. https://doi.org/10.1016/S1369-5274(99)80055-1
Muyzer, G., Waal, E.C.D.E., Uitierlinden, A.G., 1993. Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA 59, 695–700.
Mwaikono, K.S., Maina, S., Sebastian, A., Schilling, M., Kapur, V., Gwakisa, P., 2016. High-throughput sequencing of 16S rRNA gene reveals substantial bacterial diversity on the municipal dumpsite. BMC Microbiol. 16, 145. https://doi.org/10.1186/s12866-016-0758-8
Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739. https://doi.org/10.1016/j.biortech.2010.11.119
Nitipan, S., Mamimin, C., Intrasungkha, N., Birkeland, N.K., O-Thong, S., 2014. Microbial community analysis of thermophilic mixed culture sludge for biohydrogen production from palm oil mill effluent. Int. J. Hydrogen Energy 39, 19285–19293. https://doi.org/10.1016/j.ijhydene.2014.05.139

Nogi, Y., Yoshizumi, M., Hamana, K., Miyazaki, M., Horikoshi, K., 2014. Povalibacter uvarum gen. nov., sp. nov., a polyvinyl-alcohol-degrading bacterium isolated from grapes. Int. J. Syst. Evol. Microbiol. 64, 2712–2717. https://doi.org/10.1099/ijs.0.062620-0

Ohimain, E.I., Izah, S.C., 2017. A review of biogas production from palm oil mill effluents using different configurations of bioreactors. Renew. Sustain. Energy Rev. 70, 242–253. https://doi.org/10.1016/j.rser.2016.11.221

Ostrem, K., Themelis, N.J., 2004. Green waste: Anaerobic Digestion for treating the organic fraction of municipal solid treating the organic fraction of municipal solid wastes. Columbia University.

Ozuolmez, D., Na, H., Lever, M.A., Kjeldsen, K.U., Jørgensen, B.B., Plugge, C.M., 2015. Methanogenic archaea and sulfate reducing bacteria co-cultured on acetate: Teamwork or coexistence? Front. Microbiol. 6, 492. https://doi.org/10.3389/fmicb.2015.00492

Payment, P., Locas, A., 2011. Water Pathogens: values and Batar correlation with Microbes indicator. J. Gr. Water 49, 4–11.

Pereira, V.J., Linden, K.G., Weinberg, H.S., 2007. Evaluation of UV irradiation for photolytic and oxidative degradation of pharmaceutical compounds in water. Water Res. 41, 4413–4423. https://doi.org/10.1016/j.watres.2007.05.056

Pérez-Pantoja, D., Donoso, R., Agulló, L., Córdova, M., Seeger, M., Pieper, D.H., González, B., 2012. Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ. Microbiol. 14, 1091–1117. https://doi.org/10.1111/j.1462-2920.2011.02613.x

Pérez, J., Muñoz-Dorado, J., De La Rubia, T., Martínez, J., 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: An overview. Int. Microbiol. 5, 53–63. https://doi.org/10.1007/s10123-002-0062-3

Poh, P.E., Chong, M.F., 2014. Upflow anaerobic sludge blanket-hollow centered packed bed (UASB-HCPB) reactor for thermophilic palm oil mill effluent (POME) treatment. Biomass and Bioenergy 67, 231–242. https://doi.org/10.1016/j.biombioe.2014.05.007

Poh, P.E., Chong, M.F., 2009. Development of anaerobic digestion methods for palm oil mill effluent (POME) treatment. Bioresour. Technol. 100, 1–9. https://doi.org/10.1016/j.biortech.2008.06.022

Poh, P.E., Yong, W.J., Chong, M.F., 2010. Palm oil mill effluent (POME) characteristic in high crop season and the applicability of high-rate anaerobic bioreactors for the treatment of pome. Ind. Eng. Chem. Res. 49, 11732–11740. https://doi.org/10.1021/ie101486w

Połka, J., Rebecchi, A., Pisacane, V., Morelli, L., Puglisi, E., 2014. Bacterial diversity in typical Italian salami at different ripening stages as revealed by high-throughput sequencing of 16S rRNA amplicons. Food Microbiol. 46, 342–356. https://doi.org/10.1016/j.fm.2014.08.023

Rainey, F.A., Donnison, A.M., Janssen, P.H., Saul, D., Rodrigo, A., Bergquist, P.L., Daniel, R.M., Stackebrandt, E., Morgan, H.W., 1994. Description of Caldicellulosiruptor saccharolyticus gen. nov., sp. nov: An obligately anaerobic, extremely thermophilic, cellulolytic bacterium. FEMS Microbiol. Lett. 120, 263–266. https://doi.org/10.1111/j.1574-6968.1994.tb07043.x

Rajala, P., Carpén, L., Vepsäläinen, M., Raulio, M., Sohlberg, E., Bomberg, M., 2015. Microbially induced corrosion of carbon steel in deep groundwater environment. Front. Microbiol. 6, 1–13. https://doi.org/10.3389/fmicb.2015.00647

Rajhi, H., Puyol, D., Mart??nez, M.C., D??az, E.E., Sanz, J.L., 2016. Vacuum promotes metabolic shifts and increases biogenic hydrogen production in dark fermentation systems. Front. Environ. Sci. Eng. 10, 513–521. https://doi.org/10.1007/s11783-015-0777-y

Rana, S., Singh, L., Wahid, Z., Liu, H., 2017. A Recent Overview of Palm Oil Mill Effluent Management via Bioreactor Configurations. Curr. Pollut. Reports 1–14.

