MORPHOLOGICAL STUDY AND EXOPOLYSACCHARIDE PRODUCTION OF MALAYSIAN *Ganoderma lucidum* MYCELIUM IN A BATCH FERMENTATION

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MORPHOLOGICAL STUDY AND EXOPOLYSACCHARIDE PRODUCTION OF MALAYSIAN *GANODERMA LUCIDUM* MYCELIUM IN A BATCH FERMENTATION

ABSTRACT

A slow-growing cultivated *Ganoderma lucidum* QRS 5120 (GLQ5) was identified from a commercial mushroom farm. The fungus was identified morphologically by standard Basidiomycete characteristics (colour, fruiting body type, hymenium pores, basidiospores, plate growth) and molecular tools. In molecular identification, the fungus was compared with top-10 similarity of National Centre for Biotechnology Information – Basic Local Alignment Search Tool (NCBI-BLAST) once the tissue culture procedure succeeded. The phylogenetic tree was then constructed using Molecular Evolutionary Genetic Analysis 10 (MEGA-X) and verified using plasmid Editor (ApE) software (100% matched with *G. lucidum* strain 39). Upon isolation and identification, preliminary studies were conducted on GLQ5 mycelium. Exopolysaccharide (EPS) and intracellular polysaccharide (IPS) in a controlled shake-flask fermentation were studied at different initial pHs (3, 4, 5, and 6). At initial pH 4, the productivity of both EPS (0.071 g/L day^{-1}) and IPS (0.046 g/L day^{-1}) were the highest possessing ovoid-pellets morphology. Such pH condition also generated 5.13 g/L of biomass, 2.64 g/L EPS and 1.46 g/L IPS higher than initial pH 5 and pH 6 conditions. Initial pH 4 also produced higher carbohydrate contents in both EPS (0.435 g/L) and IPS (0.191 g/L). Then, using the preliminary studies as the reference, response surface methodology (RSM) was done to study the interaction between initial pH, initial glucose concentration and agitation rate for production of biomass-EPS-IPS from GLQ5 in submerged fermentation (SmF). A central composite design (CCD) was applied, and the polynomial model was fitted to the experimental data. The model was significant in all parameters investigated. Initial pH showed the strongest effect ($p < 0.0001$) for biomass, EPS and IPS production, meanwhile agitation showed

significant value ($p < 0.005$) for biomass. The model was validated by applying the optimized conditions and generated 5.12 g/L of biomass (initial pH 4.01,32.09 g/L of glucose and 102.45 rpm), 2.49g/L EPS (initial pH 4, 24.25 g/L of glucose and 110 rpm) and 1.52 g/L of IPS (and initial pH 4, 40.43 g/L of glucose, 103 rpm) in 500-mL shake flask fermentation. By using the optimised media, the productive pellet morphology was determined. At day 9 (hairy-ovoid pellet), the biomass was the highest. At day 11 (hairy-sphere pellets), EPS was highest, and at day 13 (sphere pellets), IPS was the highest. Meanwhile, in a 2-L stirred-tank bioreactor (STR), 1.9-fold higher of biomass (9.75 g/L: smooth pellets) was generated and 2.1-fold higher EPS (5.43 g/L: ovoid-hairy pellets) compared to shake flask. Then, the molecular characterisation of EPS was studied using Proton Nuclear Magnetic Resonance (^{1}H NMR) and Fourier Transform Infrared Spectroscopy (FTIR). The EPS showed the characteristics of β-glycosidic linkages in FTIR at 925 cm^{-1}, 1635 cm^{-1}, 1077 cm^{-1},920 cm^{-1}, 800 cm^{-1}, and in ^{1}H NMR at δ 4.58, 3.87, 3.8. The result indicated that EPS was composition of 1-3-β-D-linkages. Finally, the bioactivity of the EPS was screened against common pathogenic bacteria. The zone of inhibition showed EPS possessed antimicrobial against *Escherichia coli* (33.32 mm), *Serratia marcescens* (24.58 mm), *Staphylococcus aureus* (39.23 mm), and *Staphylococcus epidermidis* (35.3 mm). EPS was also positive against *Aspergillus niger* for antifungal-demelanizing activity. The result showed that the antifungal-demelanizing activity of EPS is dose-dependent. Together, the research managed to design an ‘’upstreaming and downstream processing’’ blueprint for bioactive production of EPS to be applied in large-scale fungal-based bioprocessing.

Keywords: *Ganoderma lucidum*; optimisation; fungal morphology; bioactivity screening; bioproduct characterisation

KAJIAN MORFOLOGI DAN PENGELUARAN EXOPOLISAKARIDA *GANODERMA LUCIDUM* MISELIUM MALAYSIA DALAM PANAPAIAN KELOMPOK

ABSTRAK

Satu fungi tumbesaran perlahan, *Ganoderma lucidum* QRS 5120 (GLQ5) telah dikenal pasti daripada ladang cendawan komersial. Cendawan tersebut telah dikenalpasti dari segi ciri-ciri morfologi Basidiomycete yang standard (warna, jenis badan berbuah, liang hymenium, sporabasidio, tumberasan atas plat) dan alat molekul. Dalam pengenalan genetic, kulat tersebut telah dibandingkan dengan 10 teratas persammaan daripada Pusat Kebangsaan untuk Maklumat Bioteknologi – Alat Pencari Alignment Asas (NCBI – BLAST) setelah prosedur kultur tisu berjaya. Pokok filogenetik telah dibina menggunakan Analisis Genetik Evolusi Molekular ke – 10 (MEGA-X) dan disahkan mengguna perisian plasmid editor (ApE) yang menunjukkan 100% persamaan dengan *G. lucidum* strain 39). Selepas pengasingan dan pengenalpastian, kajian awal telah dijalankan atas GLQ5. Polisakarida extra (EPS) dan intra (IPS) di dalam fermentasi kelalang konikal terkawal telah dikaji dalam pH yang berbeza (3, 4, 5, dan 6). Pada pH 4, produktiviti tertinggi EPS (0.071g/L per hari) dan IPS (0.046g/L per hari) telah dicatatkan, yang menghasilkan pelet dengan morfologi bujur. Produktivi biojisim (5.13g/L), EPS (2.64g/L) dan IPS (1.46g/L) juga paling tinggi pada pH 4 berbanding dengan pH 5 dan pH 6. Selepas itu, hasil kajian awal telah digunnakan sebagai rejukan, "response surface methodology' (RSM) digunakan untuk mengkaji interaksi antara pH, kepekatan glukosa, dan kadar agitasi untuk mengetahui kadar pengeluaran biojisim-EPS-IPS daripada GLQ5 dalam fermentasi terendam. Reka bentuk komposit pusat (CCD) telah digunakan dan model polinomial dipasang pada data eksperimen. pH menunjukkan kesan yang paling kuat ($p < 0.0001$) untuk biojisim, EPS dan pengeluaran IPS, sementara itu agitasi menunjukkan nilai signifikan ($p < 0.005$) untuk biojisim. Verifikasi model ini telah

dibuktikan dengan penghasilan 5.12 g / L biojisim (awal pH 4.01,32.09 g / L glukosa dan 102,45 rpm), 2.49g / EPS L (pH 4, 24.25 g / L glukosa dan 110 rpm) dan 1.52 g / L IPS (pH 4, 40,43 g / L glukosa, 103 rpm) dalam 500 mL fermentasi kelalang konikal. Dengan menggunakan media yang dioptimumkan, morfologi pellet yang produktif telah ditentukan. Pada hari ke 9, (pelet berbentuk bulu ovoid) produktivi biojisim adalah tertinggi dan pada hari ke 11 (pelet berbulu sfera), produktivi EPS adalah tertinggi dan pada hari ke 13 (pelet sfera), produktiviti IPS adalah tertinggi. Sementara itu, di dalam bioreaktor pengacau 2-L, produktiviti biojisim terhasil adalah 1.9 kali ganda lebih tinggi (9.75 g / L: pelet licin) dan EPS terhasil adalah 2.1 kali ganda EPS yang lebih tinggi (5.43 g / L: pelet bujur telur-berbulu) berbanding dengan fermentasi kelalang konikal. Kemudian, ciri-ciri molecular EPS telah dikaji menggunakan spektroskopi proton resonans magnetic nuclear (1H-NMR) dan spektroskopi Fourier Transform Spectroscopy Infrared (FTIR). EPS-nya menunjukkan ciri-ciri lingkaran β – glikosid dalam FTIR pada 925 cm-1, 1635 cm-1, 1077 cm-1,920 cm-1, 800 cm-1, dan dalam 1H NMR pada δ 4.58, 3.87, 3.8. Hasilnya menunjukkan EPS mengandungi komposisi (1-3) -β-D-hubungan. Akhirnya, bioaktiviti EPS diuji terhadap bakteria patogen yang biasa. Zon perencatan menunjukkan EPS memiliki antimikrob terhadap Escherichia coli (33.32 mm), Serratia marcescens (24.58 mm), Staphylococcus aureus (39.23 mm), Staphylococcus epidermidis (35.3 mm). EPS juga telah diuji terhadap Aspergillus niger untuk aktiviti menghancurkan antikulat. Hasilnya menunjukkan aktiviti menghancurkan antifungal EPS bergantung kepada dos. Bersama-sama, kajian ini telah mengurus reka bentuk proses “upstreaming and downstreaming” cetak biru untuk pengeluaran bioaktif dari EPS untuk digunakan dalam bioteknologi berasaskan kulat skala besar.

Kata kunci: *Ganoderma lucidum*; pengoptimuman; morfologi kulat; pemeriksaaan bioaktif; pencirian bioproduk

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
δ	:	Delta
°C	:	Degree Celsius
µg	:	Micrograms
µm	:	Micrometres (microns)
bp	:	Base pairs
cm	:	Centimetres
cm^{-1}	:	Wavelength per distance
EPS	:	Exopolysaccharide
FTIR	:	Fourier Transform Infrared Spectroscopy
g	:	Grams
G	:	Gravitational force
H	:	Hydrogen
IPS	:	Intracellular polysaccharide
kb	:	Kilobase
L	:	Litres
mg	:	Milligrams
min	:	Minutes
mL	:	Millilitres
mm	:	Millimetres
MW	:	Molecular weight
NA	:	Nutrient Agar
Nm	:	Nanometres

NMR	:	Nuclear Magnetic Resonance
PDA	:	Potato Dextrose Agar
pH	:	Potential hydrogen
s	:	Seconds
v/v	:	Volume per volume
YE	:	Yeast Extract

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CHAPTER 1: INTRODUCTION

1.1 Research background

Ganoderma lucidum is widely used as a traditional medicine to substitute modern medication. The bioproduct (polysaccharide) from *G. lucidum* is extensively studied due to its potential as a therapeutic agent (Adotey et al., 2011; Andoh et al., 2010; Baig et al., 2015; Barbieri et al., 2017; Batbayar et al., 2011). Polysaccharide obtained from fungus has been shown to possess a vast number of biological activities (Barbieri et al., 2017; Wan-Mohtar et al., 2016a). To obtain such polysaccharide, two different methods can be used, either *via* solid substrate fermentation (SSF) or submerged fermentation (SmF). In SSF, the fungal mycelium grows through the substrate bed by colonisation. This causes the heterogeneity issue of mycelium growth within the substrate bed. Therefore, the production of polysaccharide in SSF is a time-consuming method (Wan-Mohtar et al., 2016b).

In SmF, the fungal mycelium grows as a cluster in free-flowing complex media, in which, becomes a stable form called pellets. Pellets are commonly found in the ovoid shape and are either densely packed or loosely packed with branched hyphae. Due to the pellet's morphology differences, the production of polysaccharide differs too. A densely packed pellet will produce higher exopolysaccharide (EPS) which, are secreted outside the pellet, and loosely packed pellet produces higher intracellular polysaccharide (IPS) which, are produced inside the pellet (Ubaidillah et al., 2015). Factor optimisation (pH, agitation speed, oxygen transfer rate (OTR), glucose concentration, and temperature) are crucial in SmF, as the interaction between the factors affects the mycelium growth as well as the production of polysaccharide (Ahmad et al., 2013; Wu et al., 2016a).

Hence, response surface methodology (RSM) shows promising results to optimise the factors compared to one-factor-at-a-time (OFAAT) method. RSM is used to study the interactions between the parameters in a complex manner. The most utilised method in RSM is a central composite design (CCD). For one numeric variable, CCD has 5 levels ($-\alpha$, -1, 0, +1, $+\alpha$) (Liu et al., 2011).

In this dissertation, the correlation and interaction between a set of experimental variables were studied using RSM and subsequently provided the optimised conditions. For the current investigation, a cultivated Malaysian *Ganoderma sp.* was subjected to molecular characterisation. Then, a preliminary study was conducted using OFAAT method to obtain the baseline data and the practical ranges of the selected SmF parameters, before the optimisation of mycelial growth, EPS and IPS production using RSM. The chosen parameters for RSM were – initial pH, glucose concentration and agitation rate. Upon optimisation, the EPS will be characterised for its general structure using FTIR and ^{1}H NMR. Finally, the EPS was tested for its bioactivity (antimicrobial and anti-fungal).

