ISOLATION AND CHARACTERISATION OF MICROBIAL DNA FROM RAIN TREE BARKS

MUN CHEE ONN

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF BIOTECHNOLOGY (HONOURS)

FACULTY OF HEALTH AND LIFE SCIENCES
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI, MALAYSIA

2017

NON-PLAGIARISM DECLARATION

By this letter I declare that I have written this thesis completely by myself, and that I have used no other sources or resources than the ones mentioned.

I have indicated all quotes and citations that were literally taken from publications, or that were in close accordance with the meaning of those publications, as such. All sources and other resources used are stated in the references.

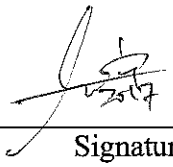
Moreover I have not handed in a thesis similar in contents elsewhere.

In case of proof that the thesis has not been constructed in accordance with this declaration, the Faculty of Health and Life Sciences has the right to consider the research thesis as a deliberate act that has been aimed at making correct judgment of the candidate's expertise, insights and skills impossible.

I acknowledge that the assessor of this item may, for the purpose of assessing this item,

- Reproduce this assessment item and provide a copy to another member of the University; and/or,
- Communicate a copy of this assessment item to a plagiarism checking service (which may then retain a copy of the assessment item on its database for the purpose of future plagiarism checking).

In case of plagiarism the examiner has the right to fail me and take action as prescribed by the rules regarding Academic Misconduct practiced by INTI International University.

MUN CHEE ONN	
Name	Signature
I13003128	10/04/2017
I.D.Number	Date

DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged, and completed under the supervision of Dr. Choong Chieh Wean.

MUN CHEE ONN	DR. CHOONG CHIEH WEAN
Student ID: I13003128	
DATE: 10 APRIL 2017	

ACKNOWLEDGEMENT

I would like to express my sincere appreciation to my honorable research supervisor, Dr. Choong Chieh Wean, for his constant guidance, wisdom, and unwavering patience throughout this project. He provided me critical advice and improvements, and it has been a greatly enriching experience to work under his authoritative guidance. Without his supervision and constant help this dissertation would not have been possible.

I am truly grateful to Dr. Geeta Selvarajah and Ms. Emily Quek Ming Poh, the examiners for their technical advice and indispensable comments on previous draft of the manuscript. I would like to express my gratitude to Faculty of Health and Life Sciences (FHLS) of INTI International University for providing modern equipment and reagents for the accomplishment of the project.

Much of the experimental work would not have been completed without the assistance of Aruna Ganesan, my friend and co-researcher. It is a proud privilege to offer my greatest appreciation to her tremendous assistance and dedication during the field work. Special thanks and appreciation are extended to Ms. Ho Li Sim from Sime Darby Technology Centre for her assistant with spectrophotometric analysis. Her efforts and collaborative attitude contributed to the success of this project.

Most importantly, I would like to thanks my family members for their support and encouragement towards the completion of my studies. Thank you.

ABSTRACT

Most microorganisms present in nature are uncultivable by standard techniques and functions of commensal bacteria remained largely uncovered. Metagenomics assessment to study microbial community on tree barks was not attempted from literature search. In this research, a metagenomic DNA extraction protocol by combining a series of lysis, extraction and purification methods was developed for high quality genomic DNA extraction of cultivable and uncultivable microorganisms present on local rain tree bark. Sodium dodecyl sulphate (SDS)-based extraction method with cetyltrimethyl ammonium bromide (CTAB) as strong DNA extraction buffer was carried out to extract metagenomes from two bark samples, namely orchid-colonised and non-orchid-colonised bark samples, followed by its qualitative (agarose gel electrophoresis) and quantitative (spectrophotometry) assessments. The quality of metagenomic DNA extracted was insufficient where the A_{260}/A_{280} ratio was in range from 1.47 to 1.81, and low A_{260}/A_{230} ratio obtained indicated the probability of contamination by humic substances and phenols. The metagenomic DNA was not suitable for cloning due to inability to be restriction digested by HindIII. The metagenomic DNA was accessed for 16S ribosomal RNA (rRNA) polymerase chain reaction (PCR) amplification and the results suggested that the purity of the metagenomics DNA was sufficient for PCR. Amplification results also indicated the presence of bacteria and fungi in high amount on the bark surface. The presence of archaea cannot be indicated due to unspecific amplification and no single band with the expected size was obtained. No difference between non-orchid and orchid-colonised bark samples in terms of amplified 16S rDNA concentration was found. Each purified PCR products had amplified rDNA in the range of 8.2 to 14.1 µg, which were sufficient for next generation sequencing (NGS) for rain tree bark metagenomics study, specifically on the ecology of microorganisms on rain tree bark.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent
°C	degree Celsius
×	multiplication (dilution factor)
×g	relative centrifugal force
µg	microgram
µL	microliter
A	absorbance
bp	basepair
CTAB	hexadecetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
EC	enzyme commission
EDTA	ethylenediaminetetraacetic acid
FRIM	Forest Research Institute Malaysia
g	gram
GB	gigabyte
h	hour
ITS	internal transcribed spacer
kbp	kilo basepair
M	molar concentration
$MgCl_2$	magnesium chloride
mg/mL	milligram per millilitre (mass per volume of solution)
min	minute
mL	millilitre
mm	millimetre

