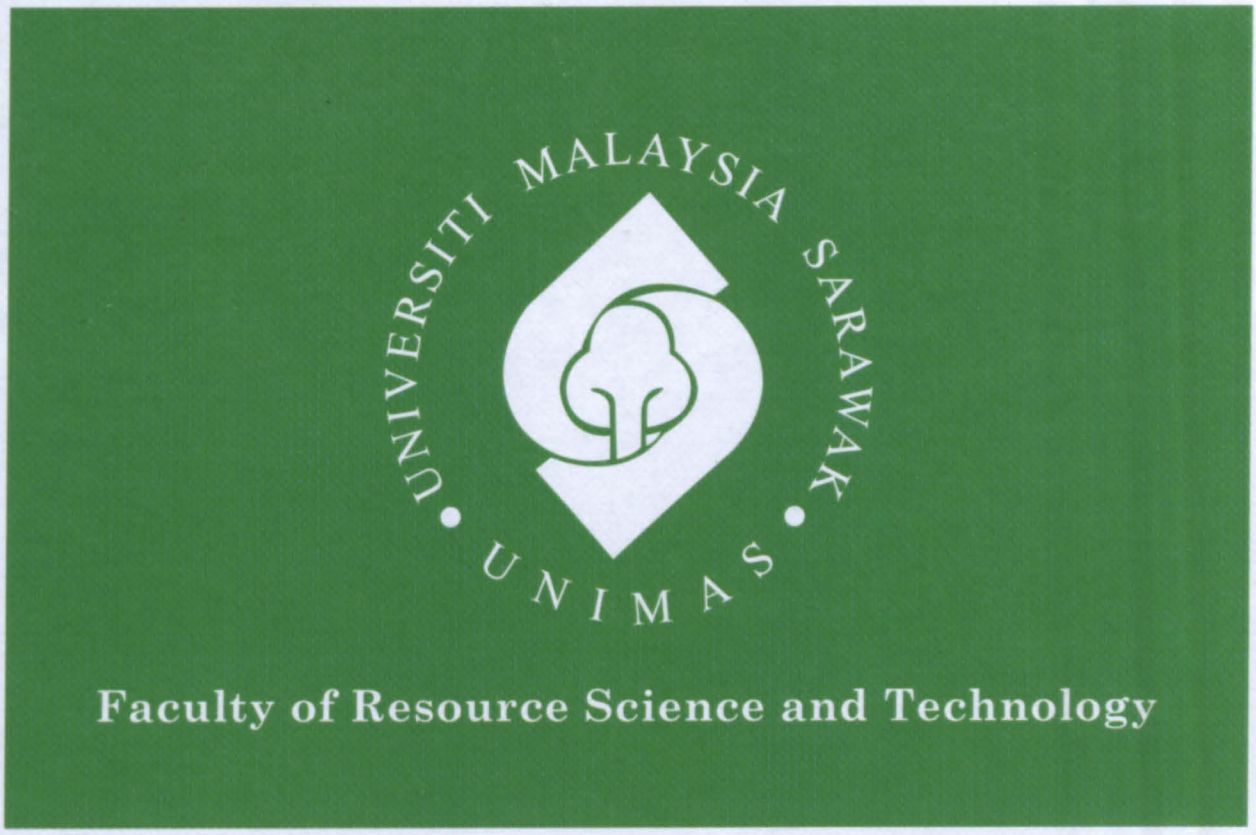

Isolation and Identification of Endophyte Fungal from Agarwood Chips

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(50515)

Bachelor of Science with Honours
(Resource Biotechnology)
2018

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The Final Year Project report is submitted in partial fulfillment of the requirement for the degree of Bachelor Science with Honours

(Resource Biotechnology)

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2018

ACKNOWLEDGEMENT

All praise to God upon His blessings, I was able to complete this research project. First and foremost, I would like to wish my deepest appreciation and gratitude to my supervisor Dr. Samuel Lihan for his valuable guidance, constructive criticism and encouragement during every stage of this project. Thank for all his support and advice throughout this research project.

I would also like to thank the staff members and the authorities of Universiti Malaysia Sarawak for providing the equipment and materials for us to complete this project. Without their help, this project would not be done smoothly.

A further gratitude from me to Mr Toh Seh Chiew, Ms Sabella binti Justin, Ms Syiqin Jamil, and Ms Habsah binti Hassan the postgraduate students of Virology Lab from Department of Molecular Biology and Mr. Iskandariah bin Abang Hassimah, the lab assistant of Virology Lab for their assistances, valuable concerns and advice to me while I am completing my research in Virology Lab. Besides, to all my laboratory members, Stanley Sait, Odealia Likong, Gedlyn Steffi, Vallerie Easter, Nurul Aainaa, and Ng Wing Woh who keep supporting and offering help during this project. Thank you for always correcting me and all the guidance throughout this project. With their help, I was able to handle the chemical and instruments correctly and in proper way.