1.2 Research objectives

The specific objectives of the present study are as follows:

- To identify cultivated Malaysian *Ganoderma sp*.
- To determine the effect of initial pH on pellet morphology, biomass, exo- and intracellular polysaccharide production of *Ganoderma lucidum* strain QRS 5120.
- To optimise the growth parameters for *Ganoderma lucidum* QRS 5120 in shake flask using response surface methodology (RSM).
- To determine the productive type of pellet morphology for exo (EPS) and intracellular polysaccharide (IPS) production in liquid cultivation of *Ganoderma lucidum* QRS 5120.

- To determine the structural linkages of EPS extracted from mycelium *Ganoderma lucidum* QRS 5120.
- To screen potential bioactivity of EPS extracted from the mycelium of *Ganoderma lucidum* QRS 5120.

1.3 Problem statement

The mushroom used in this study was a cultivated medicinal mushroom. Medicinal mushrooms are difficult to obtain from their natural environment and often are seasonal. The ability to cultivate these valuable mushrooms independent from season and external variations found in nature are limited. Thus, the optimised growth medium and submerged cultivation conditions for the mushrooms are in need.

1.4 Scope of work

This study focuses on the cultivation and enhancement of slow-growing medicinal mushroom, *Ganoderma lucidum* in submerged fermentation (SmF) to produce the bioactive compound. Initially, as a preliminary study, the initial pH was optimised. Then using response surface methodology (RSM), the interaction between initial pH, starting glucose concentration and agitation rate for production of biomass, exopolysaccharide and intracellular polysaccharide were studied for its significant effect for the bio-production.

1.5 Dissertation outline

The dissertation consists of six chapters. Chapter One describes the introduction for the study; Chapter Two outlines the literature review of the current research. Chapter Three describes the material and methods used in the study, Chapter Four details the results and discussion, Chapter Five summarises the conclusion of the research and Chapter Six proposes the future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Fungi

Numerous investigations have been carried out to differentiate between two groups of fungi, namely microfungi "the lower fungi" and macrofungi "the higher fungi" (Fazenda et al., 2008). Its morphology aspects differentiate these two groups in terms of bearing fruiting body and its filaments properties. Macrofungi carry the fruiting body and also known as filamentous fungi, whereas microfungi do not produce the fruiting body and have a very discrete form of filamentous (Fukuda et al., 1985).

2.2 Phyla

2.2.1 Basidiomycota

This Phylum Basidiomycota is easily recognisable under a light microscope or even naked eye due to its club-shaped fruiting body characteristics called basidia. These basidia, are known to be their reproductive organs and often contained with common mushrooms seen in the fields after rain, supermarket shelves, or in our lawn (Zhao et al., 2017). Another common name for these mushrooms is 'gill fungi' because of the gill structure underneath the cap. Basidiomycetes include the shelf fungus, in which the fungus clings on the wood bark like shelves, smuts and rusts, which are plant pathogens. There are also some basidiomycetes, which are deadly; *Crytococcus neoformans* causes respiratory illness (Arras et al., 2017).

2.3 Mushroom

Edible mushrooms have been consumed throughout the history of humankind, and most studied mushrooms are the medicinal mushrooms for the disease treatment. Japan was the first country to grow medicinal mushrooms, about two thousand years ago, Japanese cultivated Shiitake (*Lentinus edodes*) (Ronis et al., 2004). Then, the Oriental countries; Taiwan and China practised the cultivation of medicinal mushroom (Kunjadia

et al., 2014). There is high demand for medicinal mushroom because numerous researches have shown that most of the species that fall in medicinal mushroom category produced beneficial compounds; antimicrobial (Younis et al., 2015), antifungal (Wan-Mohtar et al., 2017), antiviral (Teplyakova et al., 2016), anti-inflammatory (Dore et al., 2007) and anticancer (Ma et al., 2013). Most of the medicinal mushroom falls under the division of Basidiomycetes (*Ganoderma* families). Therefore, improvements in the cultivation of these species are imperative.

2.3.1 *Ganoderma*

The genus *Ganoderma* includes the mushrooms that grow on woods. There are about 80 species in genus *Ganoderma*, mainly from humid regions of China, Korea, Japan, and Malaysia (Bidegain et al., 2015). *Ganoderma* is a white-rot fungus that produces enzymes that allows them to break down wood components. Environmentally, *Ganoderma* is important as they help in the degradation of wood. In traditional Asian medicine, *Ganoderma* is economically important. *G. applanatum*, *G. atrum*, *G. tsugae*, and *G. lucidum*. Since the 1980s, *Ganoderma* species have been the subject of research mushroom (Gupta et al., 2014).

2.3.2 Structure of *Ganoderma sp.* Basidiospores

The basic structure of Basidiospore is ellipsoid or ovoid. At the apex of the basidiospore, a hyaline epispore projects and form a papilla-like structure. This structure then collapses once maturity is reached and gives the spore a truncated look. Inside the spore (Endosporium) is yellowish-brown colour, the exterior (Exosporium) is smooth (before maturity) and rough (at maturity). Typically, the size of basidiospore is about $10^{-2} \times 6^{-8}$ μm (Banerjee, 1959).

2.3.3 *Ganoderma lucidum*

G. lucidum (Basidiomycota) was chosen for the current study due to its reported value in the literature. This well-known species is also termed 'Lingzi' in China and 'Reishi' in Japan and is widely used in traditional Chinese medicine (Bidegain et al., 2015). The fruiting body of *G. lucidum* contains various chemical substance, which includes triterpene and many types of polysaccharide (Jiang et al., 2014). These polysaccharides are the main core source of pharmacological activity; antifungal, antibacterial and anti-tumour. By 1988, Lingzi has been used as a fitness and health food in tropical countries. Similarly, in Taiwan and Korea, Lingzi has been used as popular medicine to treat various illness; gastric cancer, encouraging longevity, lowering risk of cancer and increasing immune system (Zhu et al., 2013).

2.3.4 The appearance of *G. lucidum*

The observable characteristic of *G. lucidum* its hymenium pores, bare stipe, brown spore print and the parasitic or saprotrophic behaviour. There are six different colours of *G. lucidum* but commonly found are the red variety. *G. lucidum* is typically seen as flat, hard, kidney-shaped caps with white to dull brown pores underneath the cap (Luo et al., 2010).

2.3.5 The natural habitat of *G. lucidum* and its life cycle

Naturally, *G. lucidum* is observed on dead and dying trees and typically found with its nearby relative, *G. tsugae*. In the wild, Lingzi matures on old logs and at the base and stumps of deciduous trees. The high concentration of carbon dioxide in its environment stimulates the fruiting body (Shrestha et al., 2016). The life cycle of *G. lucidum* starts with germination of haploid basidiospores to form monokaryotic mycelia, where each cell contains a haploid nucleus (primary mycelium). Later, when two genetically compatible monokaryons are joined, they somatogamy to produce secondary mycelium-

containing dikaryotic cells. The two haploid nuclei divide synchronously by a mechanism comprising a clamp connection, which ensures that the dikaryotic state of the cell is sustained (Cao et al., 2017).

2.3.6 Toxicity of *G. lucidum*

G. lucidum does not exhibit any cytotoxicity and is considered a safe food. This is due to its long history of oral administration. Moreover, the extract of *G. lucidum* has been tested on animal and exhibit deficient levels of toxicity, indicating its safe for human consumption (Cizmarikova, 2017).

2.3.7 Role of *G. lucidum*

G. lucidum is commonly used in Japan, China, and other countries as a source for natural drugs development (Zhao et al., 2012). Commonly, the fruiting body of *G. lucidum* is used medicinally. The role of *G. lucidum* is described in detail (Section 2.6), particularly its pharmacological properties.

2.3.8 Natural cultivation of *G. lucidum*

Production of *G. lucidum* globally was valued at 4900-5000 tonnes in the year 2001. At least 100 brands of *G. lucidum* are available in the market (Han et al., 2005). The early cultivation of this mushroom was significantly different than those employed today. The first cultivation was done by gathering the fruit bodies from the natural habitat and taking them to a region of 'fresh' substrate. Ultimately, the spores would germinate and colonise the substrate, hence giving rise to a new fruit body. *G. lucidum* mycelium has been traditionally gowning in waste from the processing of citrus fruits and molasses and in sulphite waste liquor from the pulp and paper industries. This significant improvement from ancient technology is termed as solid-state fermentation (SSF) (Son et al., 2017).

SSF is defined as when the growth of *G. lucidum* on a solid substrate proceeds in the near absence of free water ($a_w \approx 0$), but enough presence to sustain fungal growth and the metabolism (Liu et al., 2015). SSF has been tested in the solid fermentation of steamed substrate, inoculate with suitable spawn that progress under controlled temperature and humidity. In other words, it is the cultivation of fungi under controlled conditions to produce bio-products. Much work had been done to cultivate a complete fruiting body of *G. lucidum* using SSF.

SSF utilises agro-industrial residues such as grain, sawdust and wood, and cheap raw materials as a substrate for growth. During the SSF upstream process, these substrates are broken down by the extracellular enzymes of the fungus, permitting further microbial processing to yield valuable food materials. Example of such food materials is soy sauce, tempeh miso and sake (Han et al., 2005).

Process of SSF is very challenging on the term of controlling, monitoring and scaling up. Moreover, in SSF, the fruiting body composition varies according to their content of bioactive substance, thus making the traditional method of polysaccharide extraction from the fruiting body challenging. Therefore, concerning *Ganoderma sp.*, solid-liquid fermentation (SLF) replaced the production of fungal bioproducts in established agro-industries (Postemsky et al., 2017).

2.3.9 Artificial cultivation of *G. lucidum*

G. lucidum fruiting body production (SSF) or so-called the artificial cultivation is an extended procedure that usually takes several months for the first product to appear. Product quality from this route can be variable. The development of SLF methods for these organisms permits hastening of the growth reactions, resulting in a biomass yield in several days (Son et al., 2017). Therefore, researchers have focused on studying SLF conditions to accelerate *G. lucidum* growth and formation of bioproducts. In this context,

derivatives of SLF, batch or fed-batch fermentation, continuous batch fermentation, and repeated-batch fermentation (RBF) have been attempted to boost the cultivation of this species.

2.3.10 The fermentation strategies of *G. lucidum*

SLF is a process that utilises free-flowing liquid substrates such as soluble sugars, liquid media, molasses, plant-based juices, and wastewater, mainly for the extraction of bioactive metabolites. SLF has become the method of choice for *G. lucidum* cultivation, principally due to the superior characteristics of products compared with SSF (Wei et al., 2016). SLF is far less problematic (e.g., it exhibits better heat and oxygen mass transfer and improved mixing) than SSF, making it more consistent, flexible, reliable, and easier to monitor and control the key parameters (Wei et al., 2016). Furthermore, SLF yields bioactive compounds more rapidly and hygienically (sterile).

G. lucidum growth in SLF has implications, particularly for intricate and synchronised processes like the formation of clamp connections and maintenance of the dikaryotic state, resulting in moderate growth rates compared with other Basidiomycetes (Wan-Mohtar et al., 2016a). The reason for this is that the clamp connections joining the hyphae may deteriorate in a long culture period, which can affect longevity.

2.3.11 Batch fermentation

Batch fermentation technology is the most ancient and simplest technique for cultivating *G. lucidum*. Although the process can endure only from 2 to 5 days of cultivation, due to an extended lag phase, the entire process can be prolonged by up to 18 days (Raja et al., 2007). Unfortunately, this method has a growth rate that is ten times lower than that of normal filamentous fungi, a major restricting factor to the commercial cultivation of *G. lucidum* (Raja et al., 2007). Hence, to develop the most efficient

approach using SLF, all information associated with batch cultures requires careful examination to identify better methods for accelerating production.

Fed-batch fermentation technology has also been introduced to improve the traditional batch fermentation process. This method incorporates feeding according to different strategies and depending on the desired product. As an example, by using initial low glucose (10 g/L) and three different glucose levels (10, 25, and 50 g/L), this strategy was shown to enhance process efficiency for *G. lucidum* (Wei et al., 2016). Here, the fed-batch mode is a useful tool to explore substrate limitation effects and possible inhibition characteristics.

2.4 Functional properties

Various compounds isolated from macrofungi possess antibacterial, antifungal, antioxidant, antiviral, and more functional properties (Table 2.1). In general, most of these bioactive compounds are isolated from phylum Basidiomycota (also known as 'medicinal mushroom) (Sugiharto et al., 2015).

Table 2.1: Functional properties of *G. lucidum*.

	Functional properties	Explanation
1.	Anti-bacterial activity	Positive outcomes were reported against Gram-positive bacteria from using extracts of *G. lucidum*. Moreover, additives effects were observed using the aqueous extract of *G. lucidum* with four known antibiotics, and the antibacterial activity had increased. However, most of the active bioactive compound was isolated from the fruiting body. Therefore, the mycelium-based extract is considered superior, yet information available of a standardised quality is scarce. Further research on suitable quality parameters and analytical methods to produce antimicrobial-mycelium products are recommended by Karaca et al. (2017). There are relatively few studies on antimicrobial or antifungal extract using SLF.

Table 2.1: Continued.

