IDENTIFICATION OF A MALAYSIAN TIGER MILK MUSHROOM SPECIMEN AND CHARACTERISATION OF ITS MYCELIAL (1,3)-β-D-GLUCAN

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FACULTY OF SCIENCE
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IDENTIFICATION OF A MALAYSIAN TIGER MILK MUSHROOM SPECIMEN AND CHARACTERISATION OF ITS MYCELIAL (1,3)-β-D-GLUCAN

ABSTRACT

The tiger milk mushroom (*Lignosus rhinocerus*) is an important medicinal mushroom and regarded as Malaysia national heritage. Its sclerotia (tuber) has been consumed by the indigenous community (*Orang Asli*) to treat illnesses such as asthma, fever, cough, cancer and other diseases. The mushroom is rare and difficult to find in nature and has frequently been confused with another mushroom of similar anatomy. Traditionally, the cultivation of the sclerotium takes a long time and propagation of its mycelial biomass and exopolysaccharides (EPS) in a stirred-tank bioreactor is an excellent alternative technique. The present study aimed to identify the wild-Malaysian tiger milk mushroom and characterise the extracted EPS from its mycelial culture in a stirred-tank bioreactor. The mushroom was morphologically identified based on its pileus, stipe and sclerotium. The extracted fungal DNA was sequenced and found to have 99% similar to *L. rhinocerus* strains CH31 and CH2. Phylogenetically, evolutionary distance (K_{nuc}) and plasmid-matching software (ApE) for sequences of matching fungal species verified that the isolate belonged to the *L. rhinocerus* species and warranted as *Lignosus rhinocerus* strain ABI (LRSA). The strain was cultured in a 13 L stirred-tank Labfors, Infors-HT bioreactor and its mycelial β-glucan (G) was extracted for compound characterisation. The structure of EPS was studied using Fourier-Transform Infrared Spectroscopy (FT-IR) and one-dimensional (1D) and two-dimensional (2D) Nuclear Magnetic Resonance (NMR). FT-IR spectroscopy showed that G exhibited a similar β-glycosidic structure to the standard (laminarin) and the presence of characteristic bands at 3277, 2919, 1638, 1545, 1400, 1078 and 896 cm^{-1} confirmed the similarities. ^{1}H and ^{13}C NMR, as well as 2D NMR:

homonuclear correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) spectra, were used for structural elucidation of the β-glucan and confirmed the extracted material as (1,3)-β-D-glucan. These findings may facilitate the development of G production in a high-scale bioreactor using LRSA.

Keywords: *Lignosus rhinocerus*; Tiger milk mushroom; 2D NMR; Bioreactor fermentation; (1,3)-β-D-glucan.

PENGENALPASTIAN SPESIMEN CENDAWAN SUSU HARIMAU MALAYSIA DAN PENCIRIAN MISELIUM (1,3)-β-D-GLUKANNYA

ABSTRAK

Cendawan susu harimau (*Lignosus rhinocerus*) merupakan cendawan perubatan yang dianggap sebagai warisan negara Malaysia. Sklerotianya (ubi) telah digunakan oleh masyarakat peribumi (Orang Asli) untuk merawat penyakit seperti asma, demam, batuk, barah dan pelbagai penyakit lain. Cendawan ini jarang dan sukar dijumpai di alam semulajadi dan sering dikelirukan dengan cendawan lain yang mempunyai anatomi yang sama. Secara tradisional, penanaman sklerotia mengambil masa yang lama dan pembiakan miselium biomas dan eksopolisakaridanya (EPS) di dalam bioreaktor tangki teraduk adalah satu teknik alternatif yang sangat baik. Kajian ini bertujuan untuk mengenal pasti cendawan susu harimau liar Malaysia dan mencirikan EPS yang diekstrak daripada miselium yang dikultur di dalam bioreaktor tangki teraduk. Cendawan ini telah dikenalpasti secara morfologi berdasarkan pileus, stip dan sklerotium. DNA kulat yang diekstrak dijujukkan dan didapati mempunyai 99% persamaan dengan strain *L. rhinocerus* CH31 dan CH2. Secara filogenetiknya, jarak evolusi (K_{nuc}) dan perisian padanan plasmid (ApE) untuk jujukan padanan spesies kulat mengesahkan pemencilan dimiliki oleh spesies *L. Rhinocerus* dan dijamin sebagai *Lignosus rhinocerus* strain ABI (LRSA). Strain tersebut telah dikultur di dalam 13 L bioreaktor tangki teraduk Labfors, Infors-HT dan miselium β-glukan (G) telah diekstrak untuk pencirian sebatian. Struktur eksopolisakarida telah dikaji menggunakan Spektroskopi Inframerah Transformasi Fourier (FT-IR) dan satu-dimensi (1D) dan dua-dimensi (2D) Resonans Magnet Nukleus (NMR). Spektroskopi FT-IR menunjukkan bahawa G mempamerkan struktur β-glikosida yang sama dengan bahan piawai (laminarin), dan kehadiran perincian jalur pada 3277, 2919, 1638, 1545, 1400, 1078, dan 896 cm^{-1} mengesahkan persamaan tersebut. ^{1}H dan

^{13}C NMR, juga 2D NMR: spektroskopi korelasi homonukleus (COSY), spektroskopi jumlah korelasi (TOCSY), heteronukleus gandaan kuantum koherens (HMQC), dan heteronukleus gandaan ikatan koherens (HMBC) spektra, telah digunakan untuk penentuan struktur β-glukan dan bahan ekstrak disahkan sebagai (1,3)-β-D-glukan. Penemuan ini mungkin boleh memberi kemudahan dalam perkembangan penghasilan G dalam bioreaktor berskala tinggi menggunakan LRSA.

Kata kunci: *Lignosus rhinocerus*; Cendawan susu harimau; 2D NMR; Penapaian bioreaktor; (1,3)-β-D-glukan.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

K_{nuc}	:	Evolutionary distance
rpm	:	Revolutions per minute
α	:	Alpha
ApE	:	A plasmid Editor software
β	:	Beta
CD_3OD	:	Tetradeuteromethanol
COSY	:	Homonuclear correlation spectroscopy
D_2O	:	Deuterated water
DNA		Deoxyribonucleic acid
DW	:	Dry weight
EDTA	:	Ethylenediaminetetraacetic acid
EPS	:	Exopolysaccharide
ENS	:	Endopolysaccharide
FT-IR	:	Fourier-Transform Infrared Spectroscopy
G	:	Mycelial β-glucan
gDNA	:	Genomic deoxyribonucleic acid
HMBC	:	Heteronuclear multiple bond coherence
HMQC	:	Heteronuclear multiple quantum coherence
HOD	:	Double water peak
ITS	:	Internal transcribed spacer
K_2HPO_4	:	Dipotassium hydrogen orthophosphate
KH_2PO_4	:	Potassium dihydrogen phosphate
MEGA	:	Molecular Evolutionary Genetics Analysis software
$MgSO_4$	:	Magnesium sulphate

MW	:	Molecular weight
NaCl	:	Sodium chloride
NJ	:	Neighbour-joining
NMR	:	Nuclear Magnetic Resonance
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar
PRESAT	:	Pre-saturation pulse sequence
SD	:	Standard deviation
SLF	:	Submerged liquid fermentation
SSF	:	Solid-state fermentation
TOCSY	:	Total correlation spectroscopy
Tris-HCl	:	Tris hydrochloride
TSP	:	Trimethylsilylpropanoic acid

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Tiger milk mushroom, scientifically known as *Lignosus rhinocerus (L. rhinocerus*), is classified within the kingdom of fungi in the Basidiomycota division of the Polyporaceae family (Abdullah et al., 2013; Johnathan et al., 2016). The mushroom's name derived from a folklore belief that it appeared on the ground where the milk of a tigress had dropped (Fung & Tan, 2019). The tiger milk mushroom can be found in southern regions of China, Sri Lanka, Thailand, Philippines, Indonesia, Papua New Guinea, Australia and Malaysia (Cui et al., 2011; Nunez & Ryvarden, 2001). However, wild tiger milk mushroom is generally expensive and difficult to source because its natural abundance is low and can only be found within areas with approximately a 5-km radius (Fung & Tan, 2019). Furthermore, it can take months to locate the fruiting body from its emerged pileus above ground with the underground sclerotium (Yap et al., 2014).

This mushroom comprises a pileus (cap), stipe (stem) and sclerotium (tuber) (Nallathamby et al., 2017). The sclerotium of *L. rhinocerus* is the most crucial part of the mushroom. It contains medicinal compounds with multiple properties for the treatment of diseases including cancer, cough, asthma, fever and other ailments (Lau et al., 2015). Previous researches have demonstrated that the sclerotia of *L. rhinocerus* possess immunomodulatory, anti-inflammatory, antioxidative, antiproliferative, antimicrobial, antiasthmatic and antiviral activities (Abdullah et al., 2011; Johnathan et al., 2016; Lee et al., 2014; Mohanarji et al., 2012; Wong et al., 2011; Yap et al., 2013). Of note, *L.rhinocerus* has frequently been confused with *Pleurotus tuber-regium* or *Lentinus tuber-regium* due to their similarities, including centrally stipitate basidiocarps (Nallathamby et al., 2017). The stipe alone might not be enough to characterise and

phylogenetically differentiate the genus morphologically. Thus, molecular phylogenetic tree analysis is required to evaluate and classify the mushroom (Sotome et al., 2008).

The cultivation of tiger milk mushroom using solid-state fermentation (SSF) limited by factors including a longer duration required for the development of the sclerotium and fruiting body and slow mycelial growth. Besides, SSF is challenging to monitor, control and scale-up (Fazenda et al., 2008). Thus, submerged liquid fermentation (SLF) has become the preferred method for mycelial fermentation and commercial applications. The polysaccharide produced in mushroom typically consists of exopolysaccharide (EPS) and endopolysaccharide (ENS) (Supramani, Ahmad, et al., 2019). Exopolysaccharide is excreted by the mycelium for survival under stress conditions while ENS is produced within the mycelium (Liu et al., 2010; Sathiyanarayanan et al., 2017). Exopolysaccharide is a high-molecular-weight polymer with a simple monosaccharide composition (Lai et al., 2014). The molecular weight (MW) distributions of some fungal EPS range from 13 kDa to 4.3×10^6 kDa (Mahapatra & Banerjee, 2013). For *L. rhinocerus*, the high MW of its sclerotia polysaccharide was found to be more than 30 kDa (Yap et al., 2018). However, variations in MW and sugar composition of fungal EPS are dependent on many factors including strains, culture conditions and medium compositions (Rabha et al., 2012). The primary polysaccharide found in the *L. rhinocerus* cell wall is β-glucan, which comprises 65%–90% (1,3)-β-D-glucan (Bowman & Free, 2006; Lau et al., 2013a). β-glucan is made up of D-glucose monomers linked by β-glycosidic bonds and containing only glucose as a structural constituent (Ruthes et al., 2013). The biological effects of β-glucan are dependent on its primary structure, conformation and MW (Tada et al., 2009). The biological activity of β-glucan in basidiomycetes has been shown to exert a positive effect on the immune systems of both humans and animals (Rop et al., 2009). Thus, SLF of mycelial basidiomycetes is more efficient, reliable, reproducible, flexible and easier to monitor when compared with SSF of fruiting bodies, especially for the production of

mycelial biomasses and their bioactive compounds, EPS and other exobiopolymers such as polysaccharide-protein complexes (Komura et al., 2010; Leung et al., 2009). However, the properties of EPS produced in SLF for *L. rhinocerus* are not well characterised.

The present study aimed to isolate wild-Malaysian tiger milk mushroom using morphological, polymerase chain reaction (PCR) molecular sequencing, phylogenetic Molecular Evolutionary Genetic Analysis (MEGA) software and plasmid matching software methods. The isolated strain was cultured in a controlled bioreactor to generate the mycelial biomass, which was used for the extraction of (1,3)-β-D-glucan (G). Subsequently, spectrophotometric characterisation using Fourier-Transform Infrared Spectroscopy (FT-IR) and one-dimensional (1D) and two-dimensional (2D) Nuclear Magnetic Resonance (NMR) and elucidation of the structural aspects of the isolated materials were performed. To our knowledge, this study is the first to report on characterised glucan from the mycelium of *L. rhinocerus* originating from a cultured bioreactor.

1.2 Problem Statement

Wild-Malaysian tiger milk mushroom is gaining more interest in developing its industrial processes due to its medicinal properties. The mushroom is very rare and difficult to find in nature and has frequently been confused with other mushrooms of similar anatomy. The morphological characterisation of the genus needs to be carried out in detail and the molecular phylogenetic tree analysis is required to evaluate and classify the mushroom thoroughly. Besides, recent research has found that the bioactive constituents of its mycelium produced using submerged culture fermentation may be an alternative to the sclerotium. However, the structural characterisation of polysaccharide from the *L. rhinocerus* mycelium has not completely elucidated. Hence, it is essential to unfold the configuration and structural feature of the polysaccharide to confirm further that mycelium is an excellent choice as an alternative source of the sclerotium of *L. rhinocerus*.

