MICROBIAL COMMUNITY CHANGES DURING CO-COMPOSTING OF OIL PALM EMPTY FRUIT BUNCH WITH PALM OIL MILL EFFLUENT ANAEROBIC SLUDGE

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The oil palm industry in Malaysia produces residues such as oil palm empty fruit bunch (OPEFB) and palm oil mill effluent (POME) anaerobic sludge which can be transformed into high value-added products. The utilization of these residues in the form of decomposed biomass for agricultural used create interest in improving the composting process to be more efficient and sustainable. During the composting process, organic materials including lignocellulose are broken down by the bacterial communities which develop in line with the symbiotic relationship among the species present. Recently, the enhanced co-composting of OPEFB and POME anaerobic sludge process has spurred increase attention into finding how microbial diversity influence the degradation process and what microbes are involved during the process. Hence, the overall objective of this study was to elucidate the bacterial communities participating in the enhanced composting process through isolation and characterization of cultivated microbes, polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE), 16S rRNA clone library techniques and next generation sequencing (pyrosequencing). In the first study, 27 cellulolytic and hemicellulolytic bacteria strains were isolated at different stages of composting of which 23 strains were identified as closely related to *Bacillus subtilis*, *Bacillus firmus*, *Thermobifida fusca*, *Thermomonospora* sp. S22-23, *Cellulomonas* sp. ANA-WS2, *Ureibacillus thermosphaericus*, *Paenibacillus barengoltzii*, *Paenibacillus campinasensis* BL11, *Geobacillus thermodenitrificans* and *Pseudoxanthomonas byssovorax.* All of these were known to be commonly involved in lignocellulose degradation. Four strains related to *Exiguobacterium acetylicum* and *Rhizobium* sp which are previously not known as lignocellulosic degraders were found with cellulolytic and hemicellulolytic activities. Consequently, PCR-DGGE and 16s rRNA clone library methods were used to visualize the shift in microbial community and to identify the bacterial community species. PCR-DGGE showed that the bacterial community drastically shifted and the banding patterns correlate with the abundance of phyla *Actinobacteria* (high G+C content DNA bacterium) and *Firmicutes* (low G+C content DNA bacterium) during thermophilic, cooling and maturing stages as detected in the clone library. 16S rRNA clones belonging to the genera *Bacillus*, *Exiguobacterium*, *Desemzia*, and *Planococcus* were the dominant group with species closely related to *Solibacillus silvestris* was found to be major contributors to the changes in the lignocellulosic component throughout composting. Clones identified as *Thermobacillus xylanilyticus*, *Brachybact-*

erium faecium, *Cellulosimicrobium cellulans*, *Cellulomonas* sp., and *Thermobifida fusca*, which are known to be lignocellulosic-degrading bacteria, were also detected. The results were in line with identification of isolated cellulolytic and hemicellulolytic bacteria and revealed the presence of these bacteria types at different stages of composting. In depth analysis of the bacterial community structure and shift in microbial abundance using pyrosequencing showed that the largest bacterial communities were belonged to phyla *Firmicutes* and *Proteobacteria*, in which their species related to *Devosia yakushimensis* and *Planoccocus rifietoensis* were found during thermophilic, cooling and maturing stages. It is of interest to report for the first time that sequence related to *Devosia yakushimensis* was found to be a dominant bacterial species during thermophilic stage which was not discovered in the analysis of clone library. The data obtained suggests that clone libraries can overlook important groups of bacteria, hindering the link between the microbial diversity to operational performance. The results of the present study clearly demonstrate the ensuing changes that take place in the microbial community during co-composting of oil palm empty fruit bunch with palm oil mill effluent anaerobic sludge.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi kerperluan untuk ijazah Doktor Falsafah