2. Anti-fungal activity	There is one study conducted by Wan-Mohtar et al. (2017) using *Aspergillus niger* A60, showed that sulphated glucan derived from exopolysaccharide of *Ganoderma lucidum* was able to demelanize the fungal's spore, by which dormant the fungus. By that, the fungus was not able to propagate and eventually deceases.
3. Antiviral activity	Antiviral properties of *Ganoderma* genus have been studied by Piraino et al. (2014). These include the major biologically active constituents, effects and mode of action. An acidic protein-bound polysaccharide isolated from the water-soluble substance of *G. lucidum* has shown antiviral activity against herpes simplex viruses (HSVs) (Piraino et al., 2014).
4. Anticancer activity	Some research has shown that extract from Basidiomycetes can stop the cycle. Such as Ganodermanontriol, a *G. lucidum* mushroom triterpene extract, inhibited the expression of β-catenin. Extract from *G. lucidum* increases the ratio of Bax/Bcl-2 in human cancer cell by increasing Bax expression while downregulating Bcl-2 expression such as inhibits cell death, suppress chemotherapy-induced apoptosis, and regulate tumour suppressor p53(Liu et al., 2005) (Muller et al., 2004). Synthetic drugs are used to treat the common forms of cancer (e.g. prostate cancer and breast cancer) by disrupting cell growth. As known, cancerous cells grew more rapidly than other cells, synthetic drugs target those cells that are in the process of reproduction. As a result, these drugs affect not only cancerous cells, but other healthy cells that also replicate quickly, including blood-forming organs, ovaries and testes, and the hair follicles (Sheena et al., 2003). Therefore, the researcher looks for other supplements such as natural compound. To date, researchers have screened more than 23,000 natural compounds to identify those that help fight cancer while healthy cells are remained unharmed. Extracts for *G. lucidum* are the popular natural drugs and have been widely used for the joint promotion of health, particularly for the prevention of several types of cancer. Therefore, *G. lucidum,* in the form of a dietary supplement or as a derivative drug, can be used as an additional therapeutic aid in cancer patients (Trajkovic et al., 2009). The most reactive anticancer compounds are a polysaccharide, which has shown beneficial properties such as the capacity for inhibiting carcinogenesis, inducing apoptosis, and suppressing the migration of cancer cells (Qu et al., 2017).

Table 2.1: Continued.

5. Immunomodulation	Compounds from *G. lucidum* may affect both the innate and adaptive immune system via stimulation reaction. The reaction recognises β-glucan as a foreign and non-self-molecule, thus promoting immunomodulation (Navarro-Garcia et al., 2000). One report has stated that *G. lucidum* may have a potential immunomodulating effect in patients with advanced colorectal cancer (Opattova et al., 2017). However, more work is required in this area.

2.5 Bioactive compounds extracted from *G. lucidum*

The valuable health properties of *G. lucidum* are exhibited across a broad range of bioactive components present in the fruiting body, spore, and mycelium. As bioactive compounds, polysaccharide and triterpenes are the two major groups, followed by phenols, mycins, vitamins, nucleosides, steroids, amino acids, lignins and nucleotides (Sudheer et al., 2016).

2.5.1 Polysaccharide and β-glucan

Polysaccharide from *Ganoderma* is a structurally diverse class of biological macromolecules with a broad range of physiochemical characteristics of which the most-studied are those of the β-glucans. β-glucans are made up of D-glucose monomers linked by β-glucan are valuable in human nutrition as soluble fibre supplements and texturing agents (Wang et al., 2014). These compounds are commonly found as cellulose in plants, the cell wall of baker's yeast, the bran of cereal grains, fungi, bacteria and mushrooms. Medicinal mushroom-produced β-glucans are significant for their therapeutic activities, especially from species of *G. lucidum*. β-glucans extracted from *G. lucidum* gained wide attention as a health supplement due to their perceived health benefits. For instance, several glucans isolated in the early 1980s from alkali and water extracts were found to be bioactive (Trajkovic et al., 2009).

Although these extracts have been isolated from more than 200 basidiocarps and mycelial biomasses, only a few have been isolated from culture media. These highly valuable compounds have inadequately researched to date, especially regards to their production. Many attempts, including the use of SLF, have been made to increase extracellular polysaccharide (EPS) content, β-glucans (Wu et al., 2016b). The structure of β-glucans is β-1-3 D-glucopyronan with 1-15 units of β-1-6 monoglycosyl side chains (Wang et al., 2009); this constituent promises to be a new type of carcinostatic agent, which may eventually be useful in immunotherapy.

2.5.1.1 Solubilisation of water-insoluble β-glucan

The biopolymers extracted from *G. lucidum* are mostly water-insoluble (1-2)-β-D-glucans; for pharmaceutical study, this may cause challenges due to their poor water solubility. Depending on their degree of polymerisation, branching and chemical derivation, the solubility of extracted β-glucan can vary (Han et al., 2008). β-glucan depolymerisation by acidic or alkali hydrolysis, enzymatic degradation, or ultrasonic treatment, along with sulfation and phosphate, have been evaluated to overcome this problem by improving solubility in aqueous solution (Han et al., 2008). Of these, sulfation is the preferred solubilisation technique due to its positive impact on biological function and formation of sulphated β-glucan.

2.5.1.2 Sulphated β-glucan

Previously, chemically-derived polysaccharides, including sulphated and carboxymethylated polysaccharides, have received much attention due to their biological properties. These polysaccharide derivatives showed improved water solubility and altered chain conformation, resulting in the enhancement of their biological activities (Bao et al., 2001b). These bioactive polysaccharides may be developed and improved using chemical modifications, thus widening their application and ease of experimental

use. Of these, sulphated chemical agents and modification may help to generate increased bioactivity, functional properties and polysaccharide by-products (Dong et al., 2012). Sulfation is a method where the extracted β-glucan is treated as another state or structural organisation of β-glucan through the introduction of a sulphate group (Bao et al., 2001a). Methods for the sulphated modification of polysaccharides include sulphuric acid, sulphur trioxide-pyridine, chlorosulphonic acid-carboxamide, chlorosulphonic acid-pyridine and sulphur trioxide-dimethylformamide.

2.6 Physical factors in *G. lucidum*

Growing *G. lucidum* in liquid media involves four main factors; chemical, biological, physical and morphology. Biological factors are its behaviour, meanwhile physical and chemical define the environment of the biocatalyst. The optimal fermentation conditions depend on the nature of the desired product and the strain of fungus used. Moreover, the growth rate and fungal morphology depend on culture conditions.

Factors such as aeration, temperature, agitation rate or shear force, fermenter design and culture time can affect the fermentation macro-environment. These factors can contribute to the morphological and physiological behaviour of the fungus, and thus affect the performance of the bioprocess. The fungal physical condition can influence the growth and productivity of the studied strain. Some example of physical factors is shown in Table 2.2.

Table 2.2: Physical factors involved in submerged fermentation of *G. lucidum*.

Physical factors	Explanation
1. Temperature	During *G. lucidum* liquid cultivation, temperature can be controlled easily. Temperature affects growth rate, medium evaporation, dissolved oxygen (DO), pellet development, and products formation in filamentous fungal cultures. The growth of *G. lucidum* has typically been studied in SLF at a temperature between 25 and 36 °C, with most established at 30°C.
2. Agitation	A commonly used turbine in bioreactor systems, Rushton turbines chiefly provide the constant agitation for *G. lucidum*. Throughout growth and bioproduct development, agitation rate is equivalent to the impeller speed of a bioreactor, which plays a significant role in determining the fungal growth rate through mixing, heat transfer and mass. In such fungal cultures, especially at scale, there can exist noticeable mass transfer gradient throughout the bioreactor vessel, triggering both mycelial morphology and product spectrum changes. The shear force generated by agitation can affect the mycelium in numerous ways such as by damaging the cell structure, stimulating morphological change, and causing variations in growth rate and bioproduct formation. For these reasons, optimum agitation is crucial to achieving adequate oxygen transfer into the medium while avoiding shear stress.
3. Aeration	Aeration is a very important parameter for Basidiomycete culture. Aeration is measured by dissolved oxygen (DO), which becomes the most significant variable to be optimised in aerobic fermentation. Aeration in controlled by air supply and agitation speed in any controlled fermenter set-up.
4. Fermenter design	There are many types of the fermenter in the market, but only one type of fermenter can be used for fungal culture, the stirred-tank reactor (STR). STR can be divided into two types; baffled and unbaffled. Most of the manufacturer supplies Rushton impellers as it gives promising oxygen transfer rate and high growth for aerobic fungi; however, it is also well known for high shear force and causes the effect to product yield due to shear-sensitive cells. A low shear oscillatory baffled bioreactor (OBR) was reported to yield high EPS under similar culture conditions, but no morphological differences were reported. Baffles in the bioreactor are improve mixing performance. Even though the cells experience a hydrodynamic force at the turbulence region of the baffle, the force is not greater than the force in the impeller zone. The limitation of using baffle for fungal culture is when the fungal cells engulf and build up at the bioreactor's baffle, which, disrupts mixing and cell growth for long continuous cultures, especially *Ganoderma lucidum*. Therefore, further investigation in the culture of this species is needed, especially in term of fermenter design.

Table 2.2: Continued.

5. Foaming	The foam may form when a fermenter medium is subjected to aeration and agitation, which may cause a significant problem. The foam may block the exit air filters and lead to an increase in pressure at the headspace of a bioreactor. In the case of fungal culture, when the foam rises, the mycelium follows the foam and eventually starts growing on the wall and headspace. Therefore, to overcome the foaming, chemical and mechanical antifoam can be utilised in large-scale cultivation. Mechanical antifoam is preferred, as it does not decrease OTRs, whereas chemical antifoam is known to decrease OTRs, which will affect cell growth.
6. Culture time	Due to carbon source depletion in SLF, the harvesting time of fungal is variable and determines the quality of polysaccharide production. Other than polysaccharide quality, the fungal culture age also has a profound effect on polysaccharide production. Therefore, it must be taken into consideration. In this study on RBF, the culture time was considered vitally, as it is extended cultivation of *G. lucidum*.

2.7 Chemical factors in *G. lucidum* cultivation

Chemical factors have high significance effect on the evolution of biotechnology processes. They are metals, ions, additives, medium pH, medium composition, carbon source, complex media, by-products and nitrogen source and are shown in Table 2.3.

Table 2.3: Chemical factors involved in submerged fermentation of *G. lucidum*.

Chemical factors	Explanation
1. Medium pH	Initial pH of medium may affect the growth of *Ganoderma lucidum* as well as the polysaccharide production. Other than that, pH medium also affects salt solubility, ionic state of a substrate, cell membrane function, cell morphology, substrate uptake rate (glucose consumption) and bioproduct formation. In this study, the effect of initial pH was tested on *Ganoderma lucidum*. Overall, the *Ganoderma lucidum* preferred and functioned best at pH 4.
2. Medium composition	The medium composition is vital for *Ganoderma lucidum* to grow healthy. In a study conducted by Fazenda et al. (2008), a vast range of complex, synthetic media or chemically-defined media and waste substrate have been used. The optimised media must result in an increment of biomass and bioproduct formation. Complex media produces higher biomass concentration than chemically-defined media. By

Table 2.3: Continued.

	controlling the carbon: nitrogen (C: N) ratio source, the medium composition can be explored.
3. Complex media	Complex media have been used in the large-scale cultivation of *Ganoderma* sp. using waste materials. Comparing with synthetic media, the cost for mushroom mycelium cultivation can be reduced using complex media. Many researchers have suggested that complex media is the optimal condition for *Ganoderma lucidum* in liquid culture.
4. Carbon Source	Carbon source can be divided into two groups, namely; disaccharides (lactose, maltose and sucrose) and monosaccharides (fructose, galactose, and glucose). The different type of carbon sources affects the production of EPS. A study by Xiao et al., 2006, stated that molasses (complex carbon source) assisted in higher EPS production and mycelial growth comparing to simple carbon source.
5. Nitrogen source	For the growth of *Ganoderma lucidum*, the common nitrogen sources are nitrate, nitrite, ammonium salts, casein, peptone, amino acids, yeast extract, beet, and cane molasses. Due to *Ganoderma lucidum*'s natural habitat is low in nutrients except for carbon, and very low N content in woods, therefore, *G. lucidum* in the wild are N-limited. By understanding this mechanism to survive the N-limited environment, new strategies can be developed to culture fungus. Type of N source highly influences cell growth and EPS production of *G. lucidum*.
6. Metals and ions	Appropriate levels of key metals and trace elements are required for the growth and product formation of *G. lucidum*. These salts (e.g., magnesium sulphate and potassium phosphate) are required for fungal development, while trace elements (manganese, zinc, iron, copper, and molybdenum) act as co-factors for enzymes (Fazenda et al., 2008). Other ions like magnesium (Mg^{2+}) and potassium (K^{+}) are also vital for Basidiomycete growth; an Mg^{2+} is responsible for enzymatic reactions while K^{+} promotes hyphal tip extension (Fazenda et al., 2008). Magnesium sulphate (in the form of Epsom salts) is commonly used in *G. lucidum* fermentation.