1.3 Research Objectives

The specific objectives of the present study were as follows:

1. To collect and characterise a wild-Malaysian tiger milk mushroom specimen using morphological and phylogenetic analysis;
2. To characterise (1,3)-β-D-glucan extracted from mycelial biomass cultivated in a stirred-tank bioreactor using Fourier-Transform Infrared Spectroscopy (FT-IR), one-dimensional (1D) and two-dimensional (2D) Nuclear Magnetic Resonance (NMR).

CHAPTER 2: LITERATURE REVIEW

2.1 Macrofungi

The kingdom of fungi belongs to the Eukarya domain in the phylogenetic tree of all living things. To date, there are about 5% out of 1.5 million species that have been documented and described which account approximately to 74,000 different species of fungi (Sundari et al., 2018). Fungi have distinctive characteristics which are eukaryotic, have filaments called hyphae that exhibit apical growth and finally form into a network called a mycelium. Fungi are heterotrophs which required pre-formed organic nutrients as energy sources and for cellular synthesis. Besides, fungi consist of a haploid genome, contain chitin and glucans as wall components and absorb nutrients through the cell wall and plasma membrane and produce spores (Deacon, 2005). They are unique and comprise of distinctive macroscopic and microscopic features which give rise to the definition of both macro- and microfungi. Macrofungi are filamentous fungi which produce visible fruiting bodies and can be held by hands. The example of macrofungi includes brackets, mushrooms, false-truffles, puffballs and many more (Chang & Miles, 1992). While microfungi are fungi that usually have a distinct filament and no fruiting bodies or macroscopic dimensions such as mould, mildews and rusts (Fazenda et al., 2008).

Most macrofungi belong to the phylum Ascomycota or Basidiomycota. However, only a few are from phylum Zygomycota (Mueller et al., 2007). Nowadays, the study of the characterisation of fungal diversity and their correlation are possible by using DNA metabarcoding to enhance further data acquisition in biodiversity research of fungal community (Geml et al., 2014). Nevertheless, it was proposed that the potential of the macrofungi are still inadequately understood and undervalued due to the complexity of their

life cycles which mainly concern their mycelial organisation and development when compared to the well-understood microfungi (Carlile et al., 2001).

2.2 Basidiomycota

About 30,000 species of macrofungi belonging to the phylum Basidiomycota have been defined and documented, which represents approximately 37% out of the total fungi in the world (Kirk et al., 2001). The Basidiomycota has been classified under subkingdom Dikarya and can be defined as filamentous fungi that are comprised of dikaryotic hyphae in which two genetically compatible nuclei present in each cell were individually divided into different compartments (Hibbett et al., 2007). The mycelium of Basidiomycota has been described as septate-reticulate because the hyphae of Basidiomycota usually encompasses septa which resembles internal cross-walls inside the hyphae that divides into numerous compartments. A multinucleated cell is present inside each compartment and the compartments linked by septal pores which work like a door. The flow of cytoplasm and nutrients through the mycelium cell that are separated by septa pores occurs during hyphal fusion. The existence of septa in higher fungi has an advantage that it is could continue growing new hyphae from the adjacent or through the damaged compartment. The damaged area does not affect the protoplasmic streaming through the mycelial network (Moore et al., 2008; Tegelaar & Wosten, 2017). Mycelium is the dominant form of the vegetative part of fungi and the dikaryotic state of mycelium can be maintained by the continuation of the growth of new hyphae by the formation of clamp connections. All fungi that produce clamp connections are members of the Basidiomycota, but not all Basidiomycota form clamp connections (Fazenda et al., 2008). **Figure 2.1** illustrates the characteristics of clamp connection and septa of hyphae of Basidiomycota.

At appropriate seasons and optimal conditions of the mycelium, fruiting bodies or mushrooms can be initiated if the mycelia are extensive and long-lived and sometimes it

could live for decades (Kauserud et al., 2010). The features of Basidiomycota such as the complex process of clamp connections, maintenance of the dikaryotic state and modest growth rates compared with other fungi, have implications for the production of mycelium in submerged liquid fermentation. Typically, Basidiomycota has lengthier fermentation processes due to the stated reasons as compared to other -microorganisms such as ascomycetes, bacteria, or yeasts(Fazenda et al., 2008).

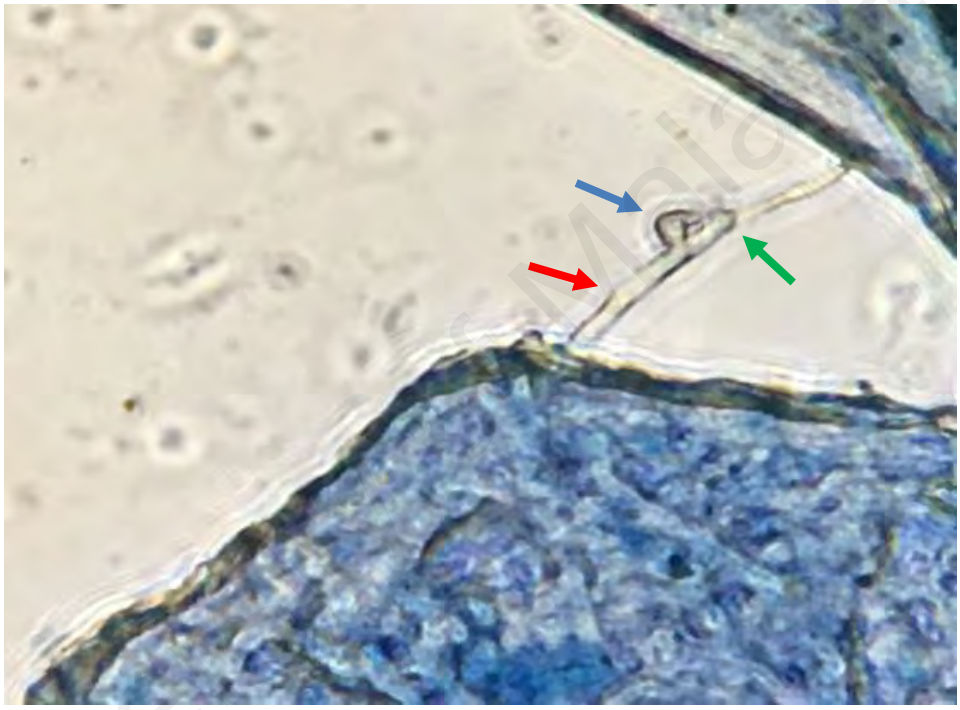

Figure 2.1: Characteristics clamp connection (blue arrow) and septa (green arrow) of Basidiomycota of *Lignosus rhinocerus* hyphae (red arrow) observed by a light microscope under 100X magnification (Figure is by author)

2.3 Habitat

Fungi are distributed worldwide and their habitats are very diverse which include both land and water. For instance, they can grow in soil or dead matter or live in symbiosis with another host such as plants, animals or other fungi. For Basidiomycota, they could grow in almost all land ecosystems, as well as aquatic habitats and prefer woody materials as their hosts or substrates, especially decaying cellulose-based plants (Hibbett et al., 2007). The most

productive time of sampling and harvesting of fungi can be determined based on local climate especially when the average temperature range between 23 °C and 32 °C and relative humidity is more than 82% (Jang & Hur, 2014). However, different fungal species have different phenologies or life cycles and also depend on other locations and heights. Sometimes, the highest richness of some fungal species only occurs for a short term and varies between years (Lodge et al., 2004).

2.4 Mushrooms

Mushrooms are macrofungi belonging to the phylum of Basidiomycota with a distinctive fruiting body (Chang & Miles, 1992). It was reported that there are approximately 140,000 mushroom species that exist on earth, but only 14,000 (10%) are fully described so far (Lindequist et al., 2005). The ethnomedicinal uses of mushrooms have benefited humankind since a long time ago back to the era of Neolithic (Poder, 2005). Several medicinal properties could be obtained from mushrooms either by direct consumption or extracting their bioactive compounds. Some of the most popular edible mushrooms like *Lentinus edodes*, *Pleurotus ostreatus*, *Flammulina velutipes*, *Chanterelle*, *Agaricus* spp., *Auricularia* spp. are consumed worldwide as food sources for the improvement of human health and their well-being (Samsudin & Abdullah, 2019). These mushrooms are known to be healthy and highly beneficial because they are low in both calories and fat but rich in proteins, minerals and dietary fibre (Manzi & Pizzoferrato, 2000). They also possess pharmacological properties such as immunomodulating, antioxidant, anticancer and anti-inflammatory activity (Hilszczańska, 2012). Examples of medicinal mushrooms include *Ganoderma lucidum, Wolfiporia cocos, Lignosus* sp., *Cookeinasulcipes* and *Schizophyllum commune* (Lee et al., 2009).

Most mushrooms grow on the ground and some of the fruiting bodies have an umbrella-shaped in which spores are produced under pileus or cap. The growth phases of mushrooms include two different types, namely vegetative and reproductive stages. The vegetative phase is when the hyphae are continually growing and branching to form a network called mycelia. Additionally, the reproductive step is the formation of the fruiting body by a process called fructification from underground mycelia. During the fructification process, the enzyme production is increased and respiration occurs during the mycelial growth (Sanchez, 2017). The hyphae absorb nutrients from substrates and penetrate to some extent. The cell walls of mushrooms are composed of β-D-glucans, proteins and chitin (Bowman & Free, 2006).

2.5 *Lignosus*

Lignosus is a genus of polypore fungi and so far it consists of up to eight described species, namely, *L.dimiticus, L.ekombitii, L.goetzii, L.rhinocerus, L.sacer, L.hainanensis, L.tigris and L.cameronensis* (Tan et al., 2013). It is a member of the Polyporaceae family belonging to the Basidiomycota in which their basidiocarps (fruiting bodies) are hard, especially the sclerotium (Choong et al., 2014). Typically, *Lignosus* species can be recognised by the large stipitate fruiting bodies that grow from the centre of the sclerotium. The fruiting bodies are white to ochraceous (pale yellow) in colour. Microscopically, the genus can be characterised by trimitic hyphal system which is binding, generative and skeletal type of hyphae that can be found in their contexts, sclerotium and stipe (Tan et al., 2013). The general shape of the spores is broadly ellipsoidal. Furthermore, all *Lignosus* species are known to sprout from underground sclerotium and not growing on a buried piece of wood or root (Choong et al., 2014; Ryvarden, 1975). *Lignosus* species have been broadly examined due to their potential therapeutic values.

2.5.1 *Lignosus rhinocerus*

Lignosus rhinocerus is a medicinal mushroom belonging to the Polyporaceae family, species of Basidiomycetes class fungi. **Table 2.1** shows the taxonomy classification of *L. rhinocerus*. The mushroom is distributed in the tropical rainforest in the regions of South China and Southeast Asia including Thailand, Malaysia, Indonesia and the Philippines (Fung & Tan, 2017).

Table 2.1: Taxonomic classification of *Lignosus rhinocerus*.

Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Polyporales
Family	Polyporaceae
Genus	*Lignosus*
Species	*Lignosus Rhinocerus*

In Malaysia, *L. rhinocerus* is known as tiger milk mushroom by the local community and has been hailed as a national treasure (Tan et al., 2012). There is a legend surrounding the naming of this mushroom. It is said that the mushroom gets its name from the fact that it grew from tigress' milk droplet (Tan et al., 2009). This mushroom has a very significant habit in blooming alone and can provide only one fruiting body at a time. This strain is classified as a rare tuber-like ancient medicinal mushroom (Nallathamby et al., 2017) because of its singularity of the sprouting habit where the interval between one fruiting body to another is spaced approximately 5-km (Fung & Tan, 2019). It has a complex macrostructure and composed by several kinds of hyphae on its flesh in which the binding of their hyphae during fruiting contributes to their hard surface and survival (Abdullah et al., 2013; Johnathan et al., 2016).

Figure 2.2 shows the wild-Malaysian tiger milk mushroom morphology which comprises of pileus (cap), stipe (stem) and sclerotium (tuber). Morphologically, *L. rhinocerus* can be distinctively characterised by its woody and formidable appearance with its umbrella-shaped fruting b in the centre emerging from a sclerotium. The pileus is concentrically zonate with an asymmetrical shape, glabrous and tea-brown in colour. The pileus and stipe of *L. rhinocerus* are woody in form while the sclerotium is a hard, dense resting body, consisting of a compact aggregated hyphal mass (Georgiou et al., 2006). The sclerotia are asexual, multicellular, specialised reproductive features holding food reserve materials for mushroom sustenance in unsuitable growth conditions (Cheung, 2013).