Lastly, all thanks and highest gratitude to my parents and family members for all their motivation, financial support and encouragement since first I started this project. Not to forget, big thanks to anyone who have been helping directly or indirectly while I was doing this project. All of these people have made a great contribution to my success in completing this project.

Thank you and regards.

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TABLE OF CONTENTS

List of Abbreviations

Agarwood	Local name for subject study plant
Aquilaria	Scientific name for subject study plant
AG	Plate labelling for agarwood chip samples
cm	Centimeter
Et al.	Et alia (and others)
g	Gram
kg	Kilogram
m	Meter
ml	Milliliter
min	Minutes
PDA	Potato Dextrose Agar
Sp.	Species
Spp.	More than one species
°C	Degree Celsius
%	Percentage
>	More than

List of Figures

List of Tables

Isolation and Identification of Endophyte Fungal from Agarwood Chips

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ABSTRACT

Agarwood is a fragrant wood from non-timber product of the genus *Aquilaria*. There are very few literatures regarding the isolation and identification of endophyte fungal from agarwood. This study is conducted to isolate and identify the endophyte fungal from agarwood chips. The species of endophyte fungal isolated were identified based on morphological characteristics. Samples of the agarwood were obtained from Ba'kelalan, Sarawak. A total of 20 agarwood chips were placed on PDA, incubated at room temperature for 7 to 14 days to allow the growth of endophyte. The identification of the isolates were done through colony growth morphology and microscopic examination. Out of 8 isolates, seven species of endophyte fungal have been identified. Three isolates AG-1, AG-15(1) and AG-15(2) were identified as *Lasiodiplodia* sp., two isolates AG-5 and AG-19 were identified as *Aspergillus niger*, two isolates AG-11(1) and AG-11(2) were identified as *Aspergillus flavus* while others AG-2, AG-6, AG-10 (1), and AG-10 (2) were identified as *Penicillium* sp., *Mucor* sp., *Fusarium solani* and *Fusarium oxysporum* respectively. The endophyte fungal isolated in this study may have the potential to be used as inoculant for agarwood induction in enhancing resin production.

Key words: agarwood, endophyte, identification, morphology

ABSTRAK

Gaharu merupakan sejenis pokok yang mengeluarkan bau wangi yang diperolehi daripada produk bukan kayu dari genus Aquilaria. Hanya sedikit sahaja kesusasteraan mengenai pengasingan dan mengenal pasti kulat endofitik dari gaharu. Kajian ini dijalankan untuk mengasingkan dan mengenal pasti kulat endofitik dari cip gaharu. Spesies kulat endofitik yang diasingkan akan dikenalpasti mengikut ciri morfologi. Sampel gaharu diperoleh dari Ba'kelalan, Sarawak. Sebanyak 20 cip gaharu diletakkan dalam PDA, diinkubasi pada suhu bilik selama 7 hingga 14 hari untuk membolehkan pertumbuhan endofitik. Pengenal pastian jenis spesies dilakukan dengan melihat morfologi pertumbuhan koloni dan melalui penggunaan mikroskop. Daripada 8 isolat, lapan species kulat endofitik telah dikenalpasti. Tiga isolat AG-1, AG-15(1) dan AG-15(2) dikenalpasti sebagai Lasiodiplodia sp., dua isolat AG-5 dan AG-19 dikenalpasti sebagai Aspergillus niger, dua isolat AG-11(1) dan AG-11(2) dikenalpasti sebagai 'Aspergillus flavus' manakala yang lain AG-2, AG-6, AG-10 (1), dan AG-10 (2) masing-masing dikenalpasti sebagai 'Penicillium sp'., 'Mucor sp'., 'Fusarium solani' dan 'Fusarium oxysporum'. Kulat endofitik yang telah diasingkan dalam kajian ini mungkin mempunyai potensi untuk digunakan sebagai inokulan untuk induksi gaharu dalam meningkatkan pengeluaran resin.

Kata kunci: gaharu, endofitik, pengenalpastian, morfologi

1.0 INTRODUCTION

1.1 Research background

'Agarwood' is an occasional and resinous product extracted from *Aquilaria* and *Gyprinos* species in the family of Thymelaeaceae (Subasinghe, Hertiarachchi & Rathnamalala, 2012). The tree of agarwood is also known as '*gaharu*' or eaglewood. Agarwood is commonly used in the production of luxury perfume, fragrance and soap manufacture (Akter *et al.*, 2013). However, this agarwood tree is also classified as the threatened species as it has high market value.