2.8 Biological factors in *G. lucidum* cultivation

Biological factors, including inoculum age and volume, prominently affect the growth and product formation of *G. lucidum*. Therefore, a proper characterisation of the inoculum is vital for potential industrial applications such as the isolation, successive selection and maintenance of cultures (Seviour et al., 2011; Stanbury et al., 1995). Additionally, these

evaluations need to be performed on the selected inoculum as they are vulnerable to contamination, spontaneous mutation, deterioration, and death (Fazenda et al., 2008). For that reason, inoculum preservation and maintenance are crucial, and it is advisable to prepare frozen stock cultures as soon as possible after isolation (Walser et al., 2001).

2.8.1 Inoculum

There are many kinds of inoculum variables that can exert a major influence on the *G. lucidum* fermentation profile, such as type, age, concentration, amount, and viability (Fazenda et al., 2008). These also affect fungal morphology, especially in pellet production and the types of pellets produced (Gibbs et al., 2000). The inoculum for *G. lucidum* can only be standardised with mycelial-based inoculum since sexual spores are formed once the fruiting body has matured (Wagner et al., 2003), compared with most readily-produced asexual spores (e.g., conidia) of other filamentous fungi which can be used as a source of standardised inoculum. Most mushroom scientists use small pieces of mycelium that are still attached to the agar plates on which the fungi were grown and inoculate these directly into the fermentation broth when using shake flasks or agar cultures. This seed culture strategy is used to inoculate a bioreactor so that the mycelium can adapt from a solid to a liquid environment, thus reducing the lag phase and lowering inoculum densities (Fazenda et al., 2010).

Nevertheless, the environmental and nutritional conditions used to prepare the seed culture must remain constant, as must those in the fermentation, to avoid a prolonged lag phase. Furthermore, *G. lucidum* inoculum standardisation is vital, such as the removal of the cut mycelia-mats at the same radial distance from the colony centre of the agar. This method ensures that all inoculum have an equal amount of mycelium at the same stage of development.

For *G. lucidum*, the mycelium can be homogenised aseptically to increase the number of active hyphal tips (Fazenda et al., 2010; Stanbury et al., 1995), using a short blending time with a sterile stainless-steel Warring Blender (Hsieh et al., 2006; Rogalski et al., 2006). The inoculum blending time should be no more than 20 seconds at a low-speed setting to avoid possible mycelial damage from the treatment.

Such treatment has helped to ensure process reproducibility, and an active inoculum will decrease the lag phase in subsequent culture provided that the inoculum is transferred at an appropriate time (i.e., true physiological state). Yang and Liau (1998b) evaluated the effect of *G. lucidum* inoculum size on its performance and stated that a seven-day shake flask inoculum was ideal (Fang et al., 2002; Fang and Zhong, 2002b). For *G. lucidum*, many scientists have chosen inoculum percentages (*v/v*) such as 17 % (Berovic et al., 2003), 10% (Wagner et al., 2003), 10 % (Sanodiya et al., 2009), 12 % (Liu et al., 2012a), and 5 % (*G. resinaceum*) (Kim et al., 2006a). Based on this literature, the maximum percentage (*v/v*) used in this study was never more than 20 %.

2.9 Morphology and rheology

The morphology of *G. lucidum* and its polysaccharide concentrations is influenced by the operating conditions of the fermenter. In turn, the rheological properties of the fermented cultures are typically determined by the concentration and morphological state of the mycelium and the EPS present, which is closely related to the broth viscosity. Fermentation cultures can behave either as Newtonian or non-Newtonian fluids in rheological terms. Here, the filamentous form of fungi tends to give rise to a highly viscous, non-Newtonian broth, whereas the pellet form of the cultured fungi produces an essentially Newtonian system with a much lower viscosity in the liquid phase. This reduced viscosity, in turn, allows better oxygen mass transfer rates within the liquid phase created by the Newtonian system at the pellet surface (Kim et al., 2003).

The submerged culture fermentation of Basidiomycetes sometimes leads to the formation of large pellets, either as a filamentous pulpy form or as discrete particles of 1 to 20 mm in diameter. Large pellets can restrict the fermentation process by limiting nutrient diffusion into the pellet and the removal of waste out of the pellet, thus causing an undesired non-homogeneous physiological state of the mycelium to develop. However, the consequences of having large pellets are advantageous to the production of higher ganoderic acid yields in *Ganoderma*, due to the limitation of oxygen in the large pellet core which would generate a lower polysaccharide concentration (Wagner et al., 2004). Conversely, smaller pellets would generate more polysaccharide.

The metabolism of *Ganoderma* relates to its morphology (Wagner et al., 2004). Pellet growth is usually triggered by variables such as sugar concentration in the medium, agitation regime and inoculum density. Pellet morphology is measured in terms of shape, surface area, hairiness or roughness (R), circularity or diameter, and compactness (Fazenda et al., 2008). The filamentous form is usually characterised by the total hyphal length and the number of actively growing tips. Pellet morphology can influence the bioproducts produced, although the relationship between *G. lucidum* pellet morphology and bioproduct production remains poorly understood (Wagner et al., 2004).

Hence, it can be stated that the manipulation and understanding of culture morphology may enhance polysaccharide production during fermentation.

2.10 Morphological analysis of *G. lucidum*

A detailed quantitative structural analysis of fungal morphology is required if a better understanding of the relationship between morphology and desired bioproducts is sought. This is an example of where the application of methods such as image analysis (IA) would permit the measurement of relevant morphological parameters (Riley et al., 2000), and IA is thus suggested for any work where the development of fungal hyphae is significant

(Tucker et al., 1992). Therefore, IA is sometimes used for the microscopic characterisation of Basidiomycetes (Park et al., 2002; Tepwong et al., 2012; Treskatis et al., 2000) and has also been developed as a fast and precise method for quantitative morphological characterisation, since parameters of an observed object such as size, total, shape, position and intensity can easily be evaluated in this way. Such morphological study was conducted by (Wan-Mohtar et al., 2016a), which stated that ovoid and starburst-like pellet gave higher exopolysaccharide production in a bioreactor.

Concerning the morphological form, cultures in which the fungus grows as pellets tend to be less viscous than those in which it grows as dispersed filaments (Fraga et al., 2014; Tang et al., 2007). This morphological form can also affect product development, particularly of EPS. According to the literature, the most favourable morphology for EPS production corresponds to a looser pellet structure (Fazenda et al., 2010; Hsieh et al., 2006; Kim et al., 2006b) compared with small pellets, as discussed in the following section.

2.10.1 *G. lucidum* morphology in EPS production

A study conducted by Wan-Mohtar et al. (2016a), showed that morphology plays a crucial role in EPS production. The pellet morphology changes throughout the time profile. It can be observed that feather-like morphology pellets (Day 12) are associated with higher EPS production. Understanding, and, if possible, controlling morphology is crucial for EPS production in SmF. Therefore, *G. lucidum* studies which evaluate this feature, both in the shake flask and bioreactor, particularly concerning their EPS-morphology relationship.

2.11 Project aim

This study aims to cultivate a wild Malaysian *Ganoderma lucidum* and identify it genetically. Upon identification, a preliminary study (initial pH optimisation) to be done as a base result for this study. Then, using statistical software, Response Surface Methodology (RSM), the interaction between different factors (initial pH, starting glucose concentration and agitation) to be optimised. Using the optimised conditions, the productive pellet morphology will be determined, and the growth profile of identified *Ganoderma lucidum* QRS 5120 will be done. Then, the exopolysaccharide will be characterised by spectral analysis using Proton Nuclear Magnetic Resonance (^{1}H-NMR) and Fourier Transform Infrared Spectroscopy (FTIR), finally, the bioactivity screening of EPS will be performed for antimicrobial and antifungal activities.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cultivation and identification

3.1.1 Cultivation of organism

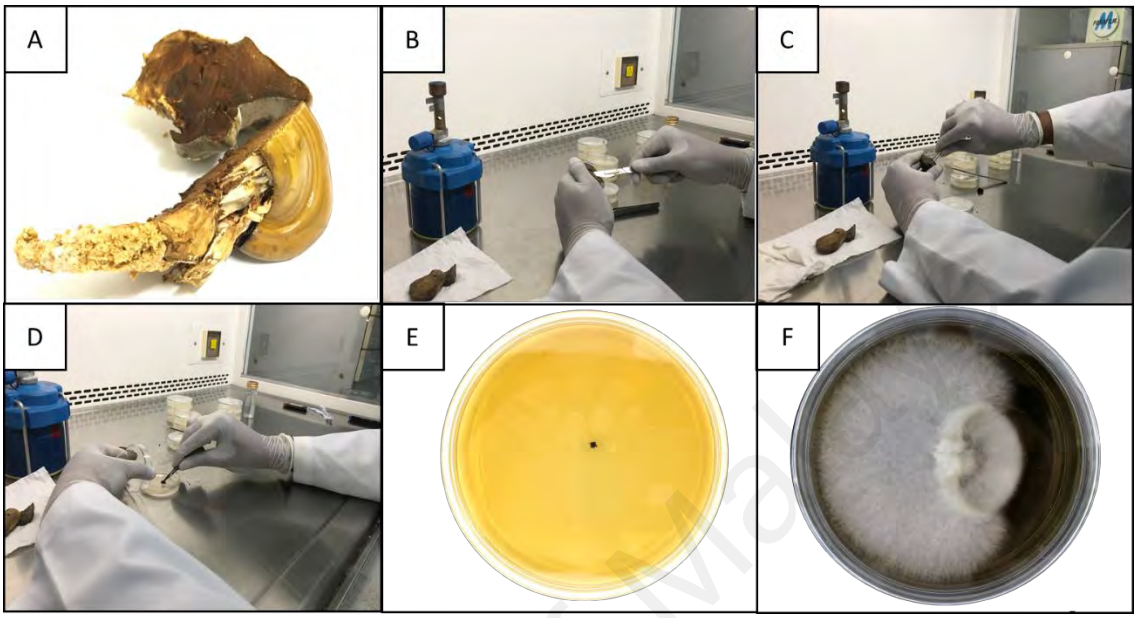

Figure 3.1: Culturing of *G. lucidum*. (A) A crack opened *Ganoderma lucidum* fruiting body. (B) Exposing the inner tissue of *G. lucidum* using a scalpel. (C) Inner tissue removal of *G. lucidum* using forceps. (D) Placing the tissue on to potato dextrose agar (PDA). (E) PDA plate with *G. lucidum*'s tissue (Day 0). (F) Mycelium of *G. lucidum* on PDA plate (Day 7)

The cultivated fruiting body of *G. lucidum* was obtained from Universiti Putra Malaysia (UPM), Mushroom unit. Then, tissue cultivation was done to obtain the mycelium. The fruiting body was washed with 99.9 ethanol for 10s and dried in laminar flow. Then, it was cracked opened using a scalpel (Figure 3.1A & 3.1B), and the inner part of the fruiting body was twisted and pulled using forceps (Figure 3.1C). Lastly, the tissue obtained was placed on malt extract agar (MEA) (Figure 3.1D & 3.1E) and kept at room temperature until the sign of mycelium growing was observed. The mycelium was then sub-cultured onto fresh MEA to obtain pure mycelium (Figure 3.1 F).

3.1.2 Identification

3.1.2.1 Preparation of mycelium for DNA extraction

Freeze-dried *G. lucidum* mycelium was prepared from mycelium grown on PDA. Sections of the mycelium were removed from agar with a scalpel, freed from traces of agar, suspended in 1 ml of pure water in an Eppendorf tube and fragmented by vigorous pipetting. 0.1g of caesium chloride (CsCl), was added and centrifuged at 13000 rpm for 10min. Then, the bulk mycelium was removed from the surface of the CsCl solution, leaving behind the agar at the bottom and a few mycelial stands dispersed in the solution. After washing twice with distilled water in an Eppendorf tube, the mycelium was freeze-dried and stored at -20°C (Liao et al., 2015).

3.1.2.2 Genome DNA (gDNA) extraction

The finely powdered mycelium (30 mg) was then resuspended and lysed using lysis buffer (500 µl) by pipetting multiple time until the suspension becomes foamy and RNAase A was added. The mixture was incubated (5 min, 37 °C). To remove the cell debris, polysaccharide and protein, NaCl solution (165 µl, 5 mol/l) were added, and the tube was inverted multiple times. Then, it was centrifuged (14000 × g, 20 min, 4 °C), and the supernatant was transferred to a fresh tube. The supernatant was mixed with chloroform (400 µl) and phenol (400 µl) and mixed by gently inverting the tube multiple times until the solution becomes cloudy. It was centrifuged (14000 × g, 20 min, 4 °C), and the aqueous phase was removed and extracted using chloroform (equal volume). Then using 95 % ethanol (2 volumes), the DNA was precipitated. To purify the DNA from a polysaccharide, lysis buffer (500µl) was added and mixed by pipetting gently. Then, NaCl (165 µl, 5 mol/l) was added and gently mixed by inverting the tubes multiple times. To extract the purified DNA, chloroform (2 volumes) was added and centrifuged (14000 × g, 10 min, 4 °C). Using ethanol (95 %), DNA was precipitated and washed three times

using ice-cold ethanol (70 %). The washed DNA was then dried and dissolved in TE buffer (50 µl) and kept in -20 °C (Hennicke et al., 2016).