Ranjard, L., Poly, F., Nazaret, S., 2000. Monitoring complex bacterial communities using culture-independent molecular techniques: Application to soil environment. Res. Microbiol. 151, 167–177. https://doi.org/10.1016/S0923-2508(00)00136-4

Rehfuss, M., Urban, J., 2005. Alcaligenes faecalis subsp. phenolicus subsp. nov. a phenol-degrading, denitrifying bacterium isolated from a graywater bioprocessor. Syst. Appl. Microbiol. 28, 421–429. https://doi.org/10.1016/j.syapm.2005.03.003

Remmas, N., Roukouni, C., Ntougias, S., 2017. Bacterial community structure and prevalence of Pusillimonas-like bacteria in aged landfill leachate. Environ. Sci. Pollut. Res. 24, 6757–6769. https://doi.org/10.1007/s11356-017-8416-8

Rupani, P.F., Rajeev, P.S., Irahim, M.H., Esa, N., 2010. Review of Current Palm Oil Mill Effluent (POME) Treatment Methods: Vermicomposting as a Sustainable Practice. World Appl. Sci. J. https://doi.org/10.5539/jas.v7n4p68

Saeed, M.O., Azizli, K., Isa, M.H., Bashir, M.J.K., 2015. Application of CCD in RSM to obtain optimize treatment of POME using Fenton oxidation process. J. Water Process Eng. 8, e7–e16. https://doi.org/10.1016/j.jwpe.2014.11.001

Saeed, M.O., Azizli, K.A.M., Isa, M.H., Ezechi, E.H., 2016. Treatment of POME using Fenton oxidation process: removal efficiency, optimization, and acidity condition. Desalin. Water Treat. 57, 23750–23759. https://doi.org/10.1080/19443994.2016.1141715

Saidudu, H., 2014. Isolation and Identification of Selected Sulphate Reducing Bacteria from Palm Oil Mill Effluent for Desulfurization Process. Universiti Teknologi Malaysia, Skudai, Malaysia.

Salihu, A., Alam, M.Z., 2012. Palm Oil Mill Effluent : A Waste or A Raw Material ? J. Appl. Sci. Res. January, 466–473.

Salminen, E., Rintala, J., 2002. Anaerobic digestion of organic solid poultry slaughterhouse waste--a review. Bioresour. Technol. 83, 13–26. https://doi.org/10.1016/S0960-8524(01)00199-7

Sánchez, O., Garrido, L., Forn, I., Massana, R., Maldonado, M.I., Mas, J., 2011. Molecular characterization of activated sludge from a seawater-processing wastewater treatment plant. Microb. Biotechnol. 4, 628–642. https://doi.org/10.1111/j.1751-7915.2011.00256.x

Sanders, H.L., 1968. Marine Benthic Diversity: A Comparative Study. Am. Nat. 102, 243–282. https://doi.org/10.1086/282541

Satyawali, Y., Balakrishnan, M., 2008. Wastewater treatment in molasses-based alcohol distilleries for COD and color removal: A review. J. Environ. Manage. 86, 481–497. https://doi.org/10.1016/j.jenvman.2006.12.024

Schmidt, O., Horn, M.A., Kolb, S., Drake, H.L., 2015. Temperature impacts differentially on the methanogenic food web of cellulose-supplemented peatland soil. Environ. Microbiol. 17, 720–734. https://doi.org/10.1111/1462-2920.12507

Selvarajan, R., Sibanda, T., Venkatachalam, S., Kamika, I., Nel, W.A.J., 2018. Industrial wastewaters harbor a unique diversity of bacterial communities revealed by high-throughput amplicon analysis. Ann. Microbiol. 68, 445–458.