Agarwood is also known due to its oleoresin production. According to Akter *et al.*, (2013), agarwood resin is obtained from pathological conditions of the wood of live trees in which it contain many aromatic substances and become a scented product. The oleoresin content in the agarwood determine the fragrant and economically value of the wood (Sangareswari *et al.*, 2016). The main active compounds found in agarwood was sesquiterpenes and other phenolic compound that lastly being expressed in the form of dark substances called resin.

According to Than (2013), fungal enter the trees when the agarwood trees is damaged either artificially or naturally. Therefore, due to the fungal attack, a dark aromatic resin will be produced by the tree which cause it to accumulate at the heartwood. Due to fungal infection, high resin in volatile organic compounds will produce in the agarwood trees which help in enhancing or retarding the growth of the fungal (Sangareswari *et al.*, 2016). Thus, fungal infection can increase resin production as a host response to damage due to fungal growth. *Aquilaria* trees are often infected by a variety of fungal. Different type of fungal accumulation in agarwood trees are *Phialophora parasitica, Torula* sp., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Cladosporium* sp., *Epicoccum granulatum,*

Cylimndrocladium, Sphaeropsis sp., *Botryodiplodia theobromae, Trichoderma* sp., *Phomopsis* sp., and *Cunninghamella echinulata* (Than, 2013).

1.2 Objectives

The objectives of this study are:

(a) To isolate the endophyte fungal from agarwood chips.

(b) To identify the species of endophyte fungal isolated from agarwood chips based on morphological characterization.

1.3 Problem statement

Agarwood has a very high commercial value due to its novel resin production. Agarwood resin is very important in the perfumery and fragrance industries. From the literature, there are not many studies related to agarwood especially in Sarawak. There are not many literature regarding the isolation and identification of endophytic fungi from agarwood can be found. Therefore, this study was conducted to isolate endophytic fungi from agarwood chips and identify them based on their morphological characteristics. An adult plant of agarwood takes a few year in order to grow and produce resin. Various endophyte fungal have been discovered to be involved with the agarwood formation (Akter *et al.*, 2013). Thus, through this study, the isolated endophytic fungi found may be the potential inoculum for the inoculation of agarwood in producing resin induction. Therefore, an alternative source of fungal inoculum is needed so that the resin can be produced in a certain period of time through the fungal infection. The factor such as plant species and endophyte fungal species involved affect the quality of the agarwood formation. By conduct this study, the potential species of endophytic fungi use as the inoculums for inoculation of agarwood to produce resin may be identified.

2.0 LITERATURE REVIEW

2.1 *Aquilaria*

2.1.1 Agarwood

Agarwood is a highly prized non-timber forest product in Southest Asia region. *Aquilaria* is the scientific name for agarwood or *'gaharu'*. According to Chua (2008), this species can be found at India, Sumatra, Singapore, Myanmar, Borneo, Peninsular, Malaysia and Phillipines. *Aquilaria* is a member of Thymelaeacea (Mohamed *et al.,* 2012). Thymelaeacea is a family of trees to perennial herbs or lianas. It has fibrous bark, leaves are typically alternate to each other. Thymelaeacea seeds are dispersed through either animal or wind-dispersed (Bates & Berry, 2010). The seed are pollinated by long-tongue insects or butterfly. According to Manning and Boatwright (2015), Thymelaecea is commonly found at Southern Africa. It usually scattered through the tropics. In their life cycle, *Aquilaria* produce seed after 7-9 years while some other species produce seed only once. Germination of the seeds take place between 16-23 days and variability of the seed is approximately 1 week. Germination is both epigeal and hypogeal type. *Aquilaria* is often found in the lowland dipterocarp and mixed dipterocarp forests. It grows up to 40 meters high and 60 cm in diameter.

Healthy wood of *Aquilaria* tree is white, soft and without scented resins (Liu *et al.,* 2013). *'Gaharu'* or agarwood is a dark resinous heartwood that forms in Aquilaria and Gyrinops trees when they become infected with a type of fungal. The formation of agarwood is mainly attributed to the defense reaction of trees either physically or chemically when they are exposed to biotic or abiotic factor (Sangareswari *et al.,* 2016). In a natural environment, agarwood forms only when affected by certain external factors such as lightning strike, animal grazing, and microbial invasion, typically around wounded or rotting parts of the trunk. A parasitic ascomycelous mold, *Phaeocremonium parasitica,* dermatitious (dark-

walled) fungus cause the formation of agarwood occurs in the trunk and roots of tree that have been infected by the fungus. According to Oller (2000), a very rich and dark resin is formed within the heartwood due to the fungus and decomposition process. Due to resin production, the mass and density of the affected wood will increase and change color from pale beige to dark brown or black. The unaffected wood of the tree is relatively light in color.