Figure 2.2: Wild-Malaysian tiger milk mushroom (Figure is by author)

The sclerotium of *L. rhinocerus* is the most important part of the mushroom in which it contains the medicinal properties to treat many ailments including cancers, cough, asthma, fever and other disorders (Chang & Lee, 2004; Lau et al., 2015). Natural tiger milk mushroom is tough to find due to its natural habitat deep in the rain forest and it takes a very long time even months to trace the mushroom by its fruiting body with its emerged cap from the underground tuber (Yap et al., 2014). Due to these limitations, the number of wild mushrooms is not enough for commercial exploitation and research purposes.

2.6 Artificial Cultivation of *Lignosus rhinocerus*

The supply of wild *L. rhinocerus* is limited by its rarity that is difficult to be discovered in nature and can only be found when the conditions are favourable for the fruiting bodies to arise from the dormant sclerotia hidden underground. The demand for the sclerotia is due to its medicinal properties and has increased tremendously. Thus, a suitable cultivation method is vital to be developed and established for *L. rhinocerus* for their commercial exploitation. There are two most common techniques to produce *L. rhinocerus*; solid-state fermentation (SSF) and submerged liquid fermentation (SLF).

Solid-state fermentation has been employed for decades to produce mushroom fruiting bodies. Additionally, research has been reported for the production of *L. rhinocerus* using SSF. However, SSF is time-consuming and labour-intensive as it requires almost one month for the formation of sclerotium and up to 12 months for the development of a fruiting body by using agro-industrial waste (Abdullah et al., 2013). Alternatively, SLF of mushrooms has significant potential and a promising substitute for the production of mushroom mycelium and metabolites.

2.7 Submerged Liquid Fermentation

Submerged culture fermentation is a process of growing microorganisms as a suspension in liquid media. These liquid media comprise of nutrients for the growth of the microorganisms. The method includes selecting a microorganism such as a bacterium or a fungus and placing it in a shake flask containing rich nutrient media for propagation of the cells. This process can be scaled up by using a larger vessel called fermenter or bioreactor.

Several studies have been reported on the production of mushroom mycelia by using submerged culture fermentation for producing biomass and EPS. There are several procedures to be performed for submerged culture fermentation. Firstly, the strain maintenance of the mycelial culture is usually performed using standard agar media such as potato dextrose agar (PDA) or malt extract agar (MEA). Next, the submerged fermentation is carried out in shaking flasks and scaling up in the stirred-tank bioreactor. The agitation is vital in SLF to make sure that there are enough oxygen supply and low shear stress on the hyphae for the cell separation to occur. The cultivation parameters which are time and temperature are strain-dependent and typically in the range between 3-18 days and 25–30 °C, respectively (Osinska-Jaroszuk et al., 2015). For media formulation of submerged culture fermentation, carbon and nitrogen sources are essential elements. The primary carbon source that is commonly used for mushroom EPS cultivation is glucose or dextrose in the range between 10-80 g/L (Elisashvili et al., 2009; Lai et al., 2014). The single-use or combination of nitrogen sources such as peptone, yeast extract, malt extract and ammonium nitrate is vital for higher yields of biomass and EPS (Jaros et al., 2018). Mineral sources include potassium phosphates and magnesium sulphate that are also used to ensure better production of mycelia. The content of media is usually adjusted to pH4-6 as pH is also one of the critical factors for the adequate

production of mushrooms (Fang & Zhong, 2002). **Figure 2.3** shows the submerged liquid fermentation (SLF) of mushroom cultivation in shake flasks.

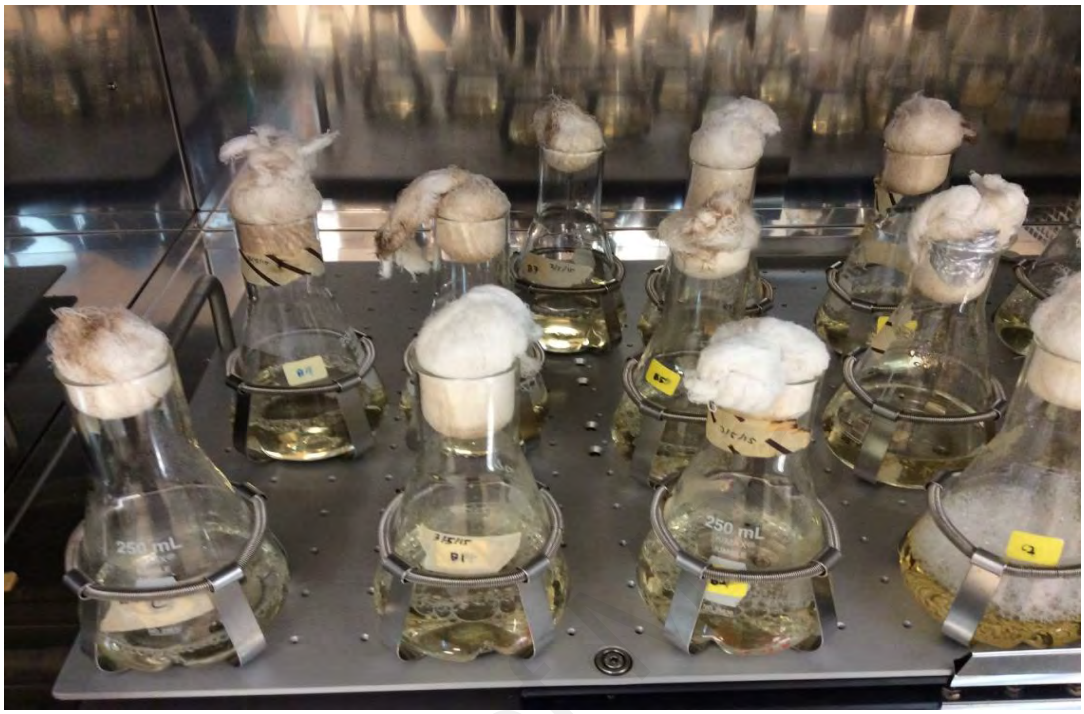

Figure 2.3: Submerged liquid fermentation of mushroom cultivation (Figure is by author)

2.7.1 Submerged Liquid Fermentation in Bioreactor

In mushroom cultivation, a stirred-tank bioreactor is often chosen to achieve the highest productivity. In stirred-tank fermenter, mixing is accomplished by mechanical agitation. Stirred-tank bioreactor is suitable to be used to produce mushroom mycelium because it offers highly flexible operating conditions, provides an efficient gas transfer to cells, readily available commercially and has been used to produce a variety of microorganisms (Spier et al., 2011). It was reported that the mycelial biomass produced in the bioreactor is higher as compared to shake flasks by two to three folds which show the usefulness of mushroom production in the stirred-tank bioreactor (Kim et al., 2002). **Figure 2.4** shows the cultivation of mushrooms in the stirred-tank bioreactor.

There are three standard modes of fermentation by which SLF takes place; batch, fed-batch and continuous fermentation (Li et al., 2011). The batch fermentation is the simplest mode of fermentation in which growth medium and microorganism are put into the fermenter at a time and removed after the fermentation is complete. The advantages of choosing batch mode are low capital cost, easy to set up, maintain and reduced risk of contamination. However, each new batch requires a new seed culture. For fed-batch fermentation, nutrients are added to the culture during the growth of the biomass to produce high cell densities and control their metabolic activities. The advantage of this technique is that it allows the operator to enhance the production process for improvement in industrial applications. However, this technology has a drawback, which it is difficult to determine the kinetic constants and to solve large numbers of equations. In continuous fermentation strategy, the process continues to run for an extended period with the addition of nutrients and harvesting the products at the same time at regular intervals. Altogether, submerged fermentation parameters such as agitation rate, temperature, dissolved oxygen rate and pH must be monitored to ensure successful production (Papagianni, 2004). Nevertheless, -several studies have been carried out to produce *L. rhinocerus* mycelial biomass and EPS using submerged culture fermentation (Lai et al., 2014; Lau et al., 2014; Ma & Yu, 2017; Rahman et al., 2012). The work done by Lai et al. (2014) reported that *L. rhinocerus* parameter conditions in a 1.5 L stirred-tank bioreactor were at 25 °C, pH6 and cultivated for 11 days which they achieved 6.4 g/L and 1.2 g/L biomass and EPS, respectively.

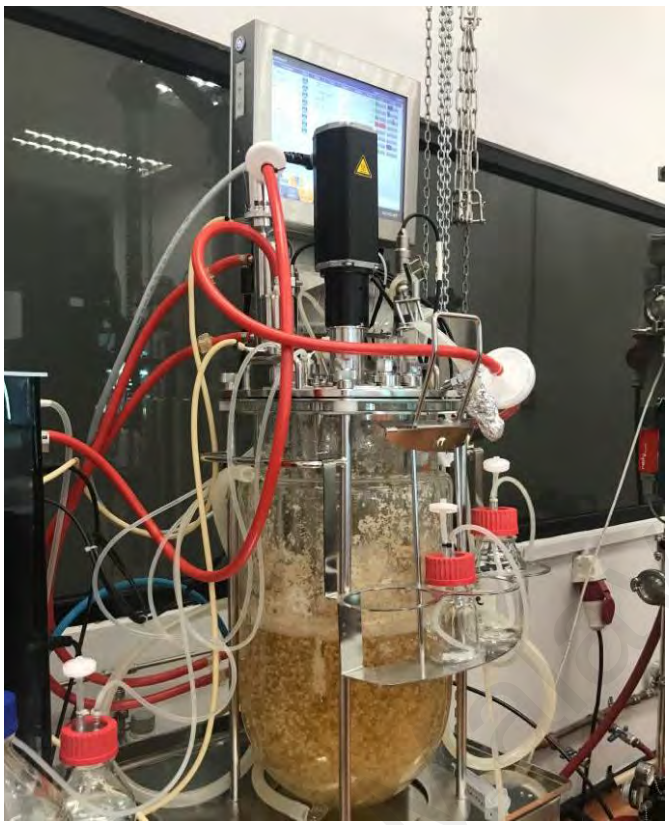

Figure 2.4: Mushroom cultivation in a 13 L stirred-tank Labfors, Infors-HT bioreactor (Figure is by author)

2.8 Functional and Medicinal Properties of *Lignosus rhinocerus*

Several studies have reported that the polysaccharide extracts from *L. rhinocerus* sclerotia possess pharmacological activities include immunomodulatory, anti-inflammatory, antiproliferative, antimicrobial and antioxidant properties (Lee et al., 2012; Lee et al., 2014; Mohanarji et al., 2012; Wong et al., 2011; Yap et al., 2013). Other than the fruiting bodies or sclerotia, the polysaccharides of mushroom can also be obtained from culture mycelia or culture broth that can give different chemical structures of compound extracts. It was reported that some polysaccharides produced in pure culture conditions were never found in fruiting bodies (Shnyreva et al., 2017). A study has proven that the sclerotium of *L. rhinocerus* was not superior to the mycelium and the mycelium using submerged culture fermentation could be an alternative in producing the health-benefits polysaccharides (Lau et al., 2013a). It was also reported that cultivated mycelium of *L. rhinocerus* is safe for

consumption (Lau et al., 2015). This is important because finding wild-growing *L. rhinocerus* for their sclerotia is very difficult due to their rarity (Lau et al., 2014). Cultivation using SSF has a drawback in terms of time-consuming for tuber and fruiting body development and slow mycelial growth. Hence, batch fermentation of SLF was chosen for this work due to its advantages such as lower cost, reduce contamination risk and shorter time.

2.8.1 Polysaccharides

Polysaccharides are long-chain polymers that comprised of more than ten monosaccharide units which are linked together by a glycosidic bond with a general chemical formula of $Cx(H_2O)y$. There are factors including different sequence of monomeric units and glycosidic linkages, different branching types, amorphous and soluble and insoluble in the water contribute to the various structures, properties and functions of the multiple polysaccharides (Mahapatra & Banerjee, 2013). Polysaccharides contribute to biological mechanisms in terms of prevention for infection, role as adhesion, immune response and signal transduction (Novak & Vetvicka, 2009).

There are three main groups of polysaccharides produced by mushrooms which are cell wall polysaccharide, ENS and EPS. The ENS is made within the mycelium cell, while EPS is excreted by the mycelium for survival under stress conditions and are either stay attached on the surface of the cells or are found in the culture broth (Liu et al., 2010; Sathiyanarayanan et al., 2017). Exopolysaccharide has more advantages as compared to ENS and cell wall polysaccharides because they can be produced on a larger scale, shorter time and easy to extract and purify (Mahapatra & Banerjee, 2013).

In Basidiomycota, the production of EPS is not growth-dependent and it is partly dissolved in the liquid media (Navarini et al., 2001). Exopolysaccharide molecules differ mainly according to size, biochemical and biological activities (Jaros et al., 2018). For

instance, EPS derived from mushrooms have various biological molecules structure with numerous physicochemical properties and the most-studied are β-glucans (Han et al., 2008).