https://doi.org/10.1007/s13213-018-1349-8
Shade, A., Peter, H., Allison, S.D., Baho, D.L., Berga, M., Bürgmann, H., Huber, D.H., Langenheder, S., Lennon, J.T., Martiny, J.B.H., Matulich, K.L., Schmidt, T.M., Handelsman, J., 2012. Fundamentals of microbial community resistance and resilience. Front. Microbiol. 3, 417. https://doi.org/10.3389/fmicb.2012.00417
Shah, H.N., Gharbia, S.E., 1992. Biochemical and chemical studies on strains designated Prevotella intermedia and proposal of a new pigmented species, Prevotella nigrescens sp. nov. Int J Syst Bacteriol 42, 542–546. https://doi.org/10.1099/00207713-42-4-542
Shannon, C.E., 1948. A Mathematical Theory of Communication. Bell Syst. Tech. J. 27, 379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x
Sharip, N.S., Ariffin, H., Hassan, M.A., Nishida, H., Shirai, Y., 2016. Characterization and application of bioactive compounds in oil palm mesocarp fiber superheated steam condensate as an antifungal agent. RSC Adv. 6, 84672–84683. https://doi.org/10.1039/c6ra13292h
Sharuddin, S.S., Ramli, N., Hassan, M.A., Mustapha, N.A., Amran, A., Mohd-Nor, D., Sakai, K., Tashiro, Y., Shirai, Y., Maeda, T., 2017. Bacterial community shift revealed Chromatiaceae and Alcaligenaceae as potential bioindicators in the receiving river due to palm oil mill effluent final discharge. Ecol. Indic. 82, 526–529. https://doi.org/10.1016/j.ecolind.2017.07.038
Sharuddin, S.S., Ramli, N., Hassan, M.A., Mustapha, N.A., Amran, A., Mohd-Nor, D., Sakai, K., Tashiro, Y., Shirai, Y., Maeda, T., 2017. Bacterial community shift revealed Chromatiaceae and Alcaligenaceae as potential bioindicators in the receiving river due to palm oil mill effluent final discharge. Ecol. Indic. 82, 526–529. https://doi.org/10.1016/j.ecolind.2017.07.038
Sharuddin, S.S., Ramli, N., Mohd-Nor, D., Hassan, M.A., Maeda, T., Shirai, Y., Sakai, K., Tashiro, Y., 2018. Shift of low to high nucleic acid bacteria as a potential bioindicator for the screening of anthropogenic effects in a receiving river due to palm oil mill effluent final discharge. Ecol. Indic. 85, 79–84. https://doi.org/https://doi.org/10.1016/j.ecolind.2017.10.020
Shi, J., Wang, Z., Stiverson, J.A., Yu, Z., Li, Y., 2013. Reactor performance and microbial community dynamics during solid-state anaerobic digestion of corn stover at mesophilic and thermophilic conditions. Bioresour. Technol. 136, 574–581. https://doi.org/10.1016/j.biortech.2013.02.073
Shi, S., Qu, Y., Ma, Q., Zhang, X., Zhou, J., Ma, F., 2015. Performance and microbial community dynamics in bioaugmented aerated filter reactor treating with coking wastewater. Bioresour. Technol. 190, 159–166. https://doi.org/10.1016/j.biortech.2015.04.075
Shin, S.G., Lee, S., Lee, C., Hwang, K., Hwang, S., 2010. Qualitative and quantitative assessment of microbial community in batch anaerobic digestion of secondary sludge. Bioresour Technol 9461–9470.
Shon, H.K., Vigneswaran, S., Snyder, S.A., 2006. Effluent organic matter (EfOM) in wastewater: Constituents, effects, and treatment. Crit. Rev. Environ. Sci. Technol. https://doi.org/10.1080/10643380600580011
Shu, D., He, Y., Yue, H., Wang, Q., 2015. Microbial structures and community functions of anaerobic sludge in six full-scale wastewater treatment plants as revealed by 454 high-throughput pyrosequencing. Bioresour. Technol. 186, 163–172. https://doi.org/10.1016/j.biortech.2015.03.072
Simpson, E.H., 1949. Measurement of diversity. Nature.

https://doi.org/10.1038/163688a0
Sluiter, a., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2012. NREL/TP-510-42618 analytical procedure - Determination of structural carbohydrates and lignin in Biomass. Lab. Anal. Proced. 17. https://doi.org/NREL/TP-510-42618
Sonune, N.A., Garode, A.M., 2015. Bioremediation Potential of Bacterial Isolates for Municipal Wastewater Treatment. Curr. World Environ. 10, 619–625. https://doi.org/10.12944/CWE.10.2.27
Sophonsiri, C., Morgenroth, E., 2004. Chemical composition associated with different particle size fractions in municipal, industrial, and agricultural wastewaters. Chemosphere. https://doi.org/10.1016/j.chemosphere.2003.11.032
Sousa, a M., Pereira, M.O., 2013. A prospect of current microbial diagnosis methods. Microb. Pathog. Strateg. Combat. them Sci. Technol. Educ. 3, 1429–1438.
Stams, A.J.M., Sousa, D.Z., Kleerebezem, R., Plugge, C.M., 2012. Role of syntrophic microbial communities in high-rate methanogenic bioreactors. Water Sci. Technol. 66, 352–362. https://doi.org/10.2166/wst.2012.192
Su, C., Lei, L., Duan, Y., Zhang, K.Q., Yang, J., 2012. Culture-independent methods for studying environmental microorganisms: Methods, application, and perspective. Appl. Microbiol. Biotechnol. 93, 993–1003. https://doi.org/10.1007/s00253-011-3800-7
Sun, Y., Wang, T., Peng, X., Wang, P., Lu, Y., 2016. Bacterial community compositions in sediment polluted by perfluoroalkyl acids (PFAAs) using Illumina high-throughput sequencing. Environ. Sci. Pollut. Res. 23, 10556–10565. https://doi.org/10.1007/s11356-016-6055-0
Suriya, J., Chandra Shekar, M., Nathani, N.M., Suganya, T., Bharathiraja, S., Krishnan, M., 2017. Assessment of bacterial community composition in response to uranium levels in sediment samples of sacred Cauvery River. Appl. Microbiol. Biotechnol. 101, 831–841. https://doi.org/10.1007/s00253-016-7945-2
Tabassum, S., Zhang, Y., Zhang, Z., 2015. An integrated method for palm oil mill effluent (POME) treatment for achieving zero liquid discharge - A pilot study. J. Clean. Prod. 95, 148–155. https://doi.org/10.1016/j.jclepro.2015.02.056
Tabatabaei, M., Zakaria, M.R., Rahim, R.A., Wright, A.D.G., Shirai, Y., Abdullah, N., Sakai, K., Ikeno, S., Mori, M., Kazunori, N., Sulaiman, A., Hassan, M.A., 2009. PCR-based DGGE and FISH analysis of methanogens in an anaerobic closed digester tank for treating palm oil mill effluent. Electron. J. Biotechnol. 12, 12–13. https://doi.org/10.2225/vol12-issue3-fulltext-4
Talib, Y., Idris, A., Aslina, S., 2016. A tannin-based agent for coagulation and fl occulation of municipal wastewater : Chemical composition , performance assessment compared to Polyaluminum chloride , and application in a pilot plant. J. Environ. Manag. https://doi.org/10.1016/j.jenvman.2016.10.033
Tan, B.H., Teng, T.T., Omar, A.K.M., 2000. Removal of dyes and industrial dye wastes by magnesium chloride. Water Res. 34, 597–601. https://doi.org/10.1016/S0043-1354(99)00151-7
Tan, K.M., Liew, W.L., Muda, K., Kassim, M.., 2015. Microbiological Characteristics of Palm Oil Mill Effluent. nternational Congress on Chemical, Biological, and Environmental Sciences (ICCBES), Kyoto, Japan.
Tan, Y.H., Goha, P.S., Lai, G.., Lau, W.., Ismail, A.F., 2014. Treatment of aerobic treated palm oil mill effluent (AT-POME) by using TiO2 photocatalytic process. J. Teknol. 70, 61–63. https://doi.org/http://dx.doi.org/10.11113/jt.v70.3436

