UNIVERSITI PUTRA MALAYSIA

ASSESSMENT OF BACTERIAL COMMUNITY DIVERSITY IN OIL PALM PLANTATION FOR ISOLATION AND CHARACTERISATION OF LIGNINOLYTIC BACTERIA

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By

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Chair: Associate Professor Nor'Aini Abdul Rahman, PhD
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The oil palm industry in Malaysia generates huge amount of oil palm biomass every year. The abundant waste of oil palm lignocellulosic biomass is the most favourable renewable source for production of bio-based products. However, the key problem hindering the effective utilisation of oil palm biomass resource is lignin which resulted in low susceptibility of lignocellulose to undergo enzymatic hydrolysis. A biological pre-treatment method using bacteria was chosen in this study to remove lignin. Ligninolytic bacteria have wider tolerance of temperature, pH and oxygen limitations compared to fungi. It is also easier to handle for large-scale growth production and genetic manipulation. Assessment of bacterial community profile in oil palm plantation soils was conducted by using 16S rRNA gene clone library approach and biodiversity index analysis of operational taxonomic units (OTUs). 16S rRNA gene clone library analysis retrieved 351 clones from three selected samples of oil palm plantation soils. The most dominant groups were *Proteobacteria* followed by *Acidobacteria* and *Bacteroidetes*. Phylogenetic analysis of dominant bacterial clone sequences revealed many potential lignin degrading bacteria from genus *Stenotrophomonas*, *Acinetobacter*, *Flavobacterium* and *Sphingomonas*.

The Shannon's diversity index (H) shows high value from 3.24 to 3.42 indicating the high level of species richness in the soil samples. Isolation and identification of ligninolytic bacteria were executed from oil palm plantation soils samples. Bacterial isolates were screened using minimum salt media (MSM) with kraft lignin (KL) as sole carbon source and methylene blue as ligninolytic dye indicator. Furthermore, the ability of strains to grow on high tolerance concentrations of KL was observed. Three bacterial isolates; SHC1, SHC2 and SHC3 were selected for further study. The identification and

characterisation of selected strains were conducted using 16S rRNA gene sequence analysis and biochemical test by Biolog. The isolate SHC1 was identified as *Bacillus cereus* and SHC2 strain as *Ochrobactrum ciceri* with 99% sequence similarity, respectively. Meanwhile, strain SHC3 was identified as *Leucobacter komagatae* at 100% identity homology.

The ability of selected isolates to degrade lignin was examined through the production of ligninolytic enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase. The submerged fermentation experiments were conducted for 7 days using KL and oil palm empty fruit bunch (OPEFB) as substrate. *B. cereus* SHC1 was found to be the highest MnP and LiP enzyme producers among other selected bacteria in this study. The maximum MnP and LiP enzyme activities production by *B. cereus* SHC1 was at 2313.4 U/L (third day) and 209.30 U/L (fifth day), respectively. The influences of culture conditions such as pH and temperature conditions were carried out to maximise the production of ligninolytic enzymes by *B. cereus* SHC1. Therefore, the optimum pH and temperature of *B. cereus* SHC1 were pH 8 and 30°C, respectively.

Thus, the isolated ligninolytic bacteria from oil palm plantation soils have proven to produce high ligninolytic enzymes from oil palm lignin residue in OPEFB. This isolated ligninolytic bacteria may have specific advantages for the lignin degradation in oil palm biomass. Further studies on the mechanism of lignin degradation from this isolated ligninolytic bacteria could provide an effective ways for utilisation of oil palm biomass into bio-based products.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENILAIAN KEPELBAGAIAN KOMUNITI BAKTERIA DALAM TANAH LADANG KELAPA SAWIT UNTUK PEMENCILAN DAN PENCIRIAN BAKTERIA PENGURAI LIGNIN