3.1.2.3 PCR amplification

The temperature of DNA dissolved in TE buffer was brought down to room temperature slowly from -20°C. Using universal primers (ITS1 and ITS4), the fungal ITS gene was amplified. First, the solution (500 µl) was added into PCR tubes. Then, 0.5 pmol of ITS1 and ITS4 were added following by deoxynucleotides triphosphates (dNTPs, 200 µM each), 0.5 U DNA polymerase, supplied PCR buffer and water. The PCR was performed as follow: 1 cycle (98 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 15 secs; 60 °C for 30 secs; 72 °C for 30 sec) for annealing and extension, and 1 cycle (72 °C for 10 min) for final extension of the amplified DNA.

3.1.2.4 PCR-amplified product purification and sequencing

The PCR products were purified and directly sequenced using a 16-capillary 3100 Genetic analysers (Applied Biosystems). BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used with protocols supplied by the manufacturer.

3.1.2.5 Data analysis

The obtained sequence of the gDNA was entered to BLAST. NCBI Nucleotide Collection (nr/not) database was selected, and the query was submitted. The sequences producing significant alignment was generated, and the top 10 hit blast was selected for Multiple Sequencing Alignment (MSA). Clustal Omega was used to obtain MSA.

3.1.2.6 Phylogenetic analysis

Using the neighbouring-joining (NJ) in Molecular Evolutionary Genetic Analysis (MEGA-X), the evolutionary distance (K_{nuc}) of identical fungal species was calculated, and then, a phylogenetic tree was generated. The species with closest K_{nuc} are considered the same species.

3.1.2.7 Verification of species

To verify the species, the sequence of the closest K_{nuc} species and the sequence of gDNA was compared for mismatches using A plasmid Editor (ApE) software.

3.2 Initial pH optimisation

3.2.1 Microorganism and medium

The obtained mycelium from Section 3.1.1 was sub-cultured onto potato dextrose agar (PDA). The plates were inoculated and incubated at 30°C under dark condition until the mycelium almost reaches the corner of the plates (10 days). Then, the plates were kept at 4 °C. The strain was preserved on PDA slants. The medium composition for seed culture and batch fermentation are maintained at same metrics at (g/L): Glucose 30, Yeast Extract 1, KH_2PO_4 0.5, $MgSO_4$ 0.5, and NH_4Cl 4, unless stated (Wan-Mohtar et al., 2016a). The pH of media was adjusted using HCl and NaOH (1M and 0.1M). The conditions for liquid fermentation for initial pH was pH 3 to pH 6, agitation 100 rpm, 30°C. Erlenmeyer flask (500 ml) was used to cultivate the mycelium in liquid with a working volume of 200 ml for 10 days. All conditions were done in triplicate.

3.2.2 Dry cell weight

The DCW from the harvested *G. lucidum* fermentation process is estimated by filtering a 200-mL sample through a pre-dried and weighed GF/C filter (Whatman Ltd., U.K.) using a Buchner funnel filter set attached to a water pump, followed by repeated washing

(three times) of the mycelial biomass with distilled water. The mycelial or pellets filter cake is dried overnight in the dryer and cooled in a desiccator for 24 h before weighing. Calculation of the DCW is done by subtraction of pre-weighed filter mass from the mass with the filtrate and multiplied by the dilution factor to get DCW in g/L. All values are taken based on averages of at least three independent trials (Wan-Mohtar et al., 2016c).

3.2.3 Intracellular polysaccharide (IPS) extraction

The Intracellular polysaccharide (IPS) of *Ganoderma lucidum* is extracted from the mycelium. IPS is obtained from the filtrate (washed mycelium). The mycelium is then mixed with 1g: 20 mL distilled water. Then it is sterilised at 121 °C for 30 minutes. After sterilising, the mixture is filtered to obtain the supernatant. Then, the crude IPS is precipitated by the addition of 1: 4 of 99.9 % (*v/v*) ethanol and left overnight at 4 °C to one volume of cell-free filtrate. The precipitate is then separated by centrifugation at 10,000 × g for 10 minutes a process that is repeated twice. The precipitate is then filtered through a pre-dried and weighted GF/ C filter paper and washed twice with 5 mL of 95 % (*v/v*) ethanol. It is then reassigned to desiccators, left to dry to constant weight, and the weight of IPS was then estimated (Ubaidillah et al., 2015).

3.2.4 Exopolysaccharide (EPS) extraction

The exopolysaccharide (EPS) of *Ganoderma lucidum* were extracted using cultivation broth. The procedure is followed with the reference from Wan-Mohtar et al. (2016a). EPS is obtained from filtered supernatants of the harvested fermentation broth. Then, the crude EPS is precipitated by the addition of four volumes of 99.9 % (*v/v*) ethanol and left overnight at 4 °C to one volume of cell-free filtrate. The precipitate is then separated by centrifugation at 10,000 × g for 10 minutes a process that is repeated twice. The precipitate is then filtered through a pre-dried and weighted GF/ C filter paper and washed

twice with 5 mL of 95 % (*v/v*) ethanol. It is then reassigned to desiccators, left to dry to constant weight, and the weight of EPS was then estimated (Wan-Mohtar et al., 2016b).

3.2.5 Phenol-sulphuric acid assay

The total carbohydrate content is determined by phenol-sulphuric acid assay. 0.1 g of DW Intracellular polysaccharide (IPS) and DW Exopolysaccharide (EPS) is diluted into 1mL of distilled water in the test tube. 0.5 mL of 4 % (*v/v*) phenol solution and 4mL of concentrated sulphuric acid are added to each test tube. The mixture is then allowed to stand at room temperature for 10 minutes. The prepared aliquots are read using spectrophotometer at 420 nm absorbance (Ubaidillah et al., 2015).

3.2.6 Morphology analysis

The morphology details of the samples collected are assessed using a light microscope (Leica) through a camera (12-megapixel iSight camera (A1687; Apple Inc., Cupertino, CA, USA) with 1.22 µpixels. The camera captured images of 4032 × 3024 pixels, each with ISO-100, 4-mm focal distance, f/2.2 aperture and 1/4-second exposure time.)

3.2.7 Microscopic Analysis

The mycelia sample is stained using lactophenol cotton blue stain. Other than that, to observe the mycelia sample as it is in the media, the mycelia sample is poured with the media into a Petri plate and sealed with parafilm tape. The plate is then observed under an inverted microscope (Leica M165 C).

3.2.8 Statistical analysis

The analysis will be carried out in the triplicate manner, and the mean (± SD) is determined GraphPad Prism 7 software (Version 7.00) and error bar will be made as

mean. If the error bar is absent, it indicates that the size of the symbol is bigger than the error value.

3.3 Optimisation of growth parameters (initial pH, initial glucose concentration and agitation rate) of *Ganoderma lucidum* QRS 5120 using response surface methodology (RSM)

3.3.1 Optimisation of growth parameters using RSM

Based on the preliminary studies, initial pH showed a high significance for the responses (mycelium biomass, EPS production, and IPS production) [Chapter 3.2]. Media composition of seed culture in shake flask was constant at (g/L): Yeast Extract 1, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_4$ 0.5, NH_4Cl_2 4, unless otherwise stated. To optimise the mycelium biomass, EPS and IPS production, CCD was used. In Table 3.1, the levels and range of the variables are shown for this study. From Table 3.1, the lowest level of variables was initial pH 4; starting glucose concentration = 10 g/L; agitation rate = 90 rpm and the highest level of variables were initial 6; starting glucose concentration = 50 g/L; agitation rate = 110 rpm.

Table 3.1: Experimental range and levels of the independent variables.

Independent variables	Range and levels		
	-1	**0**	**1**
Initial pH	4	5	6
Glucose (g/L)	10	30	50
Agitation (rpm)	90	100	110

To analyse the impact of factors and its interaction, an empirical model was established based on a second-order quadratic model for the responses as shown by Eq.3.1:

$$Y = b_0' + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j>1}^{n} b_{ij} X_i X_j \quad (3.1)$$

where Y is the predicted response, b_0' is the constant coefficient b_i is the linear coefficient, b_{ij} is the interaction coefficient, b_{ii} is the quadratic coefficient, and $X_i X_j$ are the coded values.

3.4 Determination of the productive type of pellet morphology for exo (EPS)- and intracellular (IPS) – polysaccharide production in submerged fermentation (SmF) of *Ganoderma lucidum* QRS 5120

3.4.1 Microorganism and medium

As described in Section 3.2.1.

3.4.2 Dry cell weight

As described in Section 3.2.2.

3.4.3 Intracellular polysaccharide (IPS) extraction

As described in Section 3.2.3.

3.4.4 Exopolysaccharide (EPS) extraction

As described in Section 3.2.4.

3.4.5 Morphology analysis

As described in Section 3.2.6.

3.4.6 Microscopic Analysis

As described in Section 3.2.7.

3.4.7 Statistical analysis

As described in Section 3.2.8.

3.5 Compositional analysis of exopolysaccharide

3.5.1 FTIR spectroscopy

FTIR analysis was performed using Agilent Cary 630 equipped with diamond ATR (Attenuated Total Reflectance). The sample (0.5 g) was placed on a clean window, and the pressure clamp was closed until a click was heard. Then, the data was collected using MicroLab software.

3.5.2 NMR spectroscopy

The NMR analysis was performed using 600Mhz Agilent, USA. The sample (10mg) was mixed with 500 µl of deuterium oxide (D_20-*d*). Then, the mixture was vortexed and sonicated for 15 min until the sample dissolves completely. Upon dissolving, the mixture was centrifuged at 10000 × *g* for 10 minutes. The clear supernatant was transferred to 5 mm NMR tube (Norell, Sigma Aldrich, Canada) for analysing.

3.6 Screening potential bioactivity exopolysaccharide (EPS) extracted from *Ganoderma lucidum* QRS 5120

3.6.1 Preparation of extract (sulfation of EPS) from *Ganoderma lucidum* QRS 5120.

EPS (0.25 g) was added in a test tube followed by 12.5 ml of DMSO and 0.75 g urea. 2 ml of concentrated sulfuric acid was added dropwise in the test tube. The test tube then placed in a water bath for 3 to 6 hours and vented at room temperature in a fume hood. The glucan sulphate was then filtered. 250 ml of water was added to glucan sulphate. Glucan sulphate solution was dialysed to cut off 10,000 MW. The volume was reduced to 125 ml. It was then lyophilised to dry (Wan-Mohtar et al., 2016c).

3.6.2 Antimicrobial test

Test organisms and culture media

1. Gram-positive : *Staphylococcus aureus, Staphylococcus epidermis*
2. Gram-negative: *Escherichia coli, Serratia marcescens*

Media: Nutrient Agar, Nutrient Broth

3.6.2.1 Kirby-Bauer disc diffusion assay

200µl of suspension of the test bacteria was smeared on the prepared nutrient agar. The standardised 6 mm sterile discs (blank) (Sigma-Aldrich) with an equal absorbed GS volume were soaked with a known amount of extract. It was positioned moderately onto the agar overlay, and the plates were carefully incubated overnight at 37 °C. Vancomycin was applied as the positive control, whereas ethanol was the negative control. After the incubation, the diameters (mm) of the inhibition zone were measured. Inhibition zones that were larger than 6 mm were considered positive for antimicrobial reactions.

3.6.3 Antifungal - demelanizing Test

Test organism and culture media

Fungal species: *Aspergillus niger*

Media: Potato dextrose agar (PDA), Potato dextrose broth (PDB)

3.6.3.1 Demelanizing activity

Glucan – sulphate (GS) was dissolved in 5 % DMSO solution containing 0.1 % Tween 80 (*v/v*) at (mg/mL): 200, 125, 100, 60, 30, 20, 15, 10, 2, and 1 and added to potato-dextrose medium with inoculum. A commercial fungicide, bifonazole (Sigma-Aldrich), was used as a positive control (2 mg/mL) with DMSO as a negative control. The

demelanizing activity of GS on *A. niger* A60 was tested in 96-well microtiter plates to determine the minimum demelanizing concentration (MDC). Examination of the treated mycelium after fixation with lactophenol was accomplished using a light microscope. The untreated control plate samples were also examined under the same conditions. DMSO (5 %) solution was used as a negative control (Wan-Mohtar et al., 2017).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Identification