mM	millimolar
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
Na_2HPO_4	disodium phosphate
NaOCl	sodium acetate
NGS	next generation sequencing
ng	nanogram
nm	nanometre
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	hydrogen potential
PVPP	polyvinylpolypyrrolidone
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second
SDS	sodium dodecyl sulphate
TBE	tris-borate-EDTA buffer
TE	tris and EDTA buffer
Tris-HCl	tris hydrochloride
U	enzyme unit
UV	ultraviolet
V	voltage
v/v	volume/volume percent (volume concentration)
w/v	mass/volume percent (mass concentration)

CHAPTER 1

INTRODUCTION

Rain tree, *Samanea saman* is one of the icons of tropical countries originated from South America and was brought into Peninsular Malaysia in 1876 (Forest Research Institute Malaysia [FRIM], 2004a). In Malaysia, these impressive umbrella-shaped rain trees were commonly planted in urban areas, especially along roadsides and highways providing shade for pedestrians and cyclists. Tropical country such as Malaysia has hot and rainy climate throughout the year and epiphytic orchids such as *Bulbophyllum vaginatum*, *Dendrobium crumenatum* and *Grammatophyllum speciosum* often envelop and colonise old rain tree branches for sunlight, water and nutrients, from air and falling rain (FRIM, 2004b; Go et al., 2011). Germination of orchid seeds may depend on suitable symbiont fungi (Whigham et al., 2002). The presence of orchids enveloping rain tree bark provides evidence of the existence of commensal bacteria and fungi on the tree bark, and thus one of the reasons it was selected as one of the environmental samples in this research.

Microorganisms are microscopic living organisms which were first discovered by Antonie van Leeuwenhoek as early as 1674. The study of microorganisms is known as microbiology. Diverse microbial taxa, for instant bacteria, archaea, fungi, and viruses, are easily found in the environment. Microorganisms are valuable for their enzyme producing ability and those enzymes have greatly contributed to current industrial and medical biotechnology (Gurung, Ray, Bose & Rai, 2013). Studies had revealed the presence of pathogenic and commensal microorganism associated with plants (Muller & Ruppel, 2013). Local and global diversity of microbial taxa of bacteria, archaea, fungi, and viruses in different inhabiting soil samples have been highlighted (Fierer et al., 2007). A hypothesis is proposed that such diversity might be similar on rain tree bark environment, some of which may produce beneficial enzymes with specific characteristics.

As much as 99% of microorganisms in the nature are uncultivable using standard techniques in the laboratory (Schloss & Handelsman, 2005) and bacterial diversity in nature is always underestimated. Culture-independent method is essential to understand the microbial ecology, evolution, and gene diversity study and discovery. To overcome this, metagenomics approach for analysing microbes in the environment becomes one of the remarkable methods for microbial ecology, evolution, and gene diversity study and discovery over the last decade (Thomas, Gilbert & Meyer, 2012). Metagenomics bypasses the uncultivability and genomic diversity of entire communities of microorganisms contained within an environmental sample by using expression or sequencing approach (Schloss & Handelsman, 2005; Thomas et al., 2012). Understanding of such analyses is necessary for functional gene diversity discovery in microbial communities present in complex ecosystems (Thomas et al., 2012), with the potential to isolate new drugs and enzymes with different characteristics with industrial and pharmaceutical importance (Simon & Daniel, 2011).

In recent years, applications of metagenomics assessment to explore bacterial communities in plants (Rastogi, Tech, Coaker & Leveau, 2010; Muller & Ruppel, 2013), soil (Nam, Kim, Lee, Yoon & Kim, 2015; Hiraoka et al., 2016), air (Yooseph et al., 2013), water (Uyaguari-Diaz et al., 2016) and human (Tridico, Murray, Addison, Kirkbride & Bunce, 2014) using direct and indirect DNA extraction approaches were reported. Despite this, metagenomic DNA extraction and metagenomics to study microbial community on tree bark was not attempted from literature search. Bark microbiota represents a rather unexplored area of metagenomic research. In this research, metagenomic DNA from cultivable and uncultivable microorganisms on orchid-colonised and non-colonised local rain tree barks were extracted and characterised to access the possibility of using the DNA for cloning and PCR amplification. This could gauge whether the DNA could be used for next generation sequencing (NGS) for rain tree bark metagenomics study.