2.8.2 β-glucans

β-glucans are homopolysaccharides that consist of D-glucose monomers connected by β-glycosidic linkages (Barton et al., 2016). Several forms of β-glucan are valuable in human nutrition as soluble fibre supplements and texturing agents. These compounds are commonly found in the cell wall of plants, yeasts, grains, fungi, bacteria and mushrooms. B-glucans derived from medicinal mushrooms are significant for their pharmacological activities and are the main bioactive compounds of mushrooms. Glucans from mushrooms come with different types of glycosidic bonds such as (1→3), (1→6)-β-glucans and (1→3)-α-glucans (Manzi & Pizzoferrato, 2000; Wasser, 2002). The linkage types in β-glucans depend- on the source of origin. For instance, the structure of cereal β-glucans is different from fungal β-glucans in which in cereal their D-glucosyl residues are linked to (1,3)-β- and (1,4)-β- and varied with the source of the β-glucans such as barley, oat and wheat (Wasser, 2011). Other types of β-glucans such as curdlan and cellulose have (1,3)-β-D-glucan and (1,4)-β-D-glucan, respectively (Zhu et al., 2016). Studies have reported that the significant structure of glucan in mushrooms are identified as a (1,3)-β-glucan backbone with one D-glucosyl residue linked (1,6)-β- to every third or fourth D-glucosyl unit in the (1,3)-β-glucan backbone (McCleary & Draga, 2016). The (1,3)-β-D-glucan presence in mushroom is identified as vital for their immunomodulatory and antitumour activities. Besides, they may form into a helical structure which gives gelling and thickening properties (Synytsya & Novak, 2013).

Like other polysaccharides, the medicinal properties of β-glucans are based on several features including structure, molecular weight and glycosidic linkage configuration.

These features are important and depend on a few factors such as temperature, fermentation time, agitation, pH, dissolved oxygen, carbon and nitrogen sources and many more (Gibbs et al., 2000). These growth parameters may affect the biological activities of the extracted compounds. To date, there is no consensus on the optimal growth and glucan-forming conditions for *L. rhinocerus*, as parameter manipulation may lead to an increase in the desired target either polysaccharide or biomass. In addition, the conformation of polysaccharide from the mycelium of *L. rhinocerus* has not been fully characterised until now. Therefore, it is necessary to explore the structural characteristics and chain conformation of *L. rhinocerus* mycelium.

CHAPTER 3: METHODOLOGY

3.1 Fungal Collection and Isolation

The tiger milk mushroom sample was obtained from a tropical forest in Lata Iskandar on July 7, 2018 (4.3244° N, 101.3249° E), Pahang, Malaysia. The wild mushroom was transported to the Bioreactor and Propagation Laboratory, Agro-Biotechnology Institute (ABI), Serdang, Malaysia for further analysis. Upon arrival from the site, the sclerotium was cultured on potato dextrose agar (PDA) media plates to assure viability and prevent contamination. The plates were subsequently incubated under dark conditions at 30 °C for ten days and then maintained at 4 °C (Wan-Mohtar et al., 2016). The strain was stored on PDA slants for preservation purposes.

3.2 Morphological Analysis

Morphological characterisation of fungal species was determined following the key characters described by Fung & Tan (2017). The morphological criteria include macroscopic features observation such as colour, shape, odour, size and dimension of cap, stem and sclerotia. Cultural characteristics such as colony appearances and mycelial textures on PDA plates were observed after 7-14 days of cultured.

3.3 Molecular Identification and Phylogenetic Tree Analysis

3.3.1 Deoxyribonucleic Acid Extraction of Mycelium

Fungal deoxyribonucleic acid (DNA) extraction was carried out using an improvised method (Liu et al., 2000). The mycelia of tiger milk mushroom (*L. rhinocerus* strain ABI) sample cultured on PDA plates was transferred aseptically using a sterile toothpick into lysis buffer (500 µL) in 1.5 mL Eppendorf tube [60 mM EDTA [pH 8.0], 400 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1% sodium dodecyl sulphate]. The tubes were then

placed at room temperature for 10 min, after which 150 µL of potassium acetate (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water, pH 4.8) was added and the samples were mixed briefly by vortex and then centrifuged at 11,000×*g* for 60 s. The supernatant was transferred into a new 1.5 mL Eppendorf tube, followed by adding isopropyl alcohol in a 1:1 ratio and the sample was mixed by inversion. The tube was centrifuged at 10,000×*g* for 120 s, the supernatant was removed and 70% ethanol (300 µL) was used to wash the DNA pellet before it was centrifuged again at 10,000×*g* for 60 s. The supernatant was removed and the resulting DNA pellet was air-dried.

3.3.2 Polymerase Chain Reaction Amplification

The resulting DNA pellet was dissolved in 1X Tris-EDTA (50 µL) to form a purified fungal gDNA. For fungal identification, two internal transcribed spacer (ITS) primers, which were ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTTGATATGC-3′, were used for the polymerase chain reaction (PCR). The modified PCR procedure of Liu et al. (2000) and Tamura et al. (2013) was followed using 25 µL of the reaction mixture. The PCR mixture included 0.5 pmol of both primers, 0.5 U DNA polymerase (Promega, Madison, USA), 200 µM of dNTP mix (Promega), PCR buffer (ThermoFisher Scientific, Waltham, USA) and nuclease-free water. The targeted fragments were amplified using an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) with the following procedure: 98 °C for 120 s, 25 cycles of 98 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min for the final extension.

3.3.3 Data Analysis

The resultant PCR products were separated on an agarose gel (1%) at 80 V for one hour. The PCR products purified using a PCR Purification Kit (Tiangen Biotech Co.,

China) and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Co., USA) for bidirectional sequencing. BLAST analysis was performed against sequences from the same or different species and matched to the ten closest species in the database.

3.3.4 Phylogenetic Tree and Species Verification

A phylogenetic tree diagram was established - based on the method described by Ab Kadir et al. (2016). Evolutionary distance (K_{nuc}) among sequences of the same fungal species was calculated using the neighbour-joining (NJ) method with MEGA software version X (Tamura et al., 2013). The closest K_{nuc} of the isolated commercial fungus was classified as the same species. For species verification, the sequence of the closest K_{nuc} species and the sequence of gDNA were compared for mismatches using A plasmid Editor (ApE) software. The results acquired were submitted to GenBank and analysed via BLAST search (http://blast.ncbi.nlm.nih.gov/) on the GenBank database.

3.4 Batch Fermentation

A seed culture for batch fermentation was prepared in a baffled shake flask. First, two mycelia agar squares (1 cm × 1 cm) were cut from the culture plate using a sterile scalpel in a laminar flow chamber and inoculated into 100 mL medium consisted of 4% (w/v) of glucose, 0.1% (w/v) of yeast extract, 0.2% (w/v) of peptone, 0.046% (w/v) of potassium dihydrogen phosphate (KH_2PO_4), 0.1% (w/v) of dipotassium hydrogen orthophosphate (K_2HPO_4) and 0.05% (w/v) of magnesium sulphate ($MgSO_4$). Flasks were placed on a rotary incubator shaker at 30 °C, 200 rpm speed for 11 days with an initial pH of 5.5.

The fermentation was performed in a 13 L stirred-tank bioreactor (Labfors, Infors H-T, Switzerland) with 10 L working volume. The seed culture (10% v/v) was inoculated into the medium. The medium composition was similar to the shake flask medium. The fermentation parameters were temperature (30 °C), aeration rate (1 vvm), agitation speed

(200 rpm), dissolved oxygen (30%–40%) and initial pH (5.5). The mycelium was cultured in the bioreactor for 14 days and the resulting mycelial pellets were isolated.

3.5 Analytical Methods

3.5.1 Extraction of Crude Exopolysaccharides

The EPS-derived β-glucan (G) was extracted based on the protocol of Supramani et al. (2019). The mycelia were filtered from the fermented culture broth and rinsed with distilled water. The filtrate obtained was added to 95% (v/v) ethanol at a ratio of 1:4 (g/mL) for the precipitation of macromolecules. The sample was then centrifuged at 10,000 rpm for 15 min. The supernatant was discarded and the pellet was dried in a food dehydrator at 35 °C until constant weight. The dried brown powder obtained was kept at room temperature for further analysis.

3.5.2 Measurement of Exopolysaccharides Content

The level of β-D-glucan in the sample was measured using a Megazyme kit (Cat. no. K-YBGL) based on the manufacturer's protocol. The Megazyme kit employed enzymatic hydrolysis for measurement of total glucan and acid hydrolysis for α-glucan. Approximately 100 mg of sample was used to determine the total glucan (including α-glucan, β-glucan, D-glucose in oligosaccharide, sucrose and free D-glucose).

Next, for the α-glucan (including phytoglycogen, starch, D-glucose in sucrose and free D-glucose) determination, another 100 mg of the sample was re-run accordingly. For glucose content analysis for both total and α-glucan; 0.1 mL of the supernatant was incubated with 3.0 mL of GOPOD reagent at 40 °C for 20 min. The D-glucose solution was used as standard (1 mg/mL) and the acetate buffer (200 mM, pH 5) as reagent blank; both were also incubated with GOPOD reagent. The glucose content analysis was carried out using UV-Visible spectrophotometer (Varian, Cary 50, USA) and the absorbance was measured at 510 nm. Finally, the percentage of β-D-glucan content [unit: g/100 g dry

weight (DW)] was calculated by subtracting the percentage of total glucan and α-D-glucan by using Equation (3.1) as follows:

$$\% \beta - D - glucan = \frac{Total\ \ glucan - \ \alpha - D - glucan}{Total\ glucan}\ X\ 100\% \quad (3.1)$$

3.6 Structural Characterisation of (1,3)-β-D-Glucan

3.6.1 Infrared Spectroscopy

An FT-IR spectrum of the G sample (5 mg) was taken using Agilent Cary 630 equipped with diamond ATR (Attenuated Total Reflectance) FT-IR spectrophotometer (Agilent Cary 630 equipped with diamond ATR). The wavelength was recorded in the range of 4000 – 650 cm^{-1} and analysed using a real-time Micro-Lab software.

3.6.2 Nuclear Magnetic Resonance Spectroscopy

The spectra of 1D NMR (^{1}H and ^{13}C) and 2D NMR (correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), Heteronuclear Multiple-Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC)) experiments were performed using 600-MHz NMR spectrometer (Agilent, USA). The G sample (10 mg) was mixed with 0.375 mL of tetradeuteromethanol (CD_3OD) and 0.375 mL buffer in D_2O (pH 6.0) containing TSP [0.1% (w/w)] in a 1.5-mL Eppendorf tube. The sample was vortexed for 60 s and sonicated at room temperature for 20 min before centrifuged at 10000 rpm for 10 min to obtain a clear supernatant. The supernatant (600 µL) was transferred to an NMR tube (5 mm, Norell, Sigma Aldrich, Canada) for NMR analysis. The comparison standard for ^{1}H NMR used for G was laminarin (*Laminaria digitata*, Sigma-Aldrich, Dorset, UK) and performed at 80 °C to generate a better separation of spectra. While all other experiments for G were conducted at 25 °C. A pre-saturation pulse sequence (PRESAT) experiment was performed to remove the large-signal for the HOD to determine ^{1}H NMR spectra.

3.7 Statistical Analysis

All experiments were performed in triplicate and the corresponding mean ± standard deviation (SD) was calculated using GraphPad Prism 5 software, version 5.0 and indicated as error bars. Error bars smaller than the symbol or icon size, do not appear in the figures.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Morphological Characteristics of Identified *Lignosus rhinocerus*

The different morphological stages of identified *L.rhinocerus* is shown in **Figure 4.1**. The diagram illustrates the basidiocarp of LRSA found in Lata Iskandar, Malaysia, with its underground sclerotium (**Fig. 4.1A**). Morphologically, *L. rhinocerus* can be distinctively characterised by its woody and rigid appearance with an umbrella-shaped stipitate basidiocarp that was emerging from a sclerotium. The pileus is concentrically zonate with an asymmetrical shape, glabrous and tea-brown in colour. The shape of the identified LRSA resembles *L. tigris* and *L. cameronensis* (Tan et al., 2013). However, *L. rhinocerus* has a smaller pore size (6–8 per mm) as compared to *L. tigris* (0.5–1 per mm) and *L. cameronensis* (2–3 per mm) (Fung & Tan, 2019). Furthermore, common *L. rhinocerus* has larger and distinctly broader ellipsoid basidiospores as compared to *L. tigris* and *L. cameronensis* (Yap et al., 2013).

The pileus and stipe of *L. rhinocerus* are woody in texture while the sclerotium is a hard, dense resting body, consisting of a compact aggregated hyphal mass (Georgiou et al., 2006). The sclerotia are asexual, multicellular, specialised reproductive features holding food reserve materials for mushroom sustenance in unsuitable growth conditions (Cheung, 2013). The sclerotia have an irregular spherical shape of about 4–5 cm in diameter (Abdullah et al., 2013). The surface of the sclerotium was rough and wrinkly and white to pale brown (**Fig. 4.1B**). The internal structure was white and powdery (**Fig. 4.1C**), which is in agreement with Abdullah et al. (2013).