4.1.1 Morphology

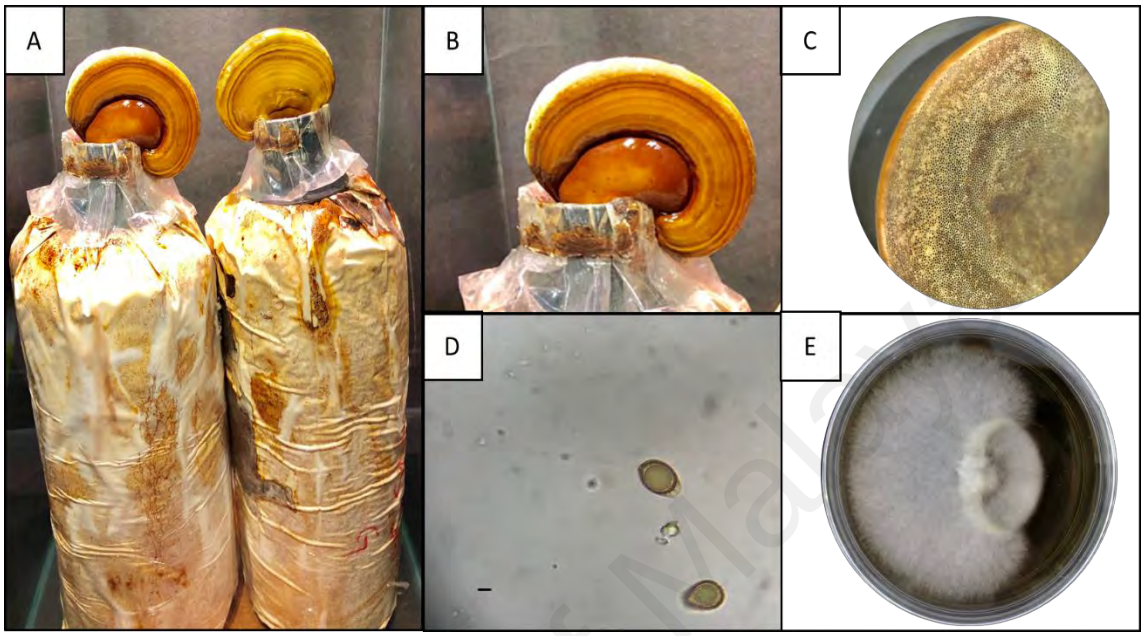

Figure 4.1: Morphological identification of *G. lucidum*. (A) *G. lucidum* obtained from a commercial mushroom farm. (B) Close-up image of *G. lucidum* fruiting body. (C) Hymenium pores at the back of *G. lucidum* fruiting body. (D) Basidiospores of *G. lucidum* (scale bar = 5 µm). (E) Mycelium of *G. lucidum* (Day 7)

Morphological identification was performed on the *G. lucidum* fruiting body, structure of basidiospores and the mycelium. Firstly, the fruiting body was analysed for its colour, shape and type. From Figure 4.1A, we can see the fruiting body is golden-yellowish in colour and Figure 4.1B; we can observe the fruiting body is kidney shaped. Then, the hymenium pores (Figure 4.1C) at the back of the fruiting body was observed and to resemble *G. lucidum*'s hymenium pores (Hennicke et al., 2016). Then, the spore's morphology was observed under a microscope at 100x magnification. In Figure 4.1D, the exosporium (hyaline) and endosporium was observed and was similar to the study conducted by Banerjee (1959). The mycelium's morphology is more compact and distinctive to each other, as shown in Figure 4.1E.

4.1.2 Gel electrophoresis

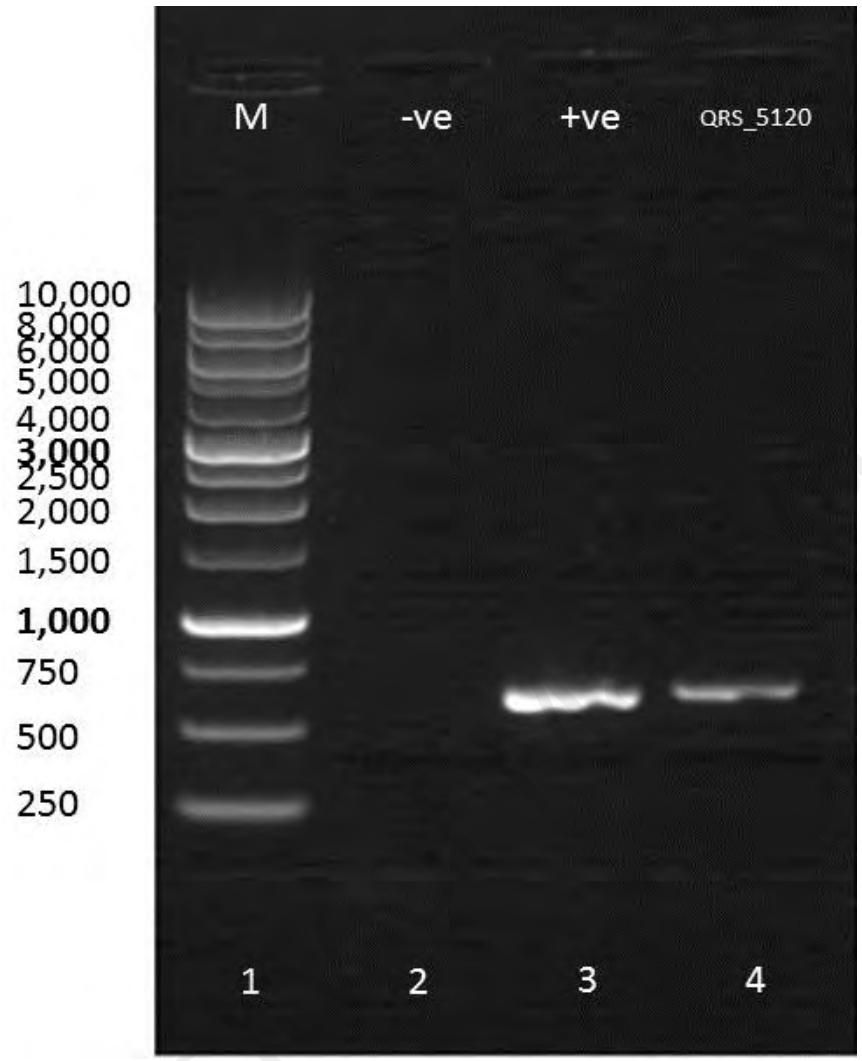

Figure 4.2: Agarose gel electrophoresis of DNA isolated from *G. lucidum* mycelium. Lane 1 corresponds to 10 kb marker. Lane 2 corresponds to the negative control (-ve), Lane 3 corresponds to a positive control (+ve) and Lane 4 corresponds to the sample (QRS 5120)

Molecular identification of a wild fungal sample is important because it distinguishes the species of wild fungal sample beforehand a study. Thus, the molecular identification was made on wild *G. lucidum*. The base pairs of wild *G. lucidum* was estimated using gel electrophoresis method. Figure 4.2 shows the Agarose gel under UV light. The marker (Lane 1) acts as the standard curve. By that, the base pairs of QRS 5120 were estimated to be 637bp (Appendix A).

4.1.3 Phylogenetic tree

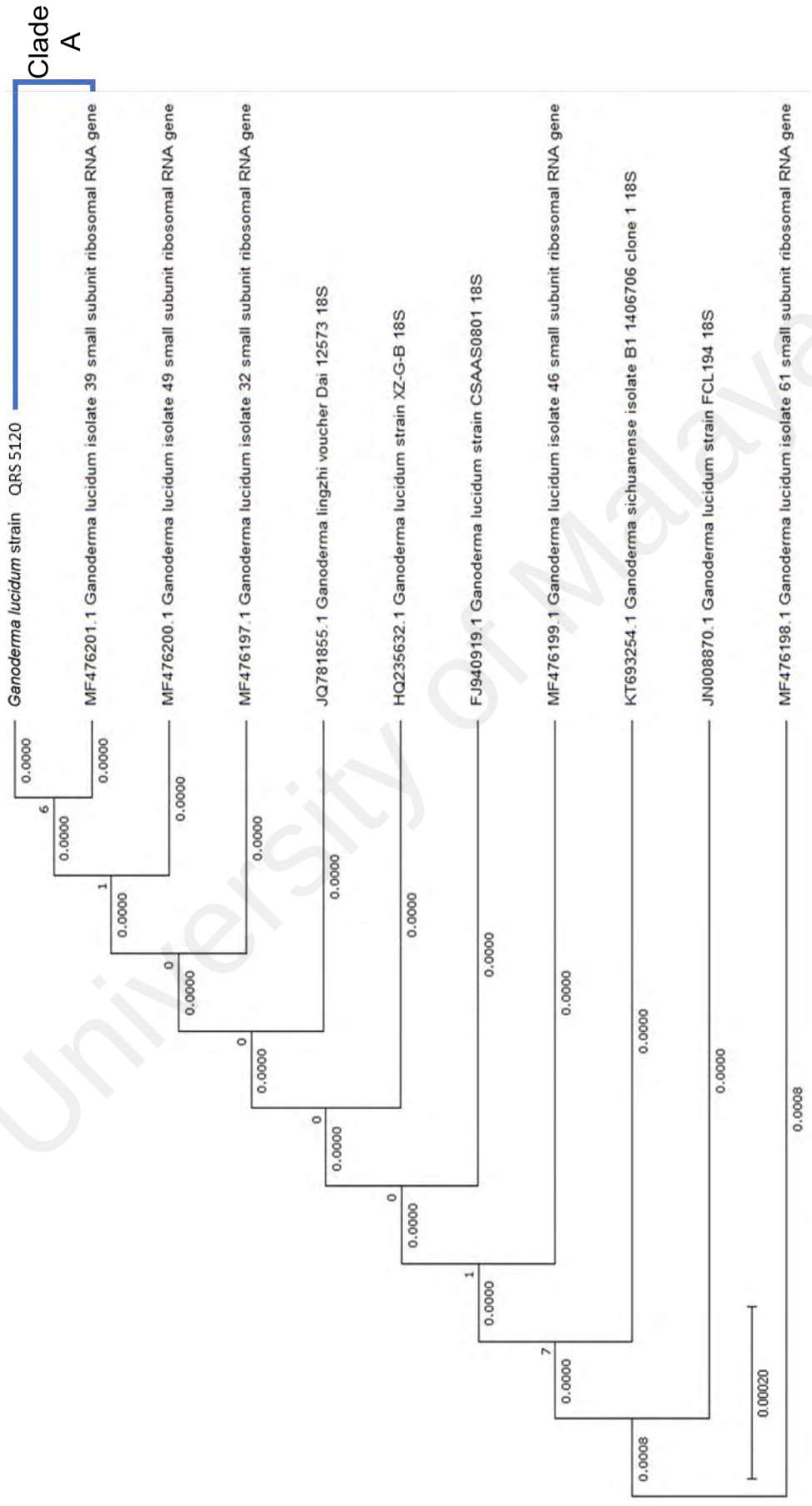

Figure 4.3: Phylogenetic tree of *G. lucidum* strain QRS_5120 with evolutionary distance. Bar = 0.00020

4.2 Verification of species

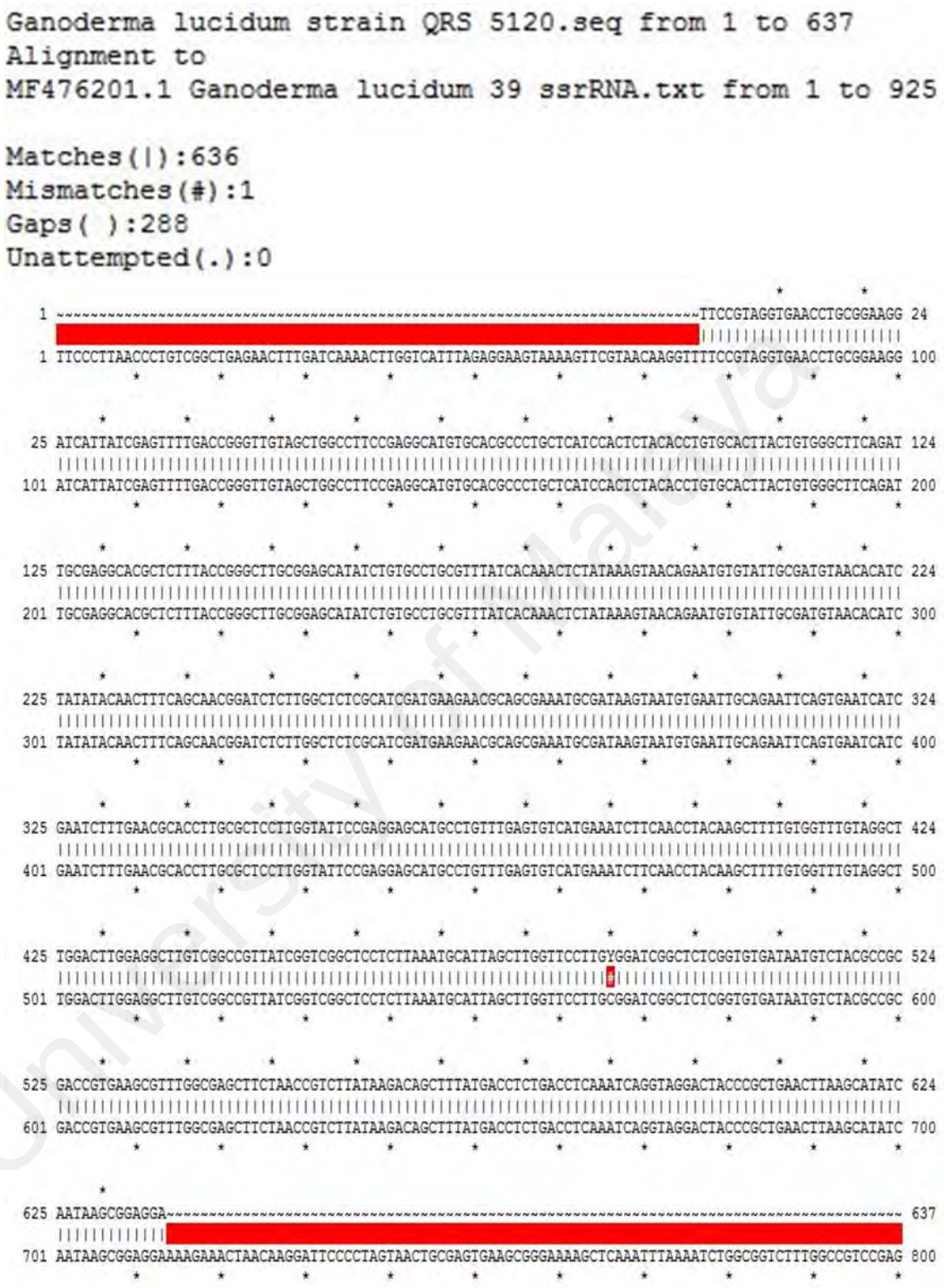

Figure 4.4: Species verification using A plasmid Editor (ApE) software

Upon sequencing the product, it was aligned with top-10 related species based on NCBI BLAST. Based on BLAST reference databases, QRS 5120 was found to be 99% *Ganoderma sp.* Detailed phylogenetic analyses (Figure 4.3), showed the evolutionary distance (K_{nuc}) values. Clade A showed *G. lucidum* QRS 5120 was closely related to *G. lucidum* isolate 39s compared to *G. lucidum* isolate 49s. In Figure 4.4, the result of ApE for the species verification is shown. From the result, out of 637 base pairs, 636 base pairs matched with *G. lucidum* 39 ssRNA. One base pair was mismatched at base pair 220 (QRS 5120) and base pair 566 (39 ssRNA). As shown, an unknown base pair was present in the DNA sequence of QRS 5120. Therefore, it is concluded as *G. lucidum* QRS 5120 with 99% similarities with *Ganoderma lucidum* 39 ssRNA.