PERUBAHAN KOMUNITI MIKROB SEMASA PENGKOMPOSAN TANDAN KOSONG KELAPA SAWIT DENGAN ENAPCEMAR ANAEROBIK EFFLUEN KELAPA SAWIT

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Industri kelapa sawit di Malaysia menghasilkan sisa seperti tandan kosong kelapa sawit dan enapcemar anaerobik effluen kelapa sawit yang boleh diubah menjadi produk nilai tambah yang tinggi. Penggunaan sisa ini dalam bentuk biojisim terurai untuk kegunaan pertanian mewujudkan kepentingan dalam mempertingkatkan proses pengkomposan yang lebih cekap and mapan. Semasa proses pengkomposan, bahan-bahan organik seperti lignosellulosa diuraikan oleh komuniti bakteria yang berkembang selaras dengan hubungan simbiotik antara spesies yang hadir. Terkini, kecekapan proses pengkomposan OPEFB bersama enapcemar anaerobik POME telah mendorong meningkatkan perhatian dalam mencari pengaruh bagaimana kelpebagaian mikroorganisma menjalankan proses penguraian dan mikroorganisma yang terlibat dalam proses tersebut. Oleh itu, objektif keseluruhan kajian ini adalah untuk menjelaskan komuniti bakteria yang terlibat dalam proses pengkomposan yang dipertingkatkan melalui pengasingan dan pencirian mikrob yang dihidupkan, tindak balas rantai polimerase-elektroforesis gel kecerunan nyahasli (PCR-DGGE) dan perpustakaan klon 16S rRNA gene dan penyusunan generasi akan datang (pyro-penyusunan. Di dalam kajian pertama, 27 bakteria pengurai selulosa dan hemiselulosa telah dipencilkan daripada peringkat proses pengkomposan yang berbeza di mana 23 bacteria telah dikenalpasti sebagai berkait rapat dengan *Bacillus subtilis*, *Bacillus firmus*, *Thermobifida fusca*, *Thermomonospora* sp. S22-23, *Cellulomonas* sp. ANA-WS2, *Ureibacillus thermosphaericus*, *Paenibacillus barengoltzii*, *Paenibacillus campinasensis* BL11, *Geobacillus thermodenitrificans* dan *Pseudoxanthomonas byssovorax*, semua yang telah diketahui umum terlibat dalam penguraian lignoselulosa. Empat bakteria yang berkaitan dengan *Exiguobacterium acetylicum* and *Rhizobium* sp yang sebelum ini tidak dikenali sebagai pengurai lignoselulosa telah ditemui dengan dan aktiviti selulose and hemiselulosa. Seterusnya, PCR-DGGE dan perpustakaan klon 16S rRNA gene telah digunakan untuk menggambarkan pangalihan komuniti mikoorganisma dan mengenalpasti spesis komuniti bakteria. PCR-DGGE menunjukkan bahawa komuniti bakteria beralih secara drastik dan keputusan corak jalur DGGE berhubung rapat dengan,banyaknya filum *Actinobacteria* (bakteria tinggi kandungan G+C DNA) dan *Firmicutes* (bakteria rendah kandungan G+ C DNA) pada peringkat termofilik serta penyejukan dan matang sebagaimana yang telah dikesan di dalam perpustakaan klon. Klon 16S rRNA kepunyaan genus *Bacillus*, *Exiguobacterium*, *Desemzia*, dan *Planococcus* ialah kumpulan dominan bersama dengan spesies yang

berkait rapat dengan *Solibacillus silvestris* didapati telah menjadi penyumbang utama kepada perubahan komponen lignoselulosa di sepanjang pengkomposan. Klon yang dikenal pasti sebagai *Thermobacillus xylanilyticus*, *Brachybacterium faecium*, *Cellulosimicrobium cellulans*, *Cellulomonas* sp., dan *Thermobifida fusca* yang dikenali sebagai bakteria pengurai lignoselulosa, juga telah dikesan. Keputusan yang diperolehi selaras dengan pemencilan bakteria pengurai selulosa dan hemiselulosa yang telah dikenalpasti dan seterusnya mendedahkan kehadiran bakteria jenis ini dipelbagai peringkat dalam proses pengkomposan. Analisis mendalam struktur komuniti bakteria dan peralihan lambakan mikrob dengan menggunakan pyro-penjujukan menunjukkan bahawa komuniti bakteria terbesar adalah kepunyaan filum *Firmicutes* dan *Proteobacteria* di mana spesies mereka yang berkaitan dengan *Devosia yakushimensis* dan *Planoccocus rifietoensis* yang didapati semasa peringkat termofilik, penyejukan dan matang. Kajian ini buat pertama kalinya melaporkan bahawa jujukan berkait rapat dengan *Devosia yakushimensis*, telah didapati menjadi sebagai dominan spesies bakteria pada peringkat termofilik yang mana ianya tidak ditemui dalam analisis perpustakaan klon. Data yang diperoleh juga menunjukkan bahawa perpustakaan klon boleh mengabaikan beberapa kumpulan penting bakteria, menghalang kupayaan untuk menghubungkan kepelbagaian mikrob kepada prestasi operasi. Hasil kajian ini jelas menunjukkan perubahan yang berlaku dalam komuniti mikrob semasa pengkomposan tandan kosong kelapa sawit bersama enapcemar anaerobik effluen kelapa sawit.