Oleh

NOR HASHIMAH BINTI ABDUL RAHMAN

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Penanaman kepala sawit di Malaysia telah menghasilkan biojisim yang sangat banyak pada setiap tahun. Sisa biojisim yang berlebihan dari lignoselulosa kelapa sawit merupakan sumber yang paling bagus untuk digunakan bagi pengeluaran bio-produk. Walaubagaimanapun, terdapat satu masalah utama iaitu lignin yang menghalang usaha untuk mengunakan sumber biojisim kelapa sawit ini. Lignin telah melambatkan proses hidrolisis enzim untuk menguraikan lignoselulosa. Oleh itu, kajian mengenai kaedah pra-rawatan biologi untuk menguraikan lignin dengan menggunakan bakteria telah dilaksanakan. Bakteria ligninolitik mempunyai toleransi yang lebih tinggi terhadap suhu, pH dan kebatasan oksigen berbanding kulat. Ianya juga lebih mudah untuk dikendalikan untuk pengeluaran berskala besar dan manipulasi genetik. Analisis profil komuniti bakteria dalam tanah ladang kelapa sawit telah dilaksanakan dengan menggunakan kaedah pengklonan genetik 16S rRNA dan analisis indeks biodiversiti. Melalui kaedah tersebut, sebanyak 351 klon daripada tiga jenis sampel tanah ladang kelapa sawit telah dikumpulkan. Kumpulan yang paling dominan yang dijumpai di dalam sampel tanah adalah *Proteobacteria* disusuli dengan *Acidobacteria* dan *Bacteroidetes*. Keputusan analisis filogenetik 16S rRNA bagi kumpulan bakteria yang dominan telah menemukan beberapa bakteria yang berpotensi untuk menguraikan lignin dari genus *Stenotrophomonas*, *Acinetobacter*, *Flavobacterium* dan *Sphingomonas*.

Keputusan dari indeks kepelbagaian Shannon telah menunjukkan nilai di antara 3.24 hingga 3.42. Berdasarkan analisis tersebut, sampel tanah kelapa sawit menunjukkan kekayaan spesis yang tinggi. Pemencilan dan pengenalpastian bakteria telah dijalankan dengan menggunakan sampel tanah ladang kelapa sawit. Bakteria terpencil telah disaringkan dengan menggunakan media MSM yang ditambah dengan lignin kraf dan metilena biru. Pemerhatian mengenai keupayaan bakteria terpencil untuk hidup di atas media yang mempunyai kepekatan lignin kraft yang tinggi telah dilaksanakan. Hasil daripada eksperimen tersebut, tiga jenis bakteria terpencil telah dipilih iaitu bakteria SHC1, SHC2 dan SHC3. Analisa pengenalan dan pencirian bakteria terpilih telah

dilaksanakan dengan menggunakan kaedah jujukan genetik 16S rRNA dan ujian biokimia oleh Biolog. Bakteria SHC1 telah dikenal pasti sebagai *B. cereus* dan bakteria SHC2 pula dikenali sebagai *O. ciceri* dengan jumlah keputusan 99% persamaan identiti. Manakala, bakteria SHC3 telah dikenal pasti sebagai *L. komagatae* dengan jumlah keputusan 100% persamaan identiti.

Justeru itu, kajian mengenai keupayaan bakteria yang terpilih dalam menghasilkan enzim ligninolitik seperti mangan peroxidase (MnP), lignin peroxidase (LiP) dan laccase telah dijalankan. Eksperimen fermentasi yang menggunakan lignin kraf dan tandan kelapa sawit kosong sebagai substrat telah dijalankan selama 7 hari berturut-turut. Bakteria *B. cereus* SHC1 telah dapat menghasilkan enzim MnP dan LiP yang paling tinggi berbanding bakteria lain dalam kajian ini. Aktiviti enzim MnP dan LiP yang paling maksimum telah dicatatkan sebanyak 2313.4 U/L (hari ketiga) dan 209.30 U/L (hari kelima) oleh bakteria *B. cereus* SHC1. Pencarian suhu dan pH yang paling optima bagi bakteria *B. cereus* SHC1 telah dilaksanakan untuk memaksimumkan pengeluaran enzim ligninolitik. Oleh itu, suhu dan pH yang optima adalah 30°C dan pH 8.