Tan, Y.J., Sun, L.J., Li, B.T., Zhao, X.H., Yu, T., Ikuno, N., Ishii, K., Hu, H.Y., 2017. Fouling characteristics and fouling control of reverse osmosis membranes for desalination of dyeing wastewater with high chemical oxygen demand. Desalination 419, 1–7. https://doi.org/10.1016/j.desal.2017.04.029

Tanikkul, P., Phalakornkule, C., Champreda, V., 2016. Comparative Granular Characteristics of Mesophilic and Thermophilic UASB Producing Biogas from Palm Oil Mill Effluent. Chem. Eng. J. 50, 205–210. https://doi.org/10.3303/CET1650035

Tiirola, M.A., Suvilampi, J.E., Kulomaa, M.S., Rintala, J.A., 2003. Microbial diversity in a thermophilic aerobic biofilm process: Analysis by length heterogeneity PCR (LH-PCR). Water Res. 37, 2259–2268. https://doi.org/10.1016/S0043-1354(02)00631-0

Van Belkum, A., Durand, G., Peyret, M., Chatellier, S., Zambardi, G., Schrenzel, J., Shortridge, D., Engelhardt, A., Dunne, W.M., 2013. Rapid clinical bacteriology and its future impact. Ann. Lab. Med. 33, 14–27. https://doi.org/10.3343/alm.2013.33.1.14

Van de Peer, Y., Van der Auwera, G., De Wachter, R., 1996. The evolution of stramenopiles and alveolates as derived by "substitution rate calibration" of small ribosomal subunit RNA. J. Mol. Evol. 42, 201–210.

Venkata Mohan, S., Raghavulu, S.V., Goud, R.K., Srikanth, S., Babu, V.L., Sarma, P.N., 2010. Microbial diversity analysis of long term operated biofilm configured anaerobic reactor producing biohydrogen from wastewater under diverse conditions. Int. J. Hydrogen Energy 35, 12208–12215. https://doi.org/10.1016/j.ijhydene.2010.08.008

Vijayaraghavan, K., Ahmad, D., Ezani Bin Abdul Aziz, M., 2007. Aerobic treatment of palm oil mill effluent. J. Environ. Manage. 82, 24–31. https://doi.org/10.1016/j.jenvman.2005.11.016

Vivas, A., Moreno, B., Garcia-Rodriguez, S., Benitez, E., 2009. Assessing the impact of composting and vermicomposting on bacterial community size and structure, and microbial functional diversity of an olive-mill waste. Bioresour. Technol. 100, 1319–1326. https://doi.org/10.1016/j.biortech.2008.08.014

Walter, A., Franke-Whittle, I.H., Wagner, A.O., Insam, H., 2015. Methane yields and methanogenic community changes during co-fermentation of cattle slurry with empty fruit bunches of oil palm. Bioresour. Technol. 175, 619–623. https://doi.org/10.1016/j.biortech.2014.10.085

Wang, J., Liu, X.D., Lu, J., 2012. Urban River Pollution Control and Remediation. Procedia Environ. Sci. 13, 1856–1862. https://doi.org/10.1016/j.proenv.2012.01.179

Wang, J., Mahmood, Q., Qiu, J.P., Li, Y.S., Chang, Y.S., Chi, L.N., Li, X.D., 2015. Zero discharge performance of an industrial pilot-scale plant treating palm oil mill effluent. Biomed Res. Int. 2015, 9. https://doi.org/10.1155/2015/617861

Wang, X., Cao, A., Zhao, G., Zhou, C., Xu, R., 2017. Microbial community structure and diversity in a municipal solid waste landfill. Waste Manag. 66, 79–87. https://doi.org/10.1016/j.wasman.2017.04.023

Weiß, S., Somitsch, W., Klymiuk, I., Trajanoski, S., Guebitz, G.M., 2016. Comparison of biogas sludge and raw crop material as source of hydrolytic cultures for anaerobic digestion. Bioresour. Technol. 207, 244–251. https://doi.org/10.1016/j.biortech.2016.01.137