CHAPTER 2

LITERATURE REVIEW

2.1 MICROBIAL ECOSYSTEM OF RAIN TREE BARKS

Bark tissue is the outermost layer of stems and roots of woody plants. It protects the vascular tissue comprising xylem and phloem from harsh and fluctuating environmental conditions such as inconsistency in temperature and relative humidity. Bark tissue of a mature woody stem composes of periderm, cortex and secondary phloem. The periderm composes of cork, cork cambium and phelloderm. Several arthropods such as woodborers, scorpions and beetles inhabit within bark tissue for reproduction and development (Aflitto et al., 2014). Feeding and inhabitation of phloem tissues of the inner bark is known as 'phloeophagy' and bark tissue provides habitat not only for wildlife but also for vast microbial taxa (Aflitto et al., 2014).

The phloem is rich in sap, a fluid found in phloem sieve tube element. Sap transports with it major carbon sources, carbohydrates, soluble sugars, and water throughout a plant. Sucrose transport activity through phloem was reported to support the growth of heterotrophic tissues (Riesmeier, Willmitzer & Frommer, 1994). Bark tissue can be easily damaged by insects, animals and epiphytic orchids. The oozing out of sap to the surface of bark tissue created an ideal environment for fungal development (Novaes, Rodrigues & Lovato, 2009). Such nutrient availability allows pathogenic bacteria to grow and multiply at high density sometimes causing infection and eventually serious diseases to host plants (Fatima & Senthil-Kumar, 2015).

Study on symbiotic relationship between plant and microorganism showed that fixed nitrogen from bacteria was exchanged with carbon source from a plant to support bacterial growth and nitrogen demand of plant tissue (Knief, Delmotte & Vorholt, 2011). Orchid-fungus relationship could be parasitism and commensalism (Rasmussen & Rasmussen, 2008) but the function of commensal bacteria remained largely uncovered (Knief et al., 2011). It is imperative to identify these unknown species so that their beneficial contribution can be assessed.

Fierer et al. (2007) highlighted a relatively wide range of microbial taxa of bacteria, archaea, fungi and viruses inhabiting different soil samples. Such diversity is expected to be found in bark ecosystem. Fungi are major decomposer of plant residues and they supply nutrients for sustaining and stimulating plant growth (Smit et al., 1999). Understanding the structure and diversity of the fungal community on tree bark is still limited.

2.2 PLANT CELL WALL POLYSACHARIDES

Plant cell wall polysaccharides consist of large biopolymers of cellulose, hemicellulose, pectin and lignin (De Vries & Visser, 2001; Pedrolli, Monteiro, Gomes & Carmona, 2009), and majority of them are found in abundance (Butt, Tahir-Nadeem, Ahmad & Sultan, 2008). The ordered structural cellulose polymers enable rigidity of the plant cell wall (De Vries & Visser, 2001). The lignin provides a plant structural integrity and protection against hydrolysis of cellulose and hemicellulose (De Gonzalo, Colpa, Habib & Fraaije, 2016). Great economic prospect was elucidated regarding degradation of these abundant materials using hydrolytic enzymes such as pectinase and xylanase (Strauss, Jolly, Lambrechts & Van Rensburg, 2001; Butt et al., 2008).

Lignin is found within the approximate range of 15 to 40% in the plant biomass dry weight and formation of cell wall, especially the bark tissue, involves lignin. Cellulose and lignin comprise the biomass of a tree. Lignin is a highly cross-linked phenolic polymer formed by polymerisation of 4-hydroxyphenylpropanoid monomers (De Gonzalo et al., 2016; Vanholme, Demedts, Morreel, Ralph & Boerjan, 2010). Protection of cell wall polysaccharides against microbial degradation by lignin imparts decay resistance (Vanholme et al., 2010). Such phenomenon increases the challenge for bioconversion of plant biomass to pulp or biofuels (Vanholme et al., 2010). Recent study reviewed on enzymatic ability of peroxidase (EC 1.11.1.19) and laccases (EC 1.10.3.2) in bacteria and fungi secretion to degrade lignin and lignocellulose compound (De Gonzalo et al., 2016).