2.1.2 Taxonomy of agarwood

Table 1 shows the taxonomy of agarwood.

Table 1: Taxonomy of agarwood

Taxonomy	Description
Domain	Eukaryote
Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoloipsida
Order	Myrtales
Family	Thymelaeaceae
Genus	Aquilaria
Species	*A. microcarppa, A. hirta, A. beccariana and A. rostrata. A. malaccensis*

Source: IUCN Red List of Threatened Species (1998)

2.1.3 The uses of agarwood

Agarwood has a lot of uses such as industrial raw material for perfumes, fragrance, and cosmetics; material for religious ritual activities and for natural healing medicines (Mega *et al.*, 2015). Agarwood has aromatic properties when it gets burned or distilled. According to Blanchette *et al.* (2010), the high value of resin produced by the agarwood makes it useful in the production of incense for religious ceremonies, perfumes in the Arab world, ornamental materials and medicinal components in oriental medicine. Besides, in Japan, Korea and Taiwan, solid pieces of agarwood are highly known as 'natural art' (Persoon,

2007). For example, agarwood is modified into beautiful wooden sculptures, beads and bracelets.

According to study done by Akter *et al.* (2013), agarwood is commonly used in East Asian as a traditional medicine. This traditional medicine help in comfort pain, arrest vomiting by warming the stomach and to relieve asthma. For instance, agarwood is famous in treating stomach pains, in pregnancy, after delivery, fever, body pains, women disease and dropsy. The agarwood is also mixed with coconut oil as liniment in which it helps to treat rheumatism and other body pain. However, in the Middle East, agarwood is known as a symbol of status, wealth and hospitality (Mamat *et al.,* 2010). For example, when the agarwood (known as 'oudh' in Arabic word) is burned, it will produce a pleasant aroma in which this show the symbol of status, wealth and hospitality. The agarwood ingredients also used in the production of toiletry products such as soap and shampoo.

Agarwood is also known with its oil production (Subasinghe *et al.*, 2012). Most of the wood is processed and turned into oil which will be used for making perfumes and cosmetic products. The oil is collected through extraction and distillation method. This process will determine the amount and quality of the oil produced. According to Persoon (2007), the production of oil from agarwood have high demand in the Arab world. Besides, the agarwood chips are processed to obtain the powder form of it and used as raw materials for the production of incense. According to Akter *et al.* (2013), the agarwood powder and dust are used to make incense sticks or coils for indoor fragrance, and are used for religious purposes by Muslims, Buddhists and Hindus. Figure 1 shows the *Aquilaria trees.*

Figure 1: *Aquilaria* trees

Source: Agriculture Information (2016)

2.1.4 Agarwood Plantation and Distribution in Malaysia

According to Elias *et al.* (2017), 19 indigenous species of *Aquilaria* in Malaysia are able to produce agarwood. This consist of 13, 11, and 13 species in Peninsular Malaysia, Sabah and Sarawak respectively. These species are identified from 5 genera which consists of *Aquilaria* (7 species), *Gonystylus* (6 species), *Wikstroema* (4 species), *Aetoxylon* (1 species) and *Enkleia* (1 species). In the rainforests of Malaysia, 5 indigenous species that can be found are *A. malaccensis, A. microcarppa, A. hirta, A. beccariana and A. rostrata.* Among the 5 indigenous species, *A. malaccensis* is the most popular source of the agarwood. According to Sarawak Timber Industry Development Corporation (STIDC) (2008), agarwood is known as the most valuable tree species in Sarawak. The most famous species identified in Sarawak are *Aquilaria malaccensis, Aquilaria beccariana,* and *Aquilaria microcarpa.* Table 2 shows the natural distribution of *Aquilaria* in Malaysia.