Wells, G.F., Park, H.D., Eggleston, B., Francis, C.A., Criddle, C.S., 2011. Fine-scale

bacterial community dynamics and the taxa-time relationship within a full-scale activated sludge bioreactor. Water Res. 45, 5476–5488. https://doi.org/10.1016/j.watres.2011.08.006
Wen, Y., Jin, Y., wang, jiayang, cai, lipeng, 2015. MiSeq Sequencing Analysis of Bacterial Community Structures in Wastewater Treatment Plants. Polish J. Environ. Stud. 24, 1809–1815. https://doi.org/10.15244/pjoes/38456
Whittaker, R.H., 1972. Evolution and Measurement of Species Diversity. Taxon 213–251. https://doi.org/10.2307/1218190
Wilkie, A.C., Riedesel, K.J., Owens, J.M., 2000. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. Biomass and Bioenergy 19, 63–102. https://doi.org/10.1016/S0961-9534(00)00017-9
Wong, Y.S., Teng, T.T., Ong, S.A., Morad, N., Rafatullah, M., 2014. Suspended growth kinetic analysis on biogas generation from newly isolated anaerobic bacterial communities for palm oil mill effluent at mesophilic temperature. RSC Adv. 4, 64659–64667. https://doi.org/10.1039/c4ra08483g
Wu, T.Y., Mohammad, A.W., Jahim, J.M., Anuar, N., 2010. Pollution control technologies for the treatment of palm oil mill effluent (POME) through end-of-pipe processes. J. Environ. Manage. 91, 1467–1490. https://doi.org/10.1016/j.jenvman.2010.02.008
Wu, T.Y., Mohammad, A.W., Jahim, J.M., Anuar, N., 2009. A holistic approach to managing palm oil mill effluent (POME): Biotechnological advances in the sustainable reuse of POME. Biotechnol. Adv. 27, 40–52. https://doi.org/10.1016/j.biotechadv.2008.08.005
Xia, S., Duan, L., Song, Y., Li, J., Piceno, Y.M., Andersen, G.L., Alvarez-Cohen, L., Moreno-Andrade, I., Huang, C.-L., Hermanowicz, S.W., 2010. Bacterial community structure in geographically distributed biological wastewater treatment reactors. Environ. Sci. Technol. 44, 7391–7396. https://doi.org/10.1021/es101554m
Xia, Y., Yang, C., Zhang, T., 2018. Microbial effects of part-stream low-frequency ultrasonic pretreatment on sludge anaerobic digestion as revealed by high-throughput sequencing-based metagenomics and metatranscriptomics. Biotechnol. Biofuels 11, 47. https://doi.org/10.1186/s13068-018-1042-y
Xin, B., Wang, Y., Tao, F., Li, L., Ma, C., Xu, P., 2016. Co-utilization of glycerol and lignocellulosic hydrolysates enhances anaerobic 1,3-propanediol production by Clostridium diolis. Sci. Rep. 6, 19044. https://doi.org/10.1038/srep19044
Yacob, S., Hassan, M., Shirai, Y., Wakisaka, M., Subash, S., 2006. Baseline study of methane emission from anaerobic ponds of palm oil mill effluent treatment. Sci. Total Environ. 366, 187–196. https://doi.org/10.1016/j.scitotenv.2005.07.003
Yacob, S., Hassan, M.A., Shirai, Y., Wakisaka, M., Subash, S., 2005. Baseline study of methane emission from open digesting tanks of palm oil mill effluent treatment. Chemosphere 59, 1575–1581. https://doi.org/10.1016/j.chemosphere.2004.11.040
Yadvika, Santosh, Sreekrishnan, T.R., Kohli, S., Rana, V., 2004. Enhancement of biogas production from solid substrates using different techniques - A review. Bioresour. Technol. 95, 1–10. https://doi.org/10.1016/j.biortech.2004.02.010
Yamaguchi, A., Sato, O., Mimura, N., Shirai, M., 2016. Catalytic production of sugar alcohols from lignocellulosic biomass. Catal. Today 265, 199–202. https://doi.org/10.1016/j.cattod.2015.08.026

Yao, Y., Guan, J., Tang, P., Jiao, H., Lin, C., Wang, J., Lu, Z., Min, H., Gao, H., 2010. Assessment of toxicity of tetrahydrofuran on the microbial community in activated sludge. Bioresour. Technol. 101, 5213–5221. https://doi.org/10.1016/j.biortech.2010.02.051

Ye, J., Song, Z., Wang, L., Zhu, J., 2016. Metagenomic analysis of microbiota structure evolution in phytoremediation of a swine lagoon wastewater. Bioresour. Technol. 219, 439–444. https://doi.org/10.1016/j.biortech.2016.08.013

Ye, L., Zhang, T., 2013. Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. Appl. Microbiol. Biotechnol. 97, 2681–2690. https://doi.org/10.1007/s00253-012-4082-4

Yoochatchaval, W., Kumakura, S., Tanikawa, D., Yamaguchi, T., Yunus, M.F.M., Chen, S.S., Kubota, K., Harada, H., Syutsubo, K., 2011. Anaerobic degradation of palm oil mill effluent (POME). Water Sci. Technol. 64, 2001–2008. https://doi.org/10.2166/wst.2011.782

Yossan, S., O-Thong, S., Prasertsan, P., 2012. Effect of initial pH, nutrients and temperature on hydrogen production from palm oil mill effluent using thermotolerant consortia and corresponding microbial communities. Int. J. Hydrogen Energy 37, 13806–13814. https://doi.org/10.1016/j.ijhydene.2012.03.151

Zaher, K., Hammam, G., 2014. Correlation between Biochemical Oxygen Demand and Chemical Oxygen Demand for Various Wastewater Treatment Plants in Egypt to Obtain the Biodegradability Indices. Int. J. Sci. Basic Appl. Res. 13, 42–48.