4.3 Effect of initial pH on pellet morphology, biomass, exo– (EPS) and intracellular (IPS) polysaccharide production of *G. lucidum* QRS 5120 in submerged fermentation

The effect of initial pH ranging from pH 3 to pH 6 on pellet morphology, biomass, EPS and IPS production from *G. lucidum* QRS 5120 was tested.

4.3.1 Effect of initial pH on pellets morphology

The initial pH's influences the morphology of pellet formation and the pellet size (Supramani et al., 2019). This is due to the differences in hydrogen ions in liquid effects the cell membranes function (Wu et al., 2016a).

4.3.1.1 Macroscopic

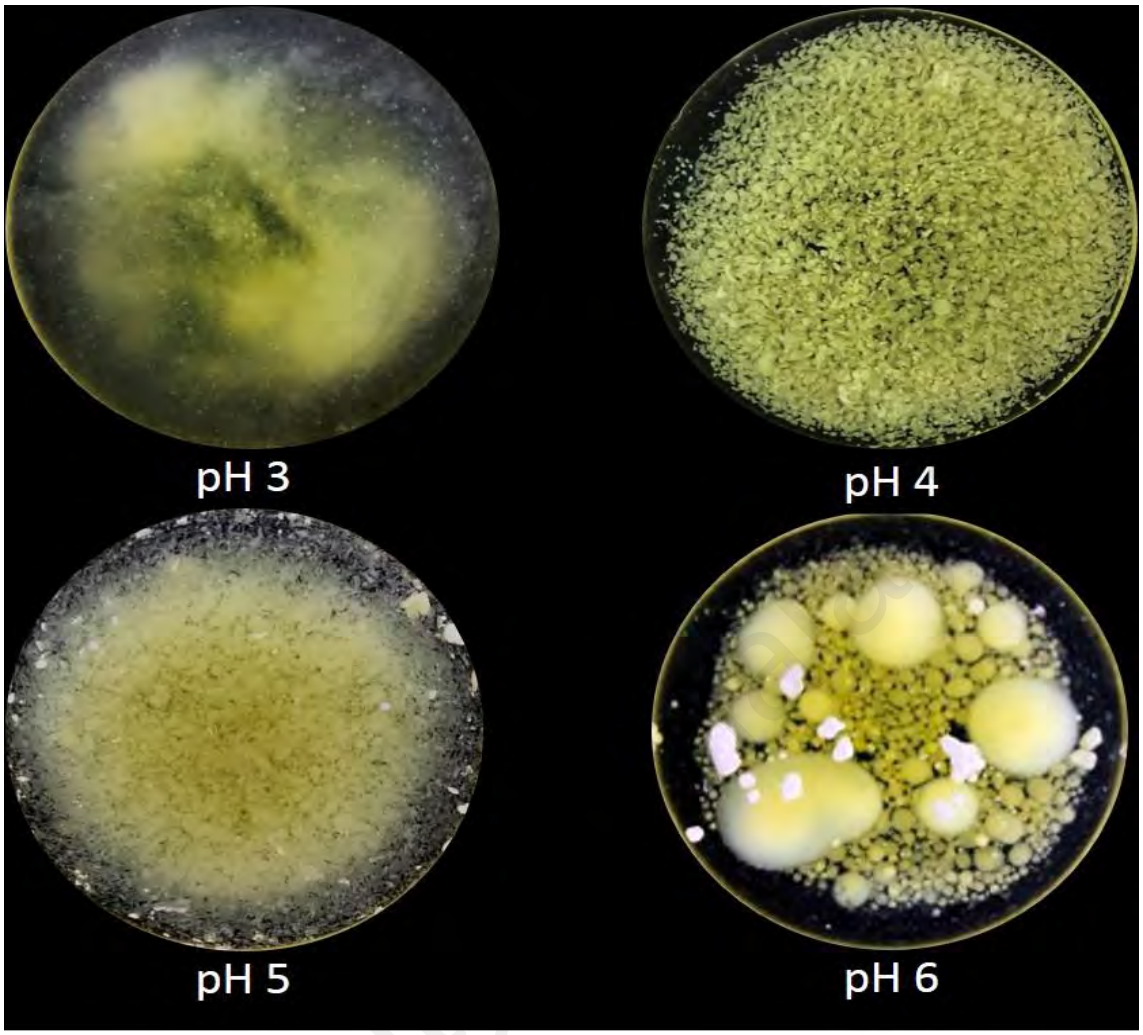

Figure 4.5: Morphological observation of *G. lucidum* QRS 5210 in different initial pH conditions at day 10 of cultivation

The effect of initial pH was studied as a preliminary test. The media component was maintained as described in Section 3.2.1, and the pH was adjusted to pH 3, pH 4, pH 5, and pH 6. From Figure 4.5, we can observe that initial pH effects the pellets morphology. Initial pH 3 showed highly dispersed mycelium; initial pH 4 showed small-hairy pellets, initial pH 5 showed small-dispersed pellets and initial pH 6 showed large smooth pellets. Pellets size plays an essential role in nutrient uptakes and polysaccharide production. The smaller the size of mycelium pellets, the larger the surface area of the mycelium pellets and therefore, more nutrients can be utilised for the polysaccharide production.

4.3.1.2 Microscopic

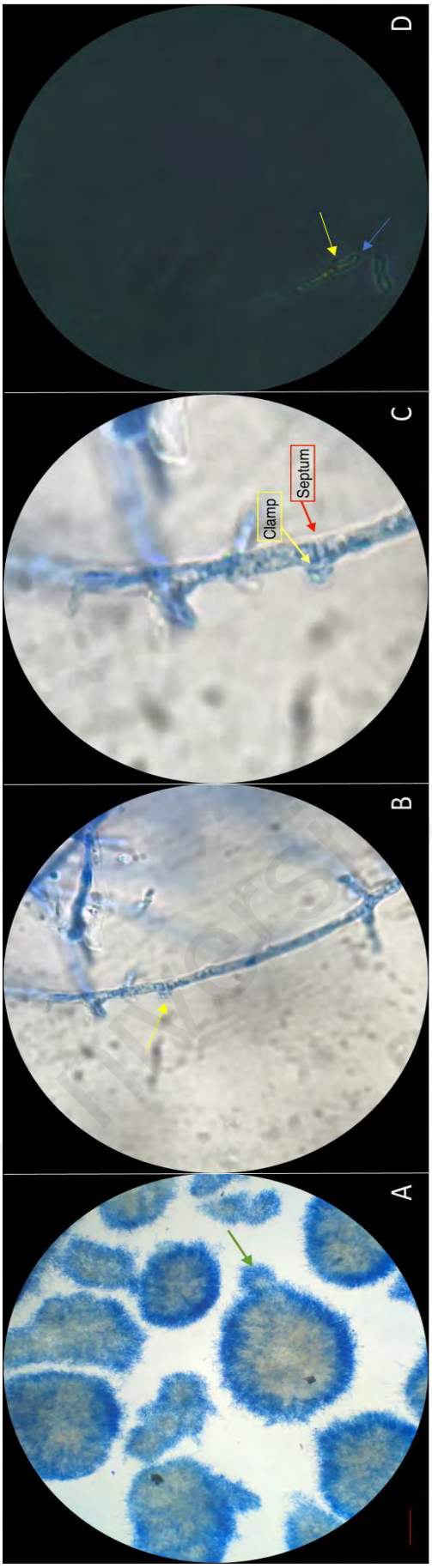

Figure 4.6: Microscopic observation of the pellets and the clamp connections (yellow arrow), subsequent pellet formation (green arrow), hyphal tip (blue arrow), and septum (red arrow) of *G. lucidum*. Image A was observed using a light microscope (40× magnification) and image B and C were taken at 1000 × magnification. Image D was observed using an inverted light microscope (400 × magnification). Bar = 30µm

Microscopic analysis was conducted to study the structure of mycelium. The basic mycelium structure of basidiomycetes consists of three structures, namely; crozier/clamp connection, septum and hyphal tip (Wan-Mohtar et al., 2016a). In Figure 4.6 A, the secondary pellet was observed (green arrow). This showed that the pellets propagate by forming a secondary pellet that detaches and forms a new pellet. Figure 4.6 B, C and D (yellow arrow), indicates the clamp connection/crozier. Clamp connection is essential to maintain dikaryotic state of the mycelium (Fazenda et al., 2008).

4.3.2 Effect of initial pH on biomass, EPS and IPS production

Table 4.1: Production of biomass, EPS and IPS at different initial pH conditions.

Initial pH	Biomass (g/L)	EPS (g/L)	IPS (g/L)
pH 4	5.13 ± 0.2	2.64 ± 0.3	1.46 ± 0.2
pH 5	4.20 ± 0.1	1.42 ± 0.01	0.6 ± 0.2
pH 6	3.90± 0.1	1.31 ± 0.1	0.32 ± 0.01

Initial pH plays a major role in submerged fermentation. According to Wang et al. (1995) and Fang et al. (2002), culture pH critically influences cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates and product biosynthesis. Therefore, a preliminary study was conducted to investigate the effect of initial pH on biomass, EPS and IPS production. Table 4.1 shows the concentration of biomass, EPS and IPS produced at different initial pH. The result obtained showed *Ganoderma lucidum* QRS 5120 preferred acidic environment. At pH 4, the biomass, EPS and IPS were the highest compared to pH 5 and 6. This statement was also supported by Wan-Mohtar et al. (2016a) and Liu et al. (2012).

4.3.3 Effect of initial pH on carbohydrate content in EPS and IPS

Table 4.2: Carbohydrate contents from exo and intracellular polysaccharide in different initial pH condition.

Initial pH	Carbohydrate content (EPS, g/L)	Carbohydrate content (IPS, g/L)
pH 4	0.435 ± 0.03	0.191 ± 0.003
pH 5	0.278 ± 0.01	0.105 ± 0.003
pH 6	0.269 ± 0.01	0.098 ± 0.005

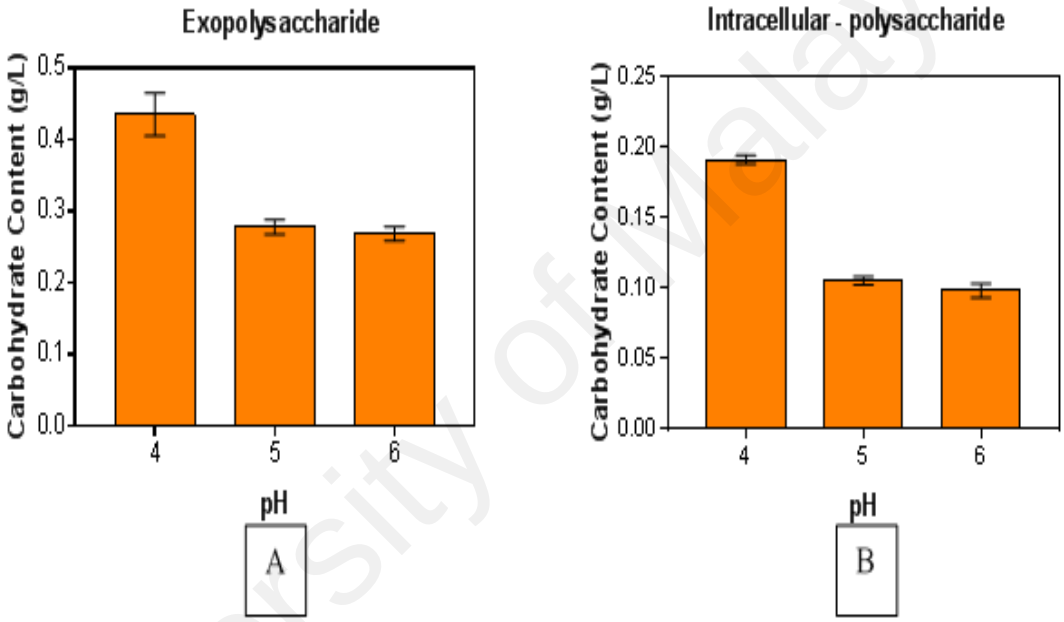

Figure 4.7: The total carbohydrate content in EPS and IPS. (A) Carbohydrate content in EPS and (B) carbohydrate content in IPS

Other than the effect on biomass, EPS and IPS concentration, pH also affected the carbohydrate content in both EPS and IPS. As shown in Table 4.2 and Figure 4.7, pH 4 produces the highest amount of carbohydrate in both EPS (Figure 4.7A) and IPS (Figure 4.7B). A study conducted by Fang et al. (2002), pH 3.5 favours higher exopolysaccharide and intracellular polysaccharide production. Therefore, lower pH favours high polysaccharide production.