Bakteria ligninolitik yang dipencilkan daripada tanah ladang kelapa sawit telah terbukti dapat menghasilkan enzim ligninolitik yang tinggi dari sisa kepala sawit OPEFB. Bakteria ligninolitik ini mungkin mempunyai kelebihan istimewa untuk menguraikan sisa lignin dalam biojisim kelapa sawit. Kajian pada masa akan datang untuk memahami mekanisme bagi menguraikan lignin kepada monomer aromatik boleh memberikan cara yang lebih berkesan untuk penggunaan biojisim kelapa sawit kepada produk berasaskan bio.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
$AlCl_3.6H_2O$	Aluminium chloride hexahydrate
BLAST	Basic Local Alignment Search Tool
bp	base pairs
$CaCI_2$	Calcium chloride
cm	Centimeter
$CoCl_2.6H_2O$	Cobalt (II) chloride hexahydrate
$CuSO_4.5H_2O$	Copper(II) sulphate pentahydrate
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide nucleoside triphosphate
Dc_{xy}	Distance of heterologous coverage
g/L	gram per litre
GC-MS	Gas Chromatography–Mass Spectrometry
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric acid
HCI	Hydrochloric acid
K	Potassium
K_2HPO_4	Dipotassium phosphate
kb	kilobase
Lac	Laccase
LB	Luria-Bertani
LiP	Lignin peroxidase

LLCs	Lignin-derived low molecular weight compounds
MF	Mesocarp fibres
mg/L	milligram per litre
$MgSO_4$	Magnesium sulphate
min	minute
ml/L	millilitre per litre
mM	millimolar
MnP	Manganese peroxidase
$MnSO_4.H_2O$	Manganese (II) sulphate monohydrate
MSM	Minimal salt media
MSM-KL	Minimal salt media-kraft lignin
N	Nitrogen
Na_2HPO_4	Disodium phosphate
Na_2MoO_4	Sodium molybdate
NaCl	Sodium chloride
NADH	reduced nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NH_4NO_3	Ammonium nitrate
nm	Wavelength
OD	optical density
OPEFB	oil palm empty fruit bunch
OTUs	Operational Taxonomic Units
P	Phosphorus
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction

PKS	palm kernel shells
pmol	picomolar
RDP	Ribosomal Database Project
RFLP	Restriction Fragment Length Polymorphism
rev/min	revolutions per minute
rpm	rotation per minute
rRNA	ribosomal ribonucleic acid
U/L	units per litre
UV-Vis	Ultraviolet-Visible spectrophotometer
V	Volts
v/v	volume for volume
VA	Vanillic acid
VL	Vanillin
$ZnCl_2$	Zinc chloride
λ	lambda

CHAPTER 1

INTRODUCTION

Oil palm plantations in Malaysia are occupying a very huge area which covers more than 4.69 million hectares of land across the country. Nearly 1 tonne of crude palm oil (CPO) is produced from 5.8 tonnes of fresh fruit bunch (FFB) (Moradi *et al*., 2012; Singh *et al*., 2010). The oil palm industry produces enormous amount of lignocellulosic biomass residue in Malaysia which included oil palm empty fruit bunches (OFEFB), palm kernel shells (PKS), mesocarp fibres (MF), oil palm fronds and trunks (Zainudin *et al*., 2014; Singh *et al*., 2010). By 2020, Malaysia is expected to produce about 100 million dry tonnes of lignocellulosic biomass (Agensi Inovasi Malaysia, 2013). Oil palm biomass residue was found to be highly potential to be industrialised as a raw material in related productions (Sulaiman *et al*., 2012). Therefore, Malaysian government has established The National Biomass Strategy 2020 to capitalise on its oil palm biomass by channelling it into higher value downstream uses such as biofuel and other industrial applications (Agensi Inovasi Malaysia, 2013). The concerted effort and specific plan to utilise oil palm lignocellulosic biomass can enhance Malaysia's economy in term of high income, great products and many job opportunities (Agensi Inovasi Malaysia, 2013).

However, lignin is the major barrier to the proficient conversion of lignocellulosic biomass to biofuel (Abdullah *et al*., 2011). It is the most structurally complex, possessing a high molecular weight and the most recalcitrant which consisted of various biologically stable linkages in lignocellulose that prevents penetration of degrading enzymes to obtain cellulose and hemicellulose (Pèrez *et al.*, 2002). Therefore, an ideal pre-treatment is important to degrade and remove lignin content from lignocellulosic oil palm biomass. The existing pre-treatment for the removal of lignin in enzymatic hydrolysis consisted of physical, chemical and biological approaches. The physical and chemical pre-treatment techniques such as hydrothermal, steam explosion, acid hydrolysis, aqueous ammonia, sodium hydroxide and oxidative delignification has been employed in the current oil palm industry (Zakaria *et al*., 2015; Chaturdevi and Verma, 2013; Brodeur *et al*., 2011; Ibrahim *et al*., 2011). However, these methods are high in cost and require extensive energy usages (Akhtar *et al*., 2015; Misson *et al*., 2009).