Zahrim, A.Y., Nasimah, A., Hilal, N., 2014. Pollutants analysis during conventional palm oil mill effluent (POME) ponding system and decolourisation of anaerobically treated POME via calcium lactate-polyacrylamide. J. Water Process Eng. 4, 159–165. https://doi.org/10.1016/j.jwpe.2014.09.005

Zainal, N.H., Jalani, N.F., Mamat, R., Astimar, A.A., 2017. A review on the development of palm oil mill effluent (POME) final discharge polishing treatments. J. Oil Palm Res. 29, 528–540. https://doi.org/10.21894/jopr.2017.00012

Zainudin, M.H.M., Hassan, M.A., Md Shah, U.K., Abdullah, N., Tokura, M., Yasueda, H., Shirai, Y., Sakai, K., Baharuddin, A.S., 2014. Bacterial community structure and biochemical changes associated with composting of lignocellulosic oil palm empty fruit bunch. BioResources 9, 316–335.

Zainudin, M.H.M., Hassan, M.A., Tokura, M., Shirai, Y., 2013. Indigenous cellulolytic and hemicellulolytic bacteria enhanced rapid co-composting of lignocellulose oil palm empty fruit bunch with palm oil mill effluent anaerobic sludge. Bioresour. Technol. 147, 632–635. https://doi.org/10.1016/j.biortech.2013.08.061

Zakaria, M.R., Hirata, S., Hassan, M.A., 2015. Hydrothermal pretreatment enhanced enzymatic hydrolysis and glucose production from oil palm biomass. Bioresour. Technol. 176, 142–148. https://doi.org/10.1016/j.biortech.2014.11.027

Zakharova, Y.R., Galachyants, Y.P., Kurilkina, M.I., Likhoshvay, A. V., Petrova, D.P., Shishlyannikov, S.M., Ravin, N. V., Mardanov, A. V., Beletsky, A. V., Likhoshway, Y. V., 2013. The Structure of Microbial Community and Degradation of Diatoms in the Deep Near-Bottom Layer of Lake Baikal. PLoS One 8, e59977. https://doi.org/10.1371/journal.pone.0059977

Zamanzadeh, M., Parker, W.J., Verastegui, Y., Neufeld, J.D., 2013. Biokinetics and bacterial communities of propionate oxidizing bacteria in phased anaerobic sludge digestion systems. Water Res 1558–1569.

Zhang, B., Xu, X., Zhu, L., 2018. Activated sludge bacterial communities of typical wastewater treatment plants: distinct genera identification and metabolic potential differential analysis. AMB Express 8, 14. https://doi.org/10.1186/s13568-018-0714-0

Zhang, B., Xu, X., Zhu, L., 2017. Structure and function of the microbial consortia of activated sludge in typical municipal wastewater treatment plants in winter. Sci. Rep. 7, 17930. https://doi.org/10.1038/s41598-017-17743-x

Zhang, X., Qu, Y., Ma, Q., Zhang, Z., Li, D., Wang, J., Shen, W., Shen, E., Zhou, J., 2015. Illumina MiSeq sequencing reveals diverse microbial communities of activated sludge systems stimulated by different aromatics for indigo biosynthesis from Indole. PLoS One 10, 1–14. https://doi.org/10.1371/journal.pone.0125732

Zhang, Y., Kelly, W.R., Panno, S. V., Liu, W.T., 2014. Tracing fecal pollution sources in karst groundwater by Bacteroidales genetic biomarkers, bacterial indicators, and environmental variables. Sci. Total Environ. 490, 1082–1090. https://doi.org/10.1016/j.scitotenv.2014.05.086

Zhao, J., Zhao, X., Chao, L., Zhang, W., You, T., Zhang, J., 2014. Diversity change of microbial communities responding to zinc and arsenic pollution in a river of northeastern China. J. Zhejiang Univ. B 15, 670–680. https://doi.org/10.1631/jzus.B1400003

Zhong, F., Wu, J., Dai, Y., Yang, L., Zhang, Z., Cheng, S., Zhang, Q., 2014. Bacterial community analysis by PCR-DGGE and 454-pyrosequencing of horizontal subsurface flow constructed wetlands with front aeration. Appl. Microbiol. Biotechnol. 99, 1499–1512. https://doi.org/10.1007/s00253-014-6063-2

Zhou, X., Wu, F., 2012. Dynamics of the diversity of fungal and Fusarium communities during continuous cropping of cucumber in the greenhouse. FEMS Microbiol. Ecol. 80, 469–478. https://doi.org/10.1111/j.1574-6941.2012.01312.x

Zunaira, S., Hanim, A., 2017. A Century of Growth. Star.

# APPENDICES

## APPENDIX A

Preparation of Gradients Solution in Denaturing Gradient Gel Casting

**Table A The gradients used for DGGE gel casting**

| Gradient (%) | 0 | 30 | 65 |
|---|---|---|---|
| Urea (g) | - | 1.89 | 4.105 |
| Formamide (mL) | - | 1.8 | 2.9 |
| 50 X TAE (mL) | 0.3 | 0.3 | 0.3 |
| 40% acrylamide solution (mL) | 3.0 | 3.0 | 3.0 |
| Ultrapure water (mL) | Up to 15 | Up to 15 | Up to 15 |
| 10% Ammonium persulfate (μL) | 135 | 135 | 135 |
| Tetramethylethylene diamine (μL) | 13.5 | 13.5 | 13.5 |