Table 2: Natural distribution of *Aquilaria* in Malaysia

Scientific Name	Natural Distribution	Features of tree
Aquilaria malaccensis	Peninsula, Sabah, Sarawak	Big trunk, tall (up to 40m) circumstance is 80cm, shining leaves surface, pointy & oval like Spanish Cherry
Aquilaria hirta	East coast of Peninsula: (Terengganu, Pahang & Johor), Sabah & Sarawak	Small (15m) furry leave underside, fruit, branch and flower included
Aquilaria beccariana	Southern Johor, Pahang, Sabah & Sarawak	Medium height (20m) second vein leave visible, grayish green, not shiny
Aquilaria rostrata	Gunung Tahan, Pahang	Small (16m) second vein leave not visible
Aquilaria microcarpa	Johor, Sabah & Sarawak	Tall (> 36m), small heart shaped leave

Source: (Elias *et al.*, 2017)

2.1.5 Agarwood Grading System and Prices

Agarwood has a significant cultural and religious value, due to its various medicinal and commercial uses, such as in fragrances and cosmetics. According to Anon (1914), there are standard grading system for agarwood use in the Federated Malay States. This system is known as the ABC Agarwood Grading Systems. According to Wyn and Anak (2010), Grade A being brown / black wood; grade B is yellow wood with flecks of brown / black and grade C is white wood with some flecks of yellow/brown. The ABC grading system is determined by the physical appearance; however, the commercial price of agarwood depends on many factors including scent, shape, weight and colour. A low grade does not consistently represent a low price, since low resin content could be made up for by an attractive shape. However, a high grade may actually be sold very cheaply if its colour or texture is disguised by low-grade wood that has yet to be scraped off. The grade of the gaharu wood in Malaysia is also determined by their density, gaharu formation and unique scent (Azah *et al.*, 2013).

Today, the grading of the gaharu is based on individual viewpoint and experience. The prices of gaharu wood chips sold in Malaysia in kilograms (kg) is shown in Table 3.

Table 3: The prices of gaharu wood chips in Malaysia (MYR/kg)

Grade	Year					
	1914[a]	1992[b]	1999[c]	2003[d]	2004[e]	2005[f]
Double super	11.19					12 000
Super						10 000
A high	4.66	790	4000	5000	8000	8000
A low		530	3200	3000		4000
B high	2	530	2500	2000	6000	4000
B low		400	1800	1500	5000	3000
C1 high	1.02	260	800	1000	5000	2000
C1 low			400	500		
C2 high	0.51		80	100		1000
C2 low			40	50		
D high	0.24		30	40		250
D low	0.12		8	4		60

Source: Wyn and Anak (2010)

2.1.6 Resin and natural products formation in agarwood

The formation of resin and other products in agarwood is due to the plant defense mechanism caused by causal agents that consist of physical, chemical and biological factor. Formation of agarwood occurs in the roots and trunk of the trees which have been infected by parasites (Akter *et al.*, 2013). Therefore, the tree produces a resin high in volatile organic compounds that aid in suppressing or delaying the infection, in a process called tylosis. According to Mohamed *et al.* (2012), the main active compounds found in agarwood was

sesquiterpenes 2-2-phenylethyl hormone derivatives. Today, many efforts have been taken to induce the formation of resin in agarwood.

One of the method to induce agarwood resin formation is by using the traditional method (Mohamed *et al.,* 2012). This traditional method includes the process of wounding the trees with large knives and hammering nails into tree trunks. The resin produce through this traditional method is due to the interruption of the capillary vessel in the plant which include the phloem and other related tissue and cells. Due to the interruption, it secretes the resin and other bioactive compounds. The resin may be accumulated at the interrupted capillary vessel. However, in this era of modernization, the method has been revolutionized using certain chemicals and microorganisms, by the creation of modern inducement kits.

Figure 2: Traditional method (Process of wounding the trees)

Source: Green Harvest Worldwide (2016)

Figure 2 shows the process of wounding the trees using nails and hammer in inducing the agarwood resin formation.

According to Ye *et al.* (2016), the use of chemical method helps in increase the quality and quantity of agarwood resin production. This method is better than traditional method. Chemical injection involved the use of chemical substances such as salicylic acid,

jasmonic acid methyl ester and ethylene into the plant chips. It enhances the production on sesquiterpenes and increase the signaling mechanism to produce the resin.

Figure 3: Chemical method (Chemical injection into the agarwood trees)
Source: www.oud-selection.com (2016)

Figure 3 shows the injection of chemical substances into the agarwood trees which helps in inducing the formation of the resins.

The use of naturally occurring microorganism such as endophytic fungal is also can help in increase the production of resin in agarwood. According to Novriyanti *et al.* (2010), fungal are considered as one of the biological agents that can induce agarwood formation. Fungal infection using fungal inoculum is preferable since the formation of development is more progressive. According to Liu *et al.* (2013), whole-tree agarwood inducing technique is one of the simplest and efficient method to induce the agarwood resin induction. This technique involved the transfusion sets whereby the agarwood inducers are injected into the xylem parts of *Aquilaria* trees. As a result, agarwood resin can be produced in a short period of time. This is due to the water transportation, whereby the inducers are transported to the whole body of the tree, which causing the formation of wound in the tree.