Mycelia differ significantly from fruiting bodies. When the sclerotia of *L. rhinocerus* were cultured on PDA media, the mycelium texture appeared furry and cottony with a

colony colour of white to beige or light yellow, as shown in **Figure 4.1D**. As described by Yap et al. (2014), the expansion of the germ tube of mycelium eventually develops into a ring or spherical shape, referred to as "tiger eyes" (**Fig. 4.1D**). Mobilisation of the growing mycelium enables nutrient uptake through cross-linking of the expanding hyphae. Tiger milk mushroom can be cultured on other types of agar media, where it presents a similar appearance to that when grown on PDA media (Abdullah et al., 2013). However, under liquid cultivation, the mycelia grow in a stable pellet structure - as illustrated in **Figure 4.1E**.

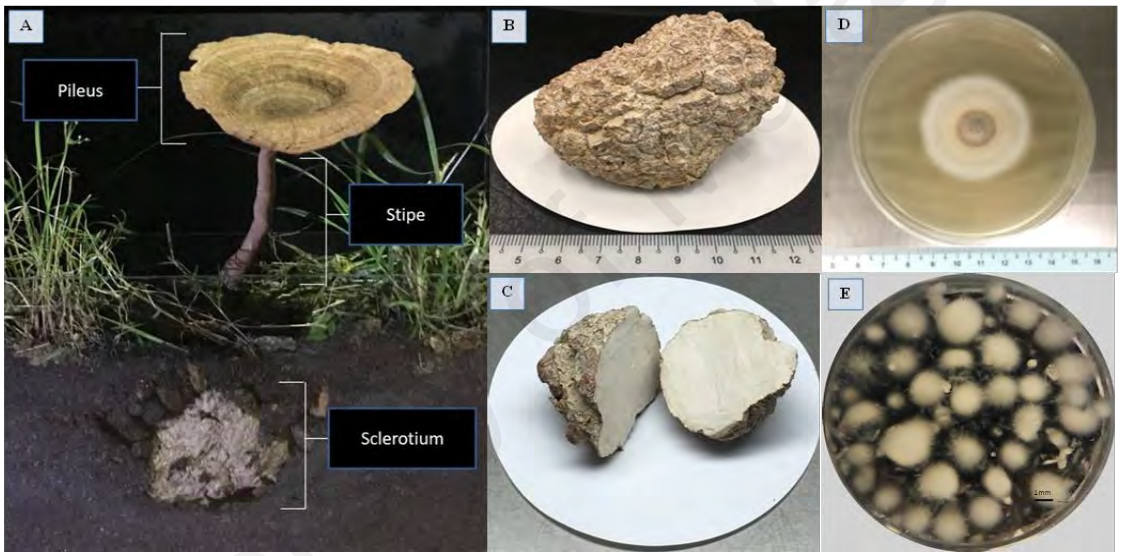

Figure 4.1: Samples of *L.rhinocerus* from different developmental stages. (A) Basidiocarp of *L.rhinocerus* isolated from a tropical forest in Lata Iskandar, Pahang, 4.3244° N, 101.3249° E, Malaysia; (B) Irregular shape sclerotium of *L. rhinocerus*; (C) Sliced sclerotium of *L.rhinocerus*; (D) Mycelium of *L.rhinocerus* strain ABI on PDA medium at day 5; (E) Mycelial pellet of *L.rhinocerus* strain ABI at day 14

4.2 Molecular Characteristics of Identified *Lignosus rhinocerus*

In the present study, LRSA was selected for the construction of a phylogenetic tree and biomolecular identification. Thus, the DNA of LRSA was extracted and PCR was performed to amplify the partial region of LRSA rDNA using ITS1 and ITS4 primers. As a result, an amplified PCR product of rRNA of approximately 515 bp in size was obtained

as shown in **Figure 4.2**. NCBI BLAST analysis was used to sequence and align the product with the top nine related species, with 14 Ganoderma species as the outgroup. The *L. rhinocerus* strain ABI sequence was found to be 99% similar to (FJ899143.1) *L. rhinocerus* strain CH31 and (FJ3380871.1) *L. rhinocerus* strain CH2 located at clade A (**Fig. 4.3**). Subsequently, the evolutionary distance (K_{nuc}) was calculated between sequences of similar fungi species using MEGA software version X for further investigation. A phylogenetic tree was constructed using the neighbour-joining (NJ) method from K_{nuc} data using the same software. The closest evolutionary distance K_{nuc} values indicated that the fungal isolate was closely related to *L. rhinocerus* (K_{nuc} 0.003) at clade A. The fungal species was verified by using a plasmid matching software (ApE) in which the isolate was found belong to the *L. rhinocerus* species (**Appendix A**), which aligned with *L. rhinocerus* strain CH2 and *L. rhinocerus* strain CH31 sequences.

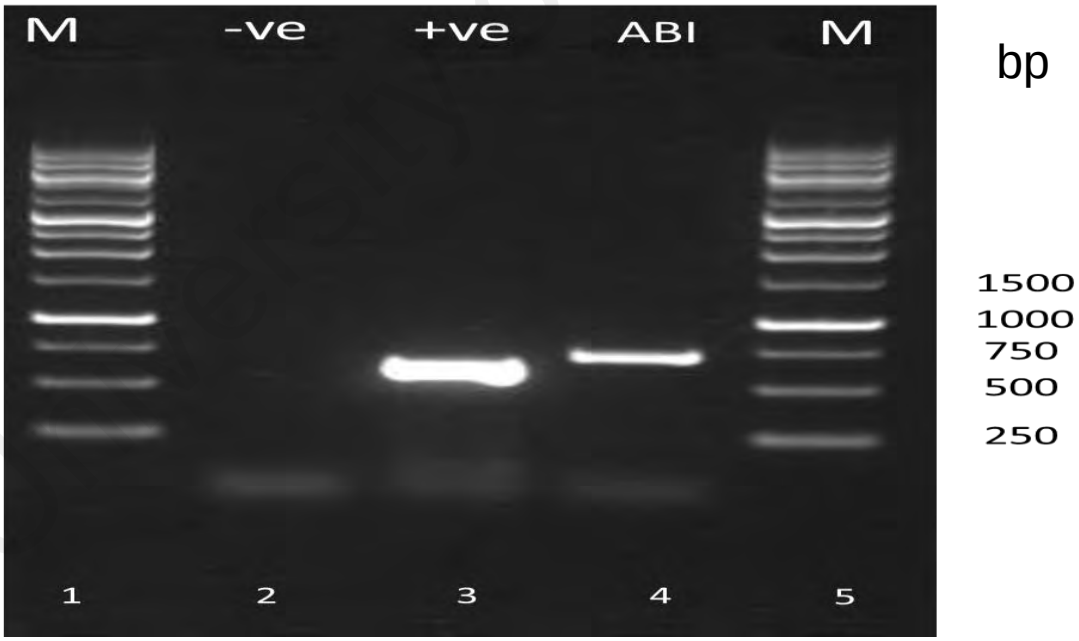

Figure 4.2: Ethidium bromide fluorescence image showing electrophoresis of *L.rhinocerus* strain ABI PCR product. The gel consists of 1% agarose and runs with 1% TE buffer at 80 V. Lanes 1 and 5 DNA marker; Lane 2 PCR negative control; Lane 3 positive control Fungal gDNA; Lane 4 PCR ABI product

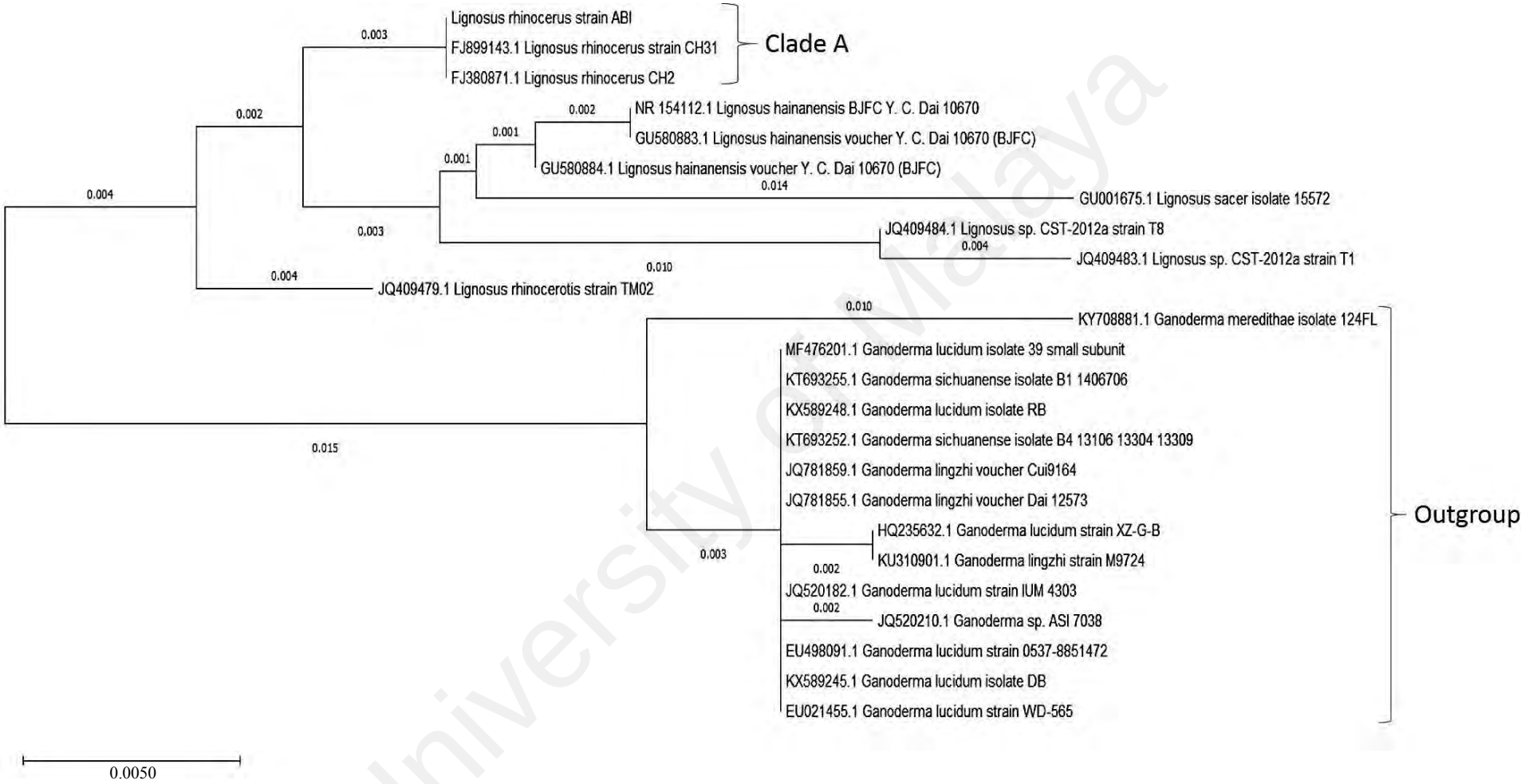

Figure 4.3: Neighbour-joining phylogenetic tree showing the relationships of *L.rhinocerus* strain ABI and top-nine BLAST species based on 18S rRNA gene sequences. The isolate located at clade A and evolutionary distance (K_{nuc}) was at 0.03 closest to *L.rhinocerus*. Bar 0.0050

4.3 β-glucan Content

In the present study, approximately 100 mg of sample was used to determine the total glucan (including α-glucan, β-glucan, D-glucose in oligosaccharide, sucrose and free D-glucose). The result for total glucan, α-glucan and β-D-glucan or D-glucose content from the mycelium of *L. rhinocerus* strain ABI was 40.49% (w/w), 4.19% (w/w) and 36.3% (w/w), respectively. Other polysaccharide monomers (63.7%, calculated by difference) identified in fungal EPS would potentially be hexoses (glucose, mannose, galactose, fucose, rhamnose, talose) but also pentoses (arabinose, ribose, xylose) (Jaros et al., 2018; Kim et al., 2000). As the structure is polysaccharide-protein complex, 59.51% would be the protein structure that holds the polysaccharide. According to McCleary & Draga (2016), medicinal mushrooms key active components were identified as 1,3:1,6-β-glucan, triterpenoids and ergosterol.