Thus, biological pre-treatment, including the use of fungi, actinomycetes, bacteria and enzymes appear to be more cost-effective and environmentally friendly. Most of biological pre-treatment of lignin degradation is extensively studied on white rot and brown rot fungi especially on *Phanerochaete chrysosporium* due to its high efficiency toward lignin degradation and powerful ligninolytic enzymes producer (Dashtban *et al*., 2010; Singh and Chen, 2008). Although fungi are the key contributors in degradation of lignin, bacteria exhibit versatile pathways to degrade aromatic substances from simple phenols to highly complex lignin (Shi *et al*., 2013). It has wider tolerance of temperature, pH and oxygen limitations compared to fungi especially for

decomposing lignin in aquatic ecosystem like pulp and paper industry (Chandra *et al*., 2007). Bacteria are easier to handle for efficient large scale growth of ligninoltyic enzymes production. Furthermore, ligninolytic enzymes from bacteria are much more amenable to genetic modification than fungi for modification of the metabolic pathways (Bugg *et al*., 2011). Several bacteria strains such as *Cupriavidus basilensis* B-8 (Shi *et al*., 2013), *Comamonas* sp. B-9 (Chen *et al*., 2012b), *Sphingomonas paucimobilis* SYK-6 (Masai *et al*., 1999) and *Streptomyces viridosporus* T7A (Ramachandra *et al*., 1988) have been reported to degrade lignin. Therefore, this study was conducted to discover lignin degrading bacteria from oil palm plantation soils that can produce ligninolytic enzymes.

The assessment of bacterial community diversity was conducted to find potential ligninolytic bacteria inside selected oil palm plantation soils. 16S rRNA gene clone library is a culture-independent approach that is proven to be an effective method for assessment of bacterial diversity in both cultivated and uncultivated bacteria from environmental samples (Izquierdo *et al*., 2010; Talia *et al*., 2012). It provides more comprehensive view of entire bacterial communities and dominant bacterial groups in soil environments (Wang *et al*., 2011). 16S rRNA gene clone library also more precise in term of faster identification, less cloning bias and more accurate than other culture-independent method such as denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) (Talia *et al*., 2012; Bruce *et al*., 2010). The exploration of biodiversity in oil palm soils could provide the basis for further studies on managing soil nutrients to increase soil quality for promoting oil palm plant growth. These bacterial profiles can also be used to strategies the beneficial bacteria inside soils which can be effective for utilisation of oil palm biomass.

Based on the discovery of potential lignin degrading bacteria from bacterial community profile in oil palm soils, the isolation of ligninolytic bacteria were carried out. Methylene blue (MB) dye was used as lignin peroxidase (LiP) enzyme indicator (Ferreira-Leitão *et al*., 2007) and kraft lignin (KL) as sole carbon source (Chandra *et al*., 2007) in isolation process. The identifications and characterisations of isolated bacteria were analysed using 16S rRNA gene sequencing analysis and biochemical test from Biolog. The production of ligninolytic enzymes by bacterial isolates were conducted using submerged fermentation process. The substrates used in submerged fermentation are KL and OPEFB. Ligninolytic enzymes activities of manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase were measured by ligninolytic enzyme assay. The optimal culture conditions of initial pH and temperature from the best selected bacteria isolate were analysed to enhance the production of ligninolytic enzymes.

In this research, the overall objective was to obtain bacterial community diversity profile and to isolate the high ligninolytic enzyme producers ofoil palm plantation soils. Hence, the specific objectives of this study were:

1) To assess bacterial community diversity profile of oil palm plantation soils by 16S rRNA gene clone library approach.

2) To isolate and characterise the ligninolytic bacteria from oil palm plantation soils.

3) To produce ligninolytic enzymes and determine the effects of initial pH and temperature of the selected bacteria isolate in submerged fermentation.

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