## APPENDIX B

Standard Curves for the Determination of Organic Acids

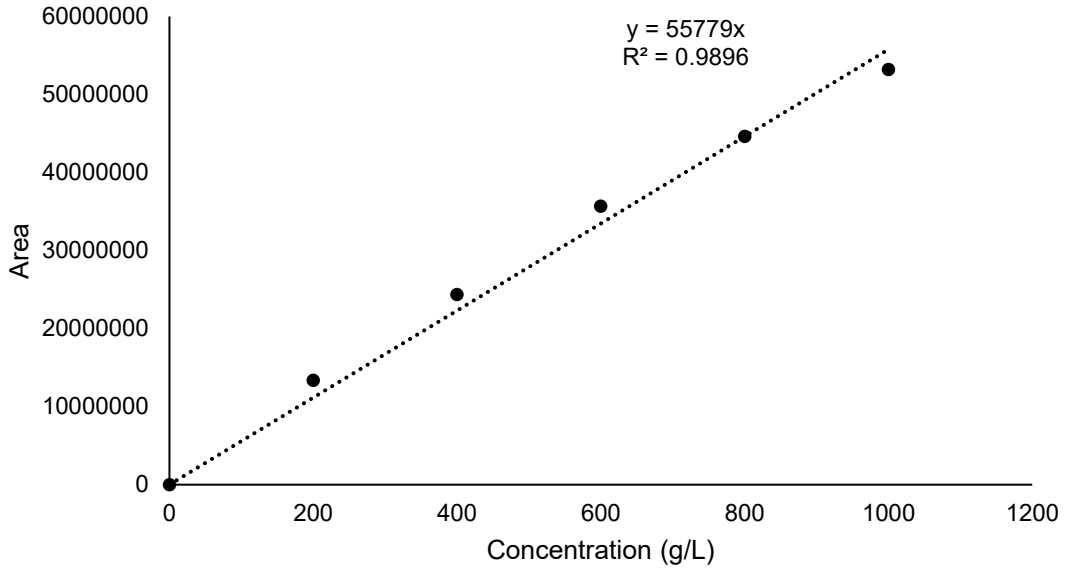



**Figure B (i) The standard curve for acetic acid**

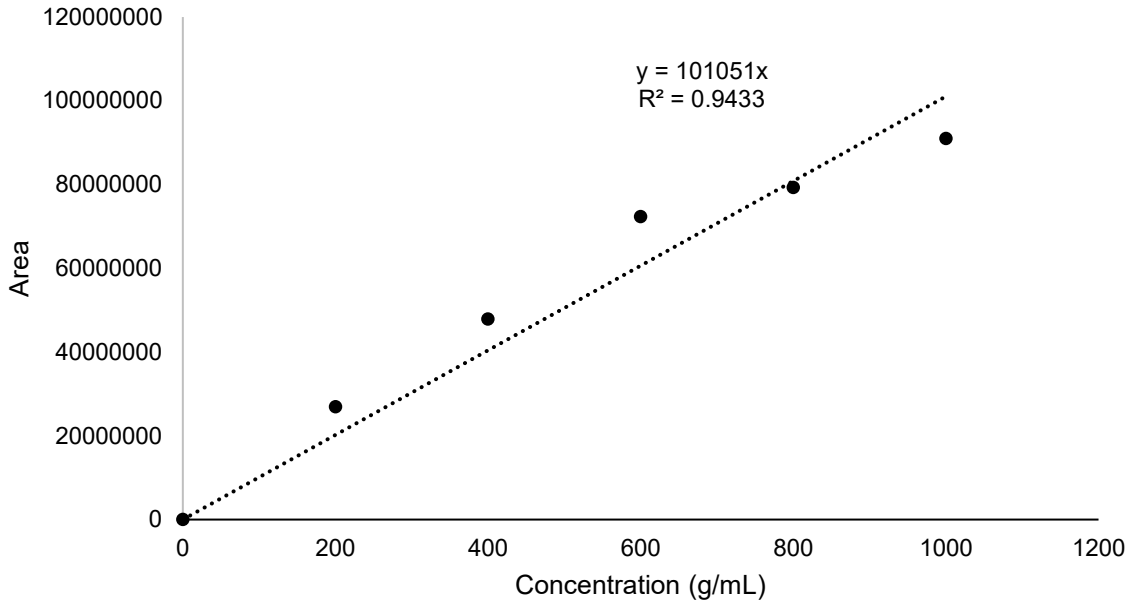



**Figure B (ii) The standard curve for lactic acid**

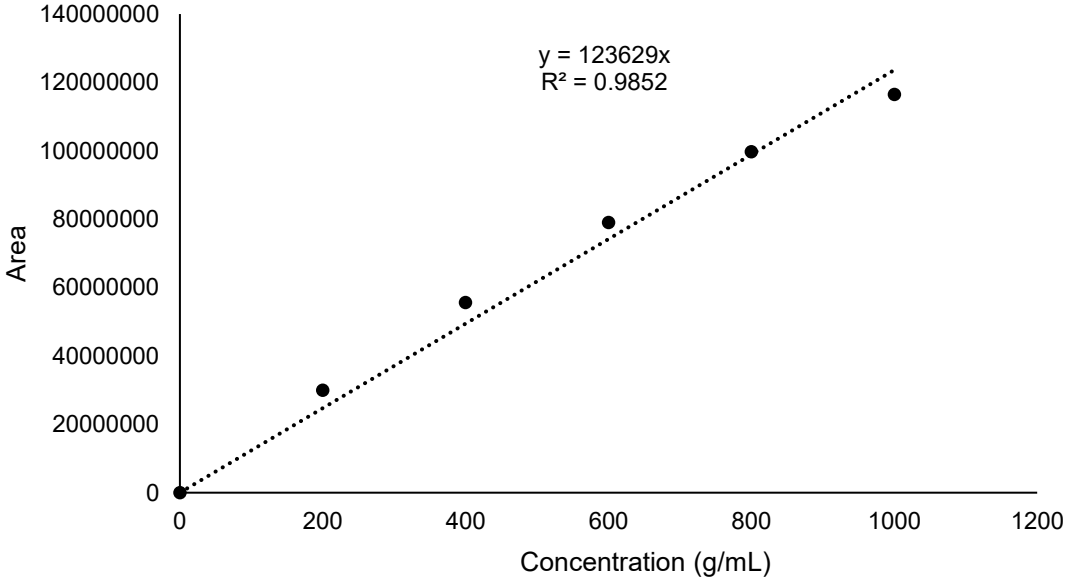



**Figure B (iii) The standard curve for propionic acid**

## LIST OF PUBLICATIONS

**Paper publications:**

**Mohd-Nor, D.**, Ramli, N., Sharuddin, S.S., Hassan, M.A., Mustapha, N.A., Amran, A., Sakai, K., Shirai, Y. and Maeda, T. 2018. *Alcaligenaceae* And *Chromatiaceae* as Reliable Bioindicators Present in Palm Oil Mill Effluent Final Discharge Treated by Different Biotreatment Processes. *Ecological Indicators* 95: 468-473.

Sharuddin, S.S., Ramli, N., Hassan, M.A., Mustapha, N.A., Amran, A., **Mohd-Nor, D.**, Sakai, K., Tashiro, Y., Shirai, Y. and Maeda, T., 2017. Bacterial community shift revealed *Chromatiaceae* and *Alcaligenaceae* as potential bioindicators in the receiving river due to palm oil mill effluent final discharge. *Ecological Indicators*, *82*, 526–529.

Sharuddin, S.S., Ramli, N., **Mohd-Nor, D.**, Hassan, M.A., Maeda, T., Shirai, Y., Sakai, K. and Tashiro, Y. 2018. Shift of low to high nucleic acid bacteria as a potential bioindicator for the screening of anthropogenic effects in a receiving river due to palm oil mill effluent final discharge. *Ecological Indicators*, *85*, 79–84.

**Mohd-Nor, D.**, Ramli, N., Sharuddin, S.S., Hassan, M.A., Mustapha, N.A., Ariffin, H., Sakai, Tashiro, Y., Shirai, Y. And Maeda, T. Dynamics of microbial populations responsible for the biodegradation during the full-scale treatment of palm oil mill effluent. Accepted in *Microbes and Environments* (Nov 2018).

**List of conference attended:**

**Mohd-Nor, D.**, Ramli, N., Hassan, M.A., Sakai, K., Tashiro, Y., Maeda, T. and Shirai, Y. 2014. Microbial community changes in different stages of palm oil mill effluent treatment. Symposium of Applied Sngineering and Sciences 2014 (SAES 2014). 10-11th November 2014, Kyushu Institute of Technology, Japan. (Poster Presentation).

**Mohd-Nor, D.