From this result, it appeared that β-D-glucan represented almost 90% of the total glucan content of the aqueous extracts of mycelium of LRSA and mostly composed of D-glucose as compared to the standard. It was reported by McCleary & Draga (2016) that prominent structural feature of mushroom species consists of (1,3)-β-glucan backbone with D-glucose as the monosaccharide. Previous studies on sclerotia of *L.rhinocerotis* (Cooke) Ryvarden (synonym: *Lignosus rhinocerus*) verified the polysaccharide extracted from the tuber consists of polysaccharide-protein complex and a glucan with glucose content of 98.6% (Lai et al., 2008). In addition, it was reported that 82 – 93% of total glucans of *L. rhinocerus* were β-D-glucan which composed of a majority of D-glucose (Lau et al., 2013a; 2013b). Together, LRSA mycelial-based glucan showed close comparability with tuber-based glucan.

4.4 Infrared Spectroscopy

FT-IR spectroscopy is a valuable technique for the structural characterisation of EPS (Prado et al., 2005) and can be used to analyse fungal glucans from various mushroom and fungal sources due to its sensitivity to the position and anomeric configuration of glycosidic linkages in glucans (Synytsya & Novak, 2014).

The FT-IR spectrum of compound G is presented in **Figure 4.4B**. By comparison with laminarin (**Fig. 4.4A**), the broad and intense absorption peak at 3277 cm^{-1} was found to represent the stretching vibration of a hydroxyl group (O-H), which indicated the presence of a polyhydroxilic compound (Liu et al., 2007). The absorption peak at 2919 cm^{-1} was assigned to the stretching vibration of C-H bonds, indicating a methylene group (CH_2) (Paulo et al., 2012). The strong absorption peak at 1638 cm^{-1} was assigned to the stretching frequency of C=O of the amide group. The spectra also showed the strong absorption at band 1638 cm^{-1} which depicted as the stretching vibration C=O of the amide group. Other major absorption bands identified at 1545 cm^{-1} and 1400 cm^{-1} were assigned to the amide group and -CH_3 stretching vibration, respectively (Ji et al., 2013). The absorption band at approximately 1078 cm^{-1} can be assigned to C-O stretching vibration and a pyranose ring (C-O-C) (Hu et al., 2017). The absorption peaks responsible for O-H, C-H and C-O groups were characteristic of the FT-IR absorption of polysaccharide (Wang & Zhang, 2009). The 'fingerprint' or anomeric region of carbohydrates is in the range of 850–1000 cm^{-1}, which verified the polysaccharide type and configuration for a specific absorption peak (Hu et al., 2017). The specific absorption peak at 896 cm^{-1} related to β configuration, which indicated that the compound G contained β-type glycosidic linkages as compared to the standard laminarin at 892 cm^{-1} (Wan-Mohtar et al., 2016).

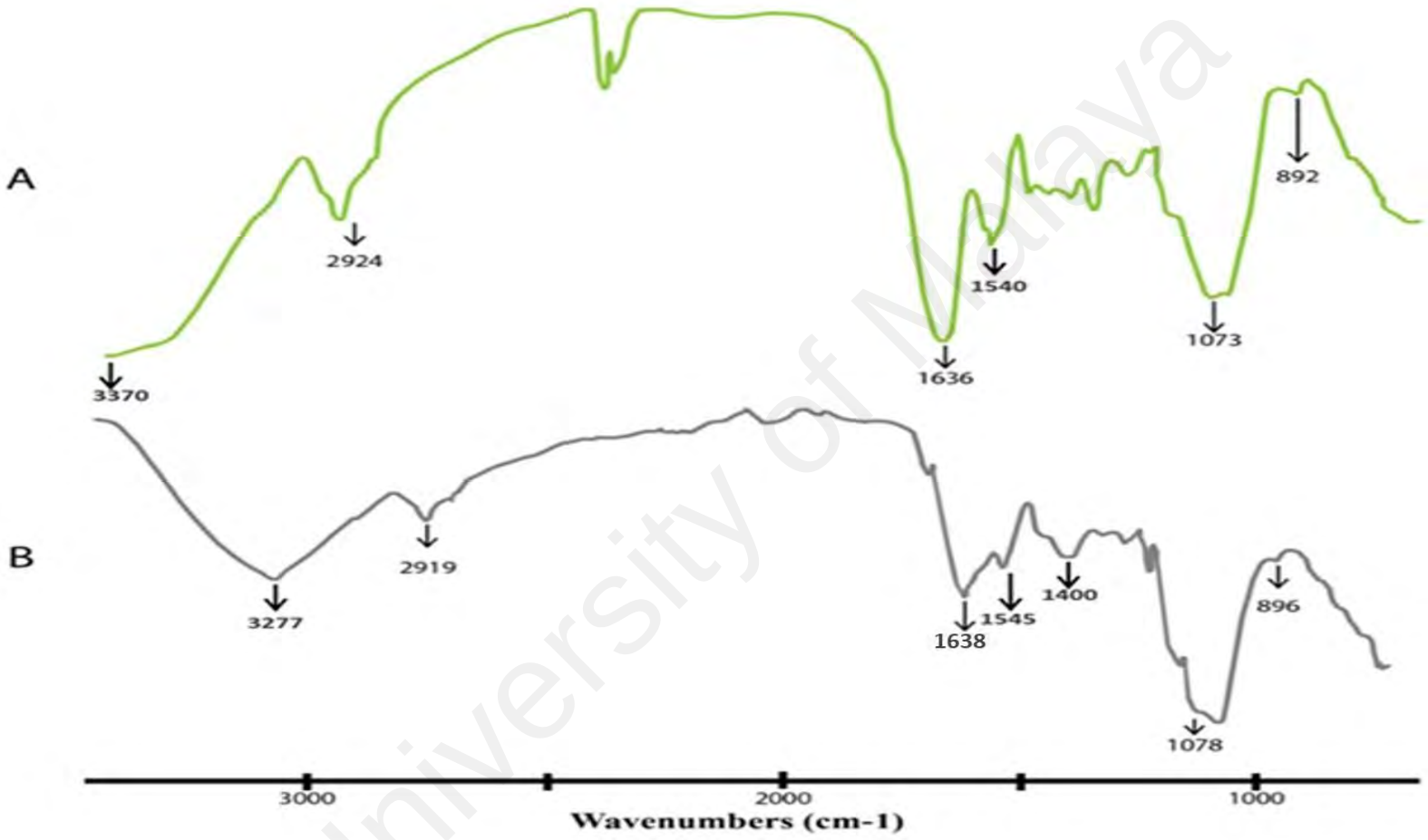

Figure 4.4: Comparison of β-glucan IR spectra. (A) Standard glucan from laminarin (*Laminaria digitata*); (B) Glucan (G) derived from *L.rhinocerus* strain ABI mycelium

4.5 Nuclear Magnetic Resonance Spectroscopy

4.5.1 One-Dimensional Nuclear Magnetic Resonance (^{1}H and ^{13}C) Analysis

The structure of G was further elucidated using NMR spectral analysis. **Figure 4.5** shows the ^{1}H NMR spectra using D_2O as a solvent. ^{1}H NMR spectra profiling presents a broad fingerprint of a biomolecule in solution (Pomin, 2012). Generally, for D-glucose or D-glucan analysis, the anomeric region appears at the most downfield region of the spectra, which is usually located between 4.0-6.0 ppm **(Fig. 4.5)** with most of the β-anomeric protons appear in the range of 4.0-5.0 ppm. In comparison, most of the α-anomeric protons appear in the range of 5.0-6.0 ppm (Hu et al., 2016). In the ^{1}H NMR spectra of EPS LRSA **(Fig. 4.5)**, a signal at 5.20 ppm with the coupling constant (*J*) of 3.73 Hz indicated the H1-α anomer. Another peak at 4.60 ppm exhibited a significantly larger *J* value (7.93 Hz) as expected for the H1-β anomer, which appeared upfield from the hydrogen of the α anomer. Another obvious signal was at 3.23 ppm, indicating the H2-β anomer (Gurst, 1991). The ^{1}H NMR spectra of LRSA had a similar pattern to the spectra obtained in previous studies for glucopyranose (Glcp), after achieving anomeric equilibrium in solution (Pomin, 2012). At equilibrium, the percentage of each anomer of D-glucopyranose was 64% for the β anomer and 36% for the α anomer, with the β-D-glucopyranose reported to be the more stable anomer (Gurst, 1991). This finding was comparable with the previous study by Ji et al. (2013) which analysed laminarin in the area of ^{1}H NMR spectrum of δ 4.49-5.5 ppm. Besides, it was also comparable with its closest counterpart *L.rhinocerotis* sclerotia (Hu et al., 2017) β-D-glucan with our *Lignosus rhinocerus* mycelium also showing β-D-glucan. Evaluation of the 'anomeric region' of ^{1}H NMR spectra in this study with those described previously specifies that they are of a similar pattern (Hu et al., 2017; Kim et al., 2000; Liu et al., 2014; Wagner et al., 2003). Thus, these spectra indicated that the glycosidic bonds in glucan (**Fig. 4.5**) was β-type. In addition, in the ^{1}H NMR spectra of compound G of LRSA, the chemical

shifts at 4.6, 3.9 and 3.8 ppm were assigned to the hydroxyl groups OH-2, OH-4 and OH-6, respectively (Supramani, Jailani, et al., 2019; Wagner et al., 2003).

Multiple compressed, overlapping and unresolved proton signals in the ^{1}H NMR spectra indicate that the application of other nuclei such as ^{13}C is vital for the characterisation of polysaccharides. **Figure 4.6** illustrates the ^{13}C NMR spectrum obtained for G compound of LRSA. It can be observed that the pattern obtained for ^{13}C NMR from this study has a similar pattern to the ^{13}C NMR achieved for D-glucose with the anomeric region is between 90-110 ppm and the β-anomer appeared the most downfield in the spectra (Brown et al., 2018; Gurst, 1991; Kim et al., 2000; Pomin, 2012). This indicated that glucan has β-configuration of D-glucosyl residues at peak 98.80 ppm for C1 (**Fig. 4.6**). The ^{13}C spectrum (**Fig. 4.6**) revealed the shifts in carbons (C1–C6) characteristic of a β-glucan: 98.8 ppm (C1), 77.1 ppm (C2), 78.8 ppm (C3), 74.2 ppm (C4), 72.5 ppm (C5) and 63.6 ppm (C6), which is in agreement with previous studies (Gonzaga et al., 2013; Liu et al., 2014; Pomin, 2012). According to Emwas et al. (2018), the quality of spectra and their subsequent interpretation in NMR are influenced by multiple factors such as sample characteristics, NMR setup and processing parameters. In the present study, the ^{13}C NMR spectrum of G showed that the chemical shifts of C1 and C3 had moved downfield as compared to those of glucose, indicating that the glycosidic bond in G was of the β-(1→3) type, which is in agreement with Ji et al. (2013).

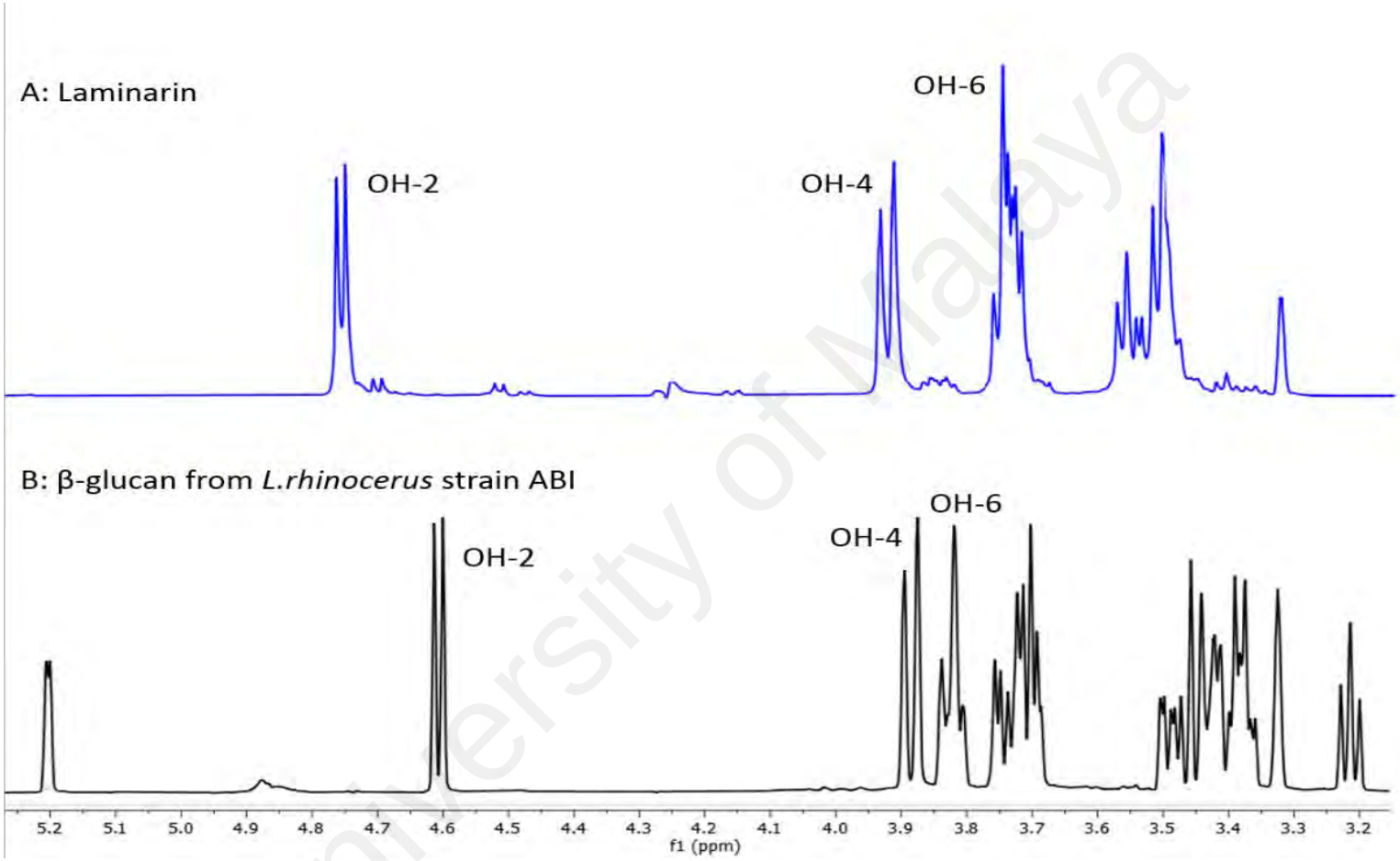

Figure 4.5: ^{1}H NMR spectra of (1,3)-β-D-glucan. (A) Standard glucan from laminarin (*Laminaria digitata*) in D_20 at 80 °C; (B) Glucan (G) derived from batch cultures of *L.rhinocerus* strain ABI in D_20 at 25 °C