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LIST OF ABBREVIATIONS

Bp	Base pair
BLAST	Basic logical alignment search tool
BOD	Biological oxygen demand
CMC	Carboxymethylcellulose
COD	Chemical oxygen demand
CPO	Crude palm oil
DGGE	Denaturing gel gradient electrophoresis
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
dNTP	deoxynucleotide triphosphate
FFB	Fresh fruit bunch
IPTG	Isopropyl β-1-D-1-thiogalactopyranoside
MPOB	Malaysian Palm Oil Board
MPOC	Malaysian Palm Oil Congress
NCBI	National Center for Biotechnology Information
OPEFB	Oil palm empty fruit bunch
OTU	Operational Taxonomical unit
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
POME	Palm oil mill effluent
Qiime	Quantitative insight into microbial ecology
RDP	Ribosomal database project
RSPO	Rountable on Sustainable of palm oil
RT-PCR	Real-time polymerase chain reaction
SREP	Small renewable energy program
rRNA	ribosomal ribonucleic acid
SEM	Scanning electron microscope

sp.	species
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

1.1 Background

With the high market price of palm oil, there is an increasing demand for increasing the supply by the industry. This leads to opening up lands where virgin forests are cleared for plantation purposes especially by major palm oil producers like Malaysia and Indonesia. As a result, the clearing of forest will not only destroy the biodiversity but also endanger the existing microflora and fauna. In addition, open burning; the most common practice that has been used to clear the forest would cause air pollution, haze and climate change. The open burning method has been claimed as one of major contributor to global warming due to the release of greenhouse gases. In order to achieve sustainability in oil palm production, several implementation guidelines have been proposed in the Roundtable on Palm Oil Production (RSPO) which was formed in August 2003 amongst which are conservation of biodiversity, land management, soil and water conservation, zero burning replanting technique, waste treatment and recycling of mill by products (Ooi and Subramaniam, 2008).

Currently, oil palm is a major plantation crop in Malaysia and the country has become the second largest producer of palm oil with a total of 16 million tones or 43% of the total world supply (Shuit *et al*., 2009). In 2006, palm oil plantation area had been reported to be 4.2 million hectares and increased to 4.98 million hectares in 2011 (Shuit *et al*., 2009; Ng *et al*., 2012). The increase in palm oil plantation is due to the growing global demand of edible oil especially this oil. With the enhancement in palm oil production, the amount of residues generates from the industry also increases. The amount of residues produced in oil palm industry are mainly palm oil mill effluent (POME) followed by oil palm empty fruit bunch (OPEFB). POME contains high amount of organic matter with chemical oxygen demand (COD) of 30,000 – 100,000 mg/L and biological oxygen demand (BOD) of 11,000 – 44,000 mg/L and also considerable amounts of plant nutrients (Gobi and Vadivelu, 2013; Singh *et al*., 2011). In common practice, anaerobic digestion is the method generally used to treat POME. Through this process, the organic matter is converted into methane biogas by a complex consortium of microorganism. POME anaerobic sludge, the by-product of anaerobic digestion consists of high amount of nutrients and has the potential to be used as a fertilizer for oil palm tree. OPEFB is the largest component of lignocellulosic waste materials produced in palm oil mill. The most common practice to eliminate this waste is through direct burning for energy generation and natural composting for production of organic fertilizer in the oil palm plantation. However the major drawback of this practice is that it releases greenhouse gases and takes a long period to decompose.

In order to encourage the sustainability of palm oil production, several approaches are developed through the utilization of plantation by-products (Shuit *et al*., 2009; Singh *et al*., 2011; Chin *et al*., 2013; Gobi and Vadivellu, 2013). It has been reported by Schuchardt *et al*., (2008) that co-composting is one the suitable methods to full-fill those guidelines implemented during the RSPO discussion which focused on reducing and recycling of waste, to reduce pollution and emission of greenhouse gases. As

reported by Yoshizaki *et al*., (2013), the economic analysis of integrated biogas energy and compost production revealed that co-composting of EFB with POME anaerobic sludge is economically viable as compared to when the biogas energy and composting technology are installed individually. Therefore, co-composting could be one of the good solutions for sustainable management in oil palm production.