**, Ramli, N., Hassan, M.A., Sakai, K., Tashiro, Y., Maeda, T. and Shirai, Y. 2015. Microbial community changes in different stages of palm oil mill effluent treatment. Asian Congress on Biotechnology 2015 (ACB 2015), 15-19th November 2015, Istana Hotel, Kuala Lumpur. (Poster presentation).

**Mohd-Nor, D.**, Ramli, N., Hassan, M.A., Sakai, K., Tashiro, Y., Maeda, T. and Shirai, Y. 2017. Bacterial community shift as potential bioindicator for monitoring the performance of palm oil mill effluent system. Symposium of Applied Sngineering and Sciences 2017 (SAES 2017). 14-15th November 2017, Universiti Putra Malaysia, Malaysia. (Poster Presentation).

## ACKNOWLEDGEMENTS

Alhamdulillah, praise to Allah S.W.T. for His mercy and guidance for which I manage to complete my research thesis entitled “Bacterial and Methanogenic Archaeal Community Changes during the Treatment of Palm Oil Mill Effluent and Biological Indicators for Final Discharge”.

Utmost gratitude and appreciation go to my enthusiastic supervisory committee chairman, Dr Norhayati Ramli. Her willingness to motivate and encourage me throughout my study contributed tremendously to the completion of this thesis. Words are inadequate in offering my thanks to my co-supervisors, Prof. Dato’ Dr Mohd Ali Hassan, Assoc. Prof. Dr Toshinari Maeda and Prof. Dr Kenji Sakai for their constant supervision and cooperation until the completion of my study.

My special gratitude also goes to all students and lecturers in Environmental Biotechnology (EB) Group, as well as lecturers, students, and staffs of Kyutech, Japan who sincerely devoted their time to help me conducting my research. I also wish to extend my heartfelt gratitude to the staffs at Universiti Putra Malaysia, Malaysia Genome Institute (MGI) and palm oil mills; FELDA Pasoh 4, FELDA Sungai Tengi, FELDA Besout and FELDA Trolak for their tremendous support and help. I also would like to express my love and gratitude to my family and colleagues for their understanding and endless support throughout the way. Lastly, I would like to thank Science and Technology Research Partnership for Sustainable Development (SATREPS) under Ministry of Education (MOE) Malaysia, Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA) for funding this study.