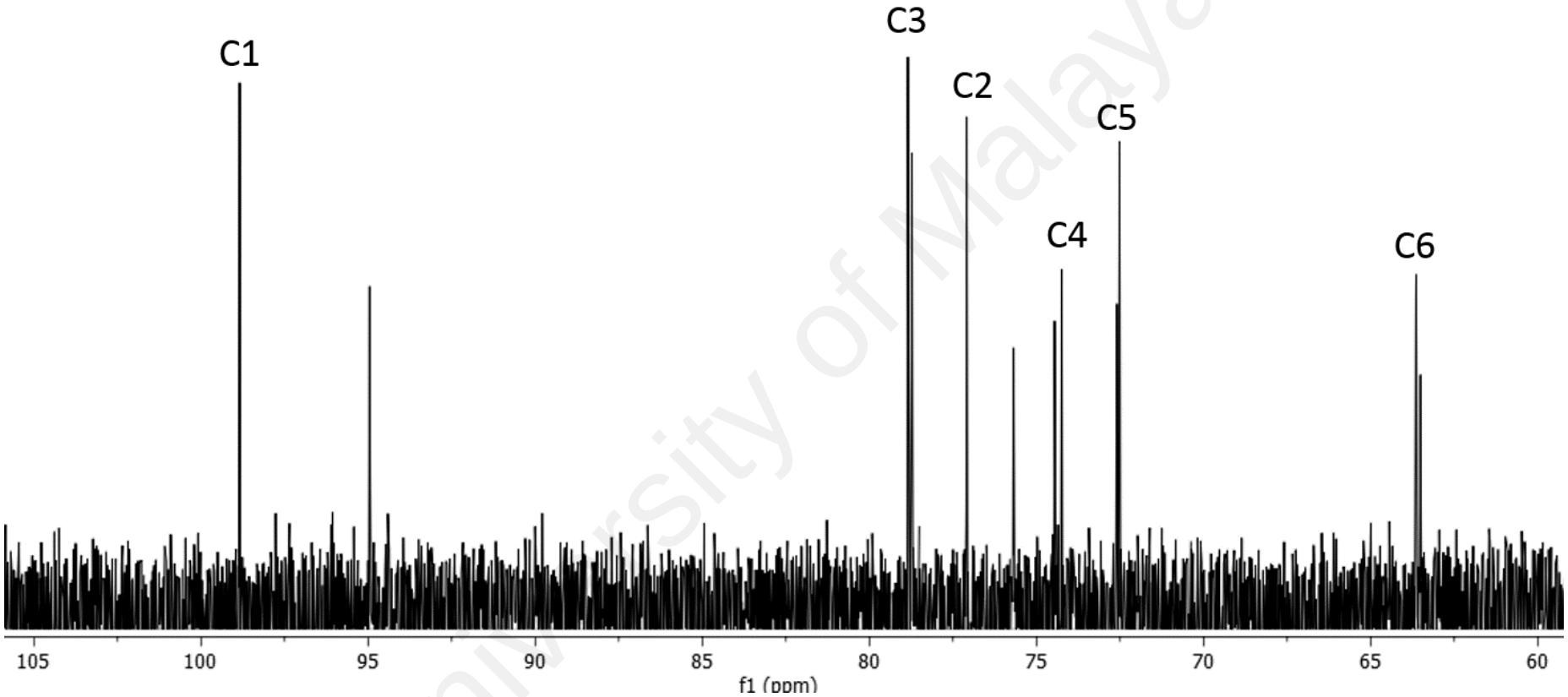

Figure 4.6: ^{13}C NMR spectra of mycelial (1,3)-β-D-glucan of *L.rhinocerus* strain ABI

4.5.2 Two-Dimensional Nuclear Magnetic Resonance (COSY, TOCSY, HMQC & HMBC) Structural Analysis

Two-dimensional (2D) NMR spectra were used to confirm the attributions recorded by ^{1}H and ^{13}C in 1D NMR spectra to reveal the characteristics of the EPS 2D NMR is a powerful tool for structure elucidation and has been shown to provide conclusive evidence for β-(1,3) linkages (Ensley et al., 1994; Lowman et al., 2011). Although many studies have used methylation analysis to determine the glycosidic linkages of β-glucans, this approach is more time-consuming and requires careful pre-treatment and interpretation of the generated data (Hakomori, 1964; Sims et al., 2018).

Figure 4.7 and **Figure 4.8** show the bidimensional COSY and TOCSY spectra with identification of the couplings between the protons ($^{1}H/^{1}H$). COSY allows the correlation of all coupled protons. Thus, the spectra reveal the interaction of $^{1}H/^{1}H$ in the two-dimensional axis. From the spectra, the COSY (**Fig. 4.7**) shows a distinct set of spots on a diagonal. The COSY permits an assignment of the chemical shifts of the anomeric proton (H1-β) coupling with its respective H2.

Meanwhile, TOCSY is a complement to COSY. TOCSY can provide correlations between all the protons that make up a spin system. The spin system is a collection of nuclei (protons) that are directly or indirectly coupled to each other. The spin system can be broken by heteroatoms and also by the loss of coupling due to lack of proton on carbon atom such as in the carbonyl group, which means it can be more than one spin system in a molecule. The TOCSY spectra (**Fig. 4.8**) show that all the protons are connected to the H1 signal which reveals that they are connected in one spin system of the (1,3)-β-D-glucan fragments. Hence, based on COSY and TOCSY spectra analyses, all ^{1}H-chemical shifts can be fully identified and assigned to the 1D ^{1}H NMR spectrum, which agrees with previous studies (Gonzaga et al., 2013; Nie et al., 2011).

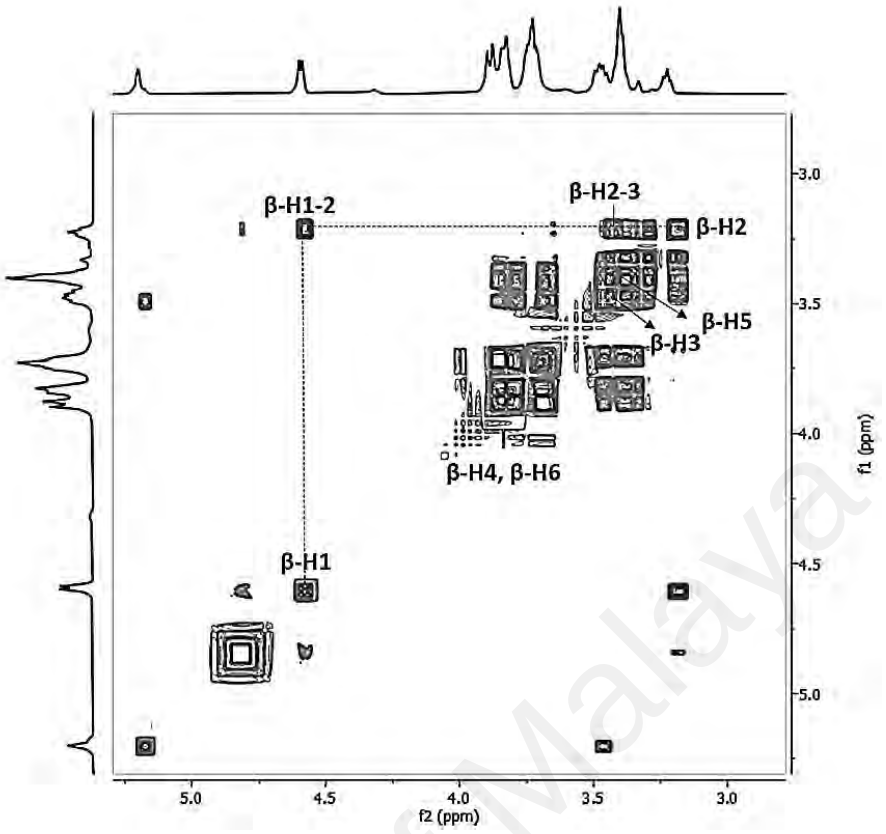

Figure 4.7: $^{1}H/^{1}H$ COSY correlation of mycelial (1,3)-β-D-glucan of *L.rhinocerus* strain ABI

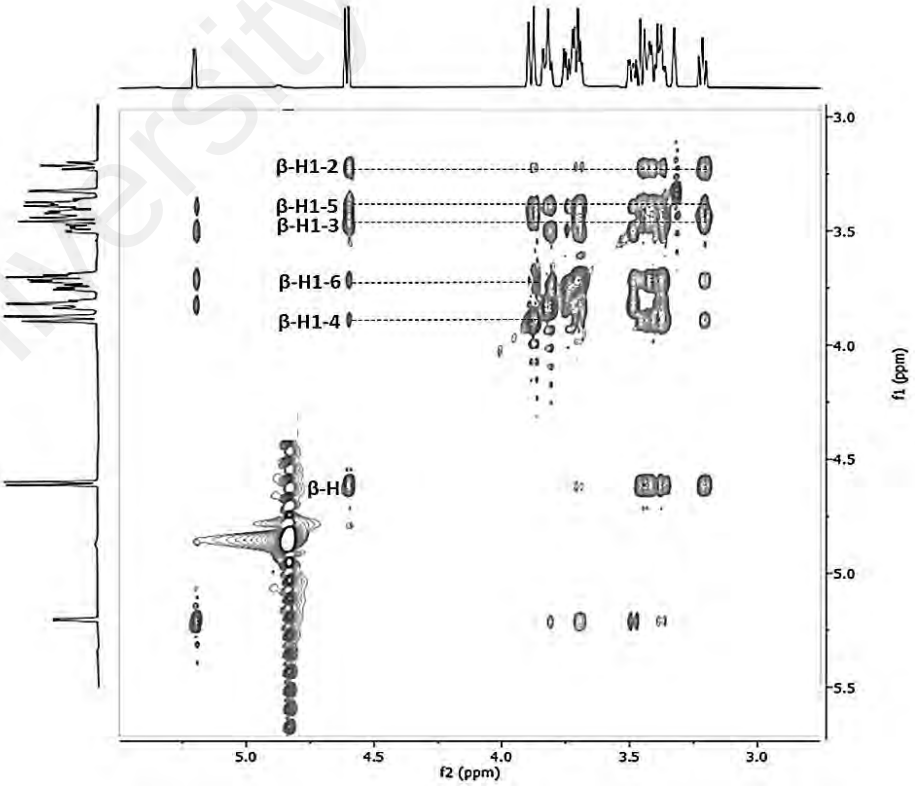

Figure 4.8: $^{1}H/^{1}H$ TOCSY correlation spectrum for a spectrum of mycelial (1,3)-β-D-glucan of *L.rhinocerus* strain ABI

Figure 4.9 represents the NMR spectra for HMQC, with a record of the couplings between carbons and hydrogens of the glycosidic ring ($^{13}C/^{1}H$). By using the carbon-related experiment for the HMQC spectrum, all ^{1}H-linked carbons signals can also be assigned through correlation with one-bonded ^{1}H-^{13}C *J*-couplings. The HMQC spectrum (**Fig. 4.9**) revealed the spectrum of distinct cross-peaks in the anomeric region of G. The C1, C2, C3, C4, C5 and C6 signals at 98.8, 77.1, 78.8, 74.2, 72.5 and 63.6 ppm cross-link to the proton signals H1, H2, H3, H4, H5 and H6 at chemical shifts 4.60, 3.23, 3.47, 3.92, 3.37 and 3.89 ppm, respectively, thus confirming the results obtained in 1D NMR. These values are fully consistent with the literature (Liu et al., 2014; Nie et al., 2011; Pomin, 2012).