Production of compost from agro-industrial waste has currently been widely used by many researchers due the cheap and abundant resources. These wastes include organic matters which mainly consist of lignocellulose materials. One of the most common problems with agricultural waste composting is the decomposition of the recalcitrant compound such as lignocellulose complex structure which requires action of many different types of enzyme (Vargas-Garcia *et al*., 2007). This obstacle may affect the compost productivity and thus, increase the cost of operation, energy and time in the compost production.

In order to overcome this problem, the application of the right microbes will ensure that efficient composting can be achieved and nurtured within the composting materials to trigger, accelerate and sustain the composting process. This strategy could be effectively viable for enhancing the compost productivity and may play an important role in the production of compost at pilot scale. Previous studies have reported that the compost productivity can be increased with the addition of inocula (Kananam *et al*., 2011; Yeoh *et al*., 2011; Amira *et al*., 2011; Kavitha *et al*., 2013)

1.2 Problem statement

Although the inoculation of effective microorganisms has been used as a method to enhance the productivity of compost, but it is difficult to be proven commercially viable in large scale operation due to potential operating cost in preparation of inocula. Generally, microorganisms are added into the compost in order to increase the productivity by reducing the composting period. However, previous research has shown that the co-composting process can be enhanced without the addition of microorganism (Baharuddin *et al*, 2010a). This system relies on the indigenous microbes present in POME anaerobic sludge which are assumed to be responsible for enhancing the compost productivity. In this technology, the use of POME anaerobic sludge as composting materials has also been proven to be economically viable through the integrated system.

As composting is a microbiologically-mediated process, the microbial communi- ties have a large influence on the overall process and thus have a direct influence on the economic viability of the composting system. While numerous studies have examined the microbial communities in composting system (Mehta *et al*., 2014; Nakasaki *et al*., 2009; Ryckeboer *et al*., 2003b), the microbial diversity and their activities in the present enhanced composting process system have not been well defined, due to the limitations in approaches including time, cost, and capability of each methods for determining the microbial diversity. Although various molecular analysis methods have been used to elucidate the microbial diversity (Baharuddin *et al*., 2009; Guo *et al*., 2007; Liew *et al*., 2009; Lim *et al*., 2009; Mehta *et al*., 2014), the data set was often small and the sequences were less than 200 bp for each data set. In addition, the estimation of species richness for other composting systems is still less available or non-conclusive and the comparisons of the microbial diversity between previous results are always contradictory.

1.3 Scope of study

In this study, the prospect of microbial diversity of enhanced co-composting of OPEFB with POME anaerobic sludge was investigated. In order to initially examine the bacteria responsible for enhancing the production of compost, the first objective was performed based on the culture-dependent method through isolation and identification of cellulolytic and hemicellulolytic bacteria at different stages of the composting process. The goal of this study was to elucidate the cellulolytic and hemicellulolytic bacteria communities, which were assumed to be responsible for accelerating the production of compost.

The second objective was based on the examination of microbial communities through culture-independent approach. In this study, correlation between microbial community structure with the changes in the biochemical properties before, during and after composting process was determined. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was used to visualize the changes in microbial community structure between the communities present at the start of compost operation to the community that ultimately established itself when the POME anaerobic sludge was continuously added throughout the composting process. In this study, microbiota analysis of 16S rRNA clone library was used to elucidate the bacterial communities during the composting process. The correlation of bacteria community succession and structure between the visualized PCR- DGGE banding pattern and the species identified through 16S rRNA clone library was determined.

The third objective was designed to take advantage of the recent advances in DNA sequencing to investigate bacterial diversity in depth and more thoroughly than previously achievable. The goal of this objective was to investigate in depth the bacterial community in order to clarify specific species involved and supported the enhanced co-composting process using 454 pyrosequencing methods. With this study, a deeper and more thorough investigation of bacterial communities and structure was carried out to explore and extend the results observed in the second objective.

1.4 Objectives

The primary objective of this research was to reveal the microbial communities participating in the co-composting process using culture-based and molecular -based methods. The specific objectives were:

1. To isolate and characterize indigenous cellulolytic and hemicellulolytic bacteria during enhanced co-composting of oil palm empty fruit bunch with palm oil mill effluent anaerobic sludge

2. To identify and characterize the bacterial community structure and biochemical changes associated with co-composting of lignocellulose oil palm empty fruit bunch by DGGE and clone library methods.

3. To assess in-depth the bacterial community succession during enhanced rapid co-composting of OPEFB and palm oil mill effluent anaerobic sludge by high-throughput pyrosequencing.

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LIST OF PUBLICATIONS

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