HMBC ($^{1}H/^{13}C$) provides correlations between protons and carbons that are separated from each other by two or three bonds or up to five-bond correlations (Vasavi et al., 2011). The cross-peaks deduced the linkage sequence of the adjacent glycosyl residues in the HMBC spectrum (**Fig. 4.10**). The cross-peak between H1(4.60 ppm) and C3 (78.8 ppm) and between H5 (3.37 ppm) and C3 (78.8 ppm) indicated that residue was linked to residue G via a β-(1→3)-linked glycosidic bond (Wu et al., 2019). Finally, by compiling the information from 1D and 2D NMR, a complete assignment of all linkage patterns was obtained, as shown in **Table 4.1**. With the results of 2D NMR, the mycelial of LRSA was confirmed to consist of (1,3)-β-D-glucan linkages.

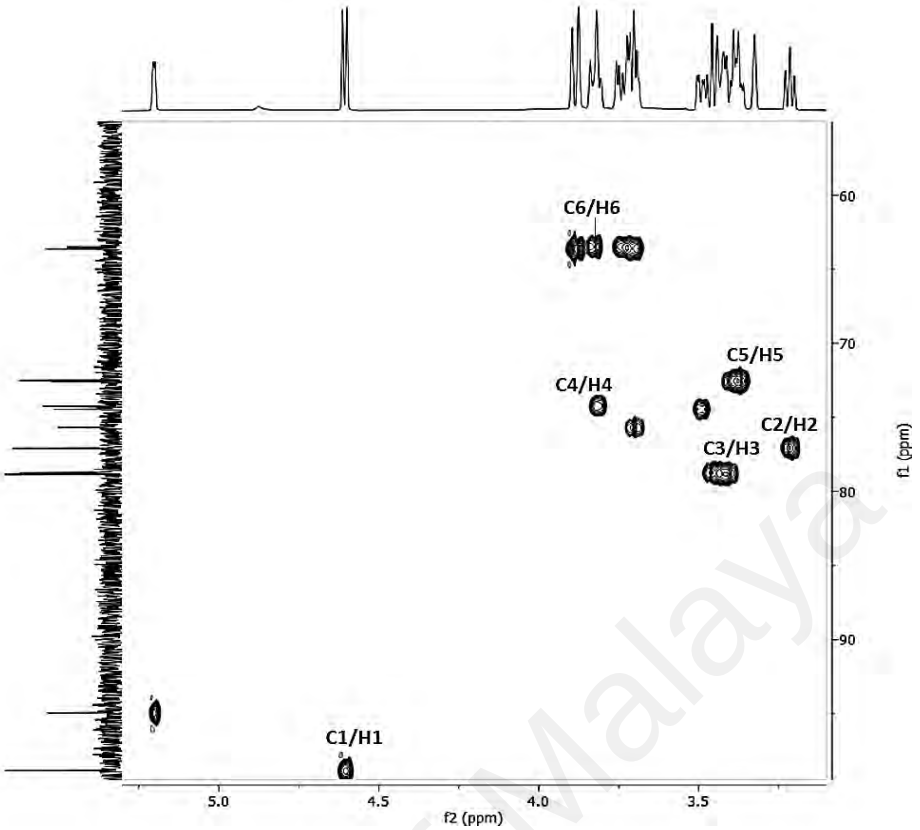

Figure 4.9: $^{1}H/^{13}C$ HMQC correlation of mycelial (1,3)-β-D-glucan of *L.rhinocerus* strain ABI

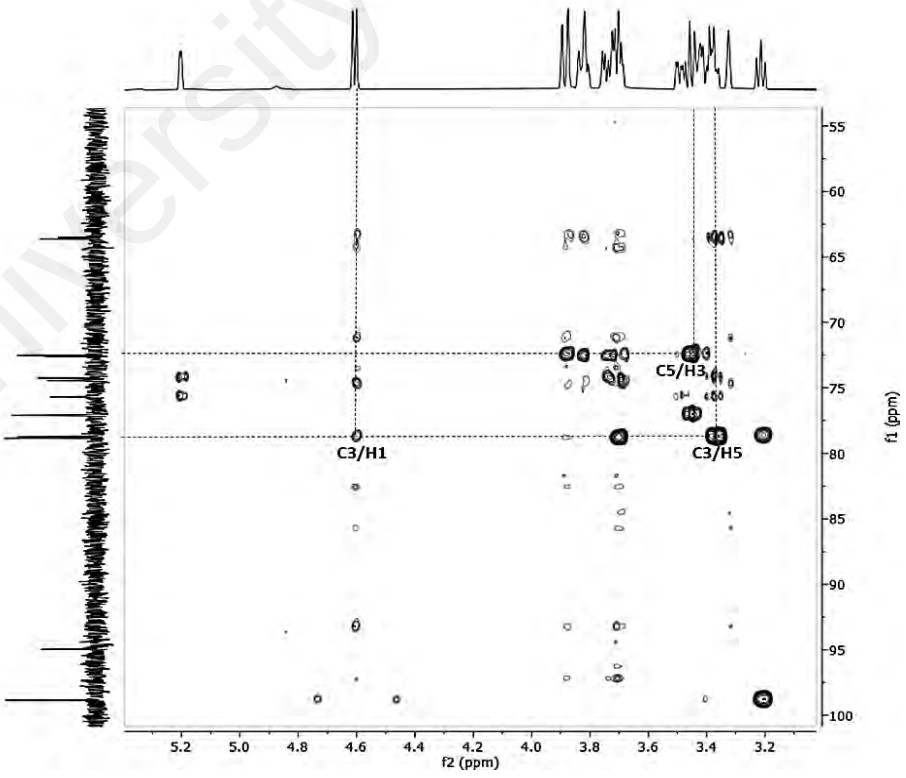

Figure 4.10: $^{1}H/^{13}C$ HMBC correlation of mycelial (1,3)-β-D-glucan of *L.rhinocerus* strain ABI

Table 4.1: Chemical shifts parts per million (ppm) of ^{1}H and ^{13}C NMR signals for G recorded in D_2O at 25 °C.

Sugar residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	H6'
(1→3)-β-D-glucan	4.60	3.23	3.47	3.92	3.37	3.89	3.69
	98.80	77.09	78.85	74.46	72.52	63.64	

4.6 Comparison of Characterised *Lignosus rhinocerus* Glucan Based on Available Literature

The tiger milk mushroom mycelial glucan characterised in the present work was compared with that of previous studies, as shown in **Table 4.2**. The present study is the first comprehensive characterisation of tiger milk mushroom in terms of strain determination using molecular identification, fungal PCR product size, sequence length, polysaccharide linkages, β-glucan content, FT-IR spectroscopy and structural characterisation using NMR. Six previous studies involving β-glucan studies have primarily considered the sclerotium of *L. rhinocerus* specifically originating from Malaysia (Choong et al., 2014; Jamil et al., 2013; Jamil et al., 2018; Lau et al., 2013a; Lau et al., 2013b; Lee et al., 2014). However, the production of sclerotia using a solid-state fermentation technique requires a long cultivation period which is economically unfeasible and susceptible to contamination (Leskosek-Cukalovic et al., 2010). Few studies have used solid-state fermentation as sclerotia cultivation takes about three to six months (Jamil et al., 2018; Kong et al., 2016; Lau et al., 2013a; Lee et al., 2012), in contrast with the present study, in which the mycelium produced in approximately 25 days. The shorter cultivation time using SLF has motivated further investigation into the potential of the mycelium as an alternative to cultivated or wild sclerotia. The production of mycelium through submerged liquid fermentation has several advantages, including a shorter cultivation time, higher yields and decreased contamination. Hence, more

efficient production of the desired products, particularly mycelial biomass and polysaccharides, can be obtained (Lau et al., 2014; Liu et al., 2014; Yang et al., 2013). The relevance of mycelium as a substitute for the sclerotia is supported by the current findings. The resultant β-D-glucan content extracted from the mycelium of *L.rhinocerus* strain ABI (36.3% w/w) was comparable with or higher than that obtained from sclerotia in previous studies (5.85%–38.93% w/w) (Choong et al., 2014; Jamil et al., 2013; Jamil et al., 2018; Kong et al., 2016; Lau et al., 2013a; Lau et al., 2013b; Lee et al., 2014). This observation also supported by the study of Lau et al. (2014), which reported that the mycelium of *L. rhinocerotis* had biological activities compared with those of the sclerotia, prompting further consideration of the mycelium as an alternative source of functional components. The current work also includes FT-IR spectroscopy analysis to investigate the EPS linkages of the mycelium of *L. rhinocerus* in contrast to previous studies. (1,3)-β-D-glucan was identified as the primary linkage in compound G and extracted from a mycelium sample, unlike the work described by Choong et al. (2014) which investigated only the extent of β-D-glucan linkage in a sclerotia sample.

Table 4.2: Comparison of characterised *Lignosus* sp. mycelial glucan based on available literature.

Species	Geographical origin	DNA source	Fungal size	Polysaccharide linkages	Total cultivation time (day)	β-glucan content (w/w) %	FT-IR (wavelength, cm^{-1})	^{1}H NMR	Reference
***L. rhinocerus* strain ABI**	Lata Iskandar, Malaysia	Mycelium (liquid fermentation)	515 bp	(1,3)-β-D-glucan	25	36.3	3277, 2919, 2850, 1638, 1630, 1032, 896	OH-2, OH-4, OH-6	*Current work*
L.rhinocerus	Lata Iskandar, Malaysia	Sclerotium (cultivated)	NA	β-D-glucan	132	63.51	NA	NA	Jamil et al. (2018)
L. tigris	Selangor, Malaysia	Sclerotium (cultivated)	NA	β- glucans	180	5.85 - 16.74	NA	NA	Kong et al. (2016)
***L. rhinocerotis* strain TM02**	Selangor, Malaysia	Sclerotium (cultivated)	NA	β- glucans	180	1.1 and 3.2	NA	NA	Lee et al. (2014)
***Lignosus* sp. strain M26/08, M49/07, M23/08**	Kuala Lumpur, Semenyih & Kuala Lipis, Malaysia.	Sclerotium (wild)	NA	β-D- glucans	NA	NA	1680, 1657, 1639, 1620, 1471	NA	Choong et al. (2014)
L. rhinocerotis	Negeri Sembilan, Malaysia	Fruiting body (cultivated), Sclerotium (cultivated), Mycelium (liquid fermentation)	NA	(1,3) and (1,6) - β-D- glucans	111	9.3 to 13.2	NA	NA	Lau et al. (2013a)
L. rhinocerotis	Negeri Sembilan, Malaysia	Sclerotium (cultivated)	NA	(1,3)-β- and (1,6)-β glucans	111	38.93	NA	NA	Lau et al. (2013b)
L. rhinocerus	Pahang, Malaysia	Fruiting body (wild)	NA	(1,3) and (1,6) - β-D- glucans	NA	33.9	NA	NA	Jamil et al. (2013)

*NA = not available. bp = base pair

CHAPTER 5: CONCLUSION

The wild-Malaysian tiger milk mushroom, *Lignosus rhinocerus* strain ABI (LRSA), was morphologically identified and verified using biomolecular techniques in which LRSA was found belonging to the *L. rhinocerus* species. The extracted EPS from the mycelium of LRSA was successfully characterised and elucidated spectrophotometrically using FT-IR and 1D and 2D NMR and was identified as a (1,3)-β-D-glucan. NMR structural analysis in the present study represents the first structural characterisation of (1,3)-β-D-glucan of *L. rhinocerus.* These findings may facilitate the development of G production on a high scale bioreactor using LRSA.

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LIST OF PUBLICATIONS

PUBLICATIONS

1. **Usuldin, S. R. A**., Mahmud, N., Ilham, Z., Ikram, N. K. K., Ahmad, R., & Wan, W. A. A. Q. I. (2020). In-depth spectral characterization of antioxidative (1,3)-β-D-glucan from the mycelium of an identified tiger milk mushroom *Lignosus rhinocerus* strain ABI in a stirred-tank bioreactor. *Biocatalysis and Agricultural Biotechnology*, *23*, Article#101455.

2. Hassan, N. A., Supramani, S., Sohedein, M. N. A., **Usuldin, S. R. A.**, Klaus, A., Ilham, Z., ... & Wan-Mohtar, W. A. A. Q. I. (2019). Efficient biomass-exopolysaccharide production from an identified wild-Serbian *Ganoderma lucidum* strain BGF4A1 mycelium in a controlled submerged fermentation. *Biocatalysis and Agricultural Biotechnology*, *21*, Article#101305.