4.4 Optimisation of growth parameters (initial pH, starting glucose concentration, agitation rate) using response surface methodology (RSM)

Using RSM, the effect of initial pH, starting glucose concentration, and agitation rate on the biomass, EPS and IPS production was investigated. CCD design, the levels of each variable and the responses are shown in Table 4.3. In total, 20 experiments were given by CCD and require the coefficient to be evaluated for each model using linear regression analysis. To estimate the significance of the model coefficient, a quadratic regression analysis using ANOVA was used. The significance of each coefficient was indicated by *p*-value. Level of significance shows the strength of the interaction between the independent variables.

Table 4.3: Experimental design matrix using RSM with CCD and responses for the mycelial biomass (DCW), EPS and IPS production from the mycelium of *G. lucidum* strain QRS 5120**.**

Run	Variables			Responses					
	Initial pH	Glucose	Agitation	Biomass (g/L)		EPS (g/L)		IPS (g/L)	
		g/L	rpm	A	P	A	P	A	P
1	4	10	90	4.6	4.52	2.2	2.31	1.2	1.28
2	6	50	110	3.8	3.92	1.2	1.13	0.2	0.14
3	6	50	90	3.1	2.97	0.9	0.91	0.2	0.25
4	5	30	100	4.1	4.12	1.1	1.31	0.8	0.85
5	5	30	90	3.9	3.6	1.3	1.15	0.7	0.73
6	5	50	100	3.8	3.96	1	0.91	0.9	0.83
7	5	30	100	4.2	4.12	1.4	1.31	0.9	0.85
8	4	50	110	5.1	4.79	2.1	2.18	1.4	1.53
9	5	30	100	3.8	4.12	1	1.31	0.9	0.85
10	4	50	90	4.9	5.04	1.9	1.96	1.5	1.44
11	5	30	100	3.9	4.12	1.3	1.31	0.7	0.85
12	5	30	110	4.2	4.3	1.4	1.37	0.9	0.77
13	5	30	100	4.2	4.12	1.5	1.31	0.8	0.85
14	5	30	100	4.1	4.12	1.2	1.31	0.8	0.85
15	4	30	100	5.2	5.26	2.9	2.61	1.7	1.57
16	6	10	110	4.1	4	1.2	1.18	0.1	0.18
17	6	30	100	4	3.74	1.3	1.41	0.3	0.33
18	4	10	110	4.8	4.97	2.5	2.53	1.5	1.47
19	5	10	100	4.1	3.74	1.2	1.11	0.8	0.77
20	6	10	90	2	2.35	1	0.96	0.3	0.19

A: Actual value; P: Predicted value

4.4.1 Optimisation of Mycelium Biomass Growth

The analysis of variance (ANOVA) for mycelium biomass production is as shown in Table 4.4. The predicted coefficient determination indicates 91.47 % ($R^2 = 0.9147$) of the variability in the response can be explained using this model. The model is significant ($p < 0.005$). The adjusted coefficient determination value (Adj. $R^2 = 0.84$) implies the significance of the model and is within reasonable agreement with the predicted R^2 value. By considering the significant terms, the model in term of actual variables of biomass was regressed and is expressed by Eq.4.1.

$$\begin{aligned}\text{Biomass} = {} & 9.515227273 - 7.661136364 \times \text{pH} + 0.126295455 \times \text{Glucose} \\ & + 0.238522727 \times \text{Agitation} + 0.386363636 \times \text{pH}^2 \\ & - 0.000659091 \times \text{Glucose}^2 - 0.001636364 \times \text{Agitation}^2 \\ & + 0.00125 \times \text{pH} \times \text{Glucose} + 0.03 \times \text{pH} \times \text{Agitation} \\ & - 0.000875 \times \text{Glucose} \times \text{Agitation} \end{aligned} \quad (4.1)$$

Table 4.4: Analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for biomass from the mycelium of *G. lucidum* strain QRS 5120.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	8.588136364	9	0.954237374	11.90767001	0.0003
A: pH	5.776	1	5.776	72.07714124	< 0.0001
B: Glucose	0.121	1	0.121	1.509926262	0.2473
C: Agitation	1.225	1	1.225	15.28644356	0.0029
A^2	0.410511364	1	0.410511364	5.122660238	0.0471
B^2	0.191136364	1	0.191136364	2.385138968	0.1535
C^2	0.073636364	1	0.073636364	0.918888259	0.3604
AB	0.005	1	0.005	0.062393647	0.8078
AC	0.72	1	0.72	8.984685196	0.0134
BC	0.245	1	0.245	3.057288712	0.1109
Residual	0.801363636	10	0.080136364		
Lack of Fit	0.666363636	5	0.133272727	4.936026936	0.0522
Pure Error	0.135	5	0.027		
Cor Total	9.3895	19			

Std. Dev. = 0.28308366896796 **R^2 = 0.91465321514845**

Mean = 4.095 **Adjusted R^2 = 0.83784110878205**

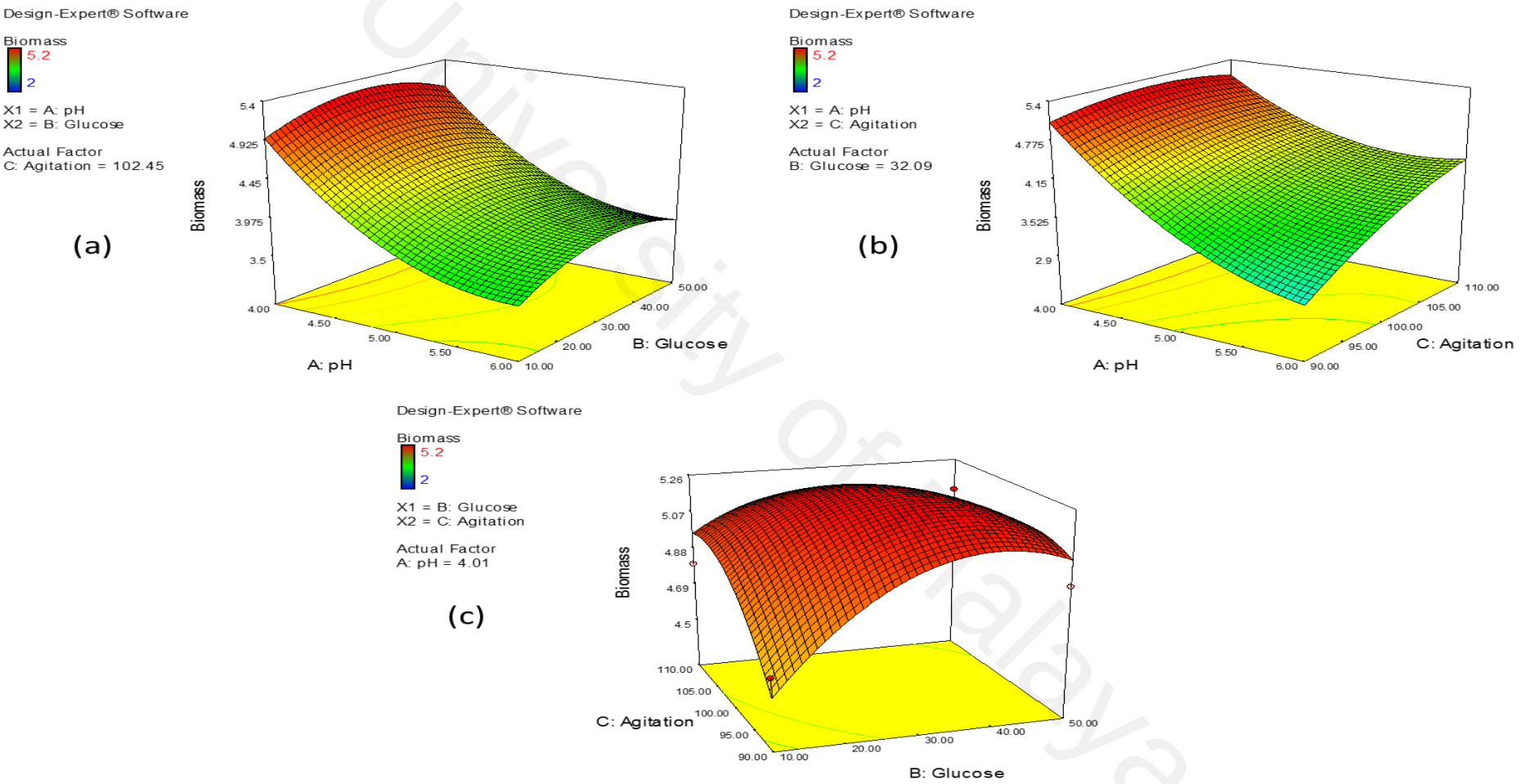

Figure 4.8: Response surface curve (3D plot) of mycelium biomass from *G. lucidum* strain QRS 5120 showing the interaction between (a) pH and glucose, (b) pH and agitation, (c) Glucose and agitation

From the model, initial pH (A) shows the strongest effect ($p < 0.0001$) on biomass meanwhile agitation (C) shows significant effect at $p < 0.05$. Both quadratic terms of initial pH (AA) and initial pH and agitation (AC) shows a significant effect at $p < 0.05$ on the yield of mycelium biomass. However, negative effects are shown by glucose (B) and quadratic terms (B^2, C^2, AB, and BC). Figure 4.8 shows the combined effect of initial pH, glucose concentration and agitation in three-dimensional plots (3D). One of the factors is at the optimum level, and the other two factors are within the experimental range. Figure 4.8A shows the effect of initial pH (A) and starting glucose concentration (B), Figure 4.8B shows the effect of A and agitation rate (C), and Figure 4.8C shows the effect of B and C on biomass production. From Figure 4.8A and 4.8B, it is obvious that increasing initial pH leads to decrease of mycelium biomass, agitation at all rate shows high mycelium biomass production and starting glucose concentration is normally distributed. The maximum mycelium biomass obtained was at initial pH 4.01, glucose concentration 32.09 g/L and at 102.45 rpm. From Figure 4.8C, shows no significant effect of B and C for mycelium biomass production.

4.4.2 Optimisation of EPS production

The analysis of variance (ANOVA) of EPS production is, as shown in Table 4.5. The predicted coefficient determination indicates 93.58 % (R^2 = 0.9358) of the variability in the response can be explained using this model. The model is significant ($p < 0.005$). The adjusted coefficient determination value (Adj. R^2 = 0.8780) implies the significance of the model and is within reasonable agreement with the predicted R^2 value. By considering the significant terms, the model in term of actual variables of biomass was regressed and is expressed by Eq. 4.2.

$$\begin{aligned}\text{EPS} = \\ &15.17090909 - 7.667045455 \times \text{pH} + 0.021931818 \times \text{Glucose} \\ &+ 0.120090909 \times \text{Agitation} + 0.695454545 \times \text{pH}^2 \\ &- 0.000761364 \times \text{Glucose}^2 - 0.000545455 \times \text{Agitation}^2 \\ &+ 0.00375 \times \text{pH} \times \text{Glucose} - 4.76916\text{E}{-16} \times \text{pH} \times \text{Agitation} \\ &- 3.80257\text{E}^{-18} \times \text{Glucose} \times \text{Agitation}\end{aligned} \quad (4.2)$$

Table 4.5: Analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for EPS production the mycelium of *G. lucidum* strain QRS 5120.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	5.364181818	9	0.596020202	16.20420717	< 0.0001
A: pH	3.6	1	3.6	97.8744439	< 0.0001
B: Glucose	0.1	1	0.1	2.718734553	0.1302
C: Agitation	0.121	1	0.121	3.289668809	0.0998
A^2	1.330056818	1	1.330056818	36.16071429	0.0001
B^2	0.255056818	1	0.255056818	6.934317845	0.0250
C^2	0.008181818	1	0.008181818	0.222441918	0.6473
AB	0.045	1	0.045	1.223430549	0.2946
AC	0	1	0	0	1.0000
BC	0	1	0	0	1.0000
Residual	0.367818182	10	0.036781818		
Lack of Fit	0.192818182	5	0.038563636	1.101818182	0.4589
Pure Error	0.175	5	0.035		
Cor Total	5.732	19			
Std. Dev. = 0.19178586543804		**R^2 = 0.93583074287889**			
Mean = 1.48		**Adjusted R^2 = 0.8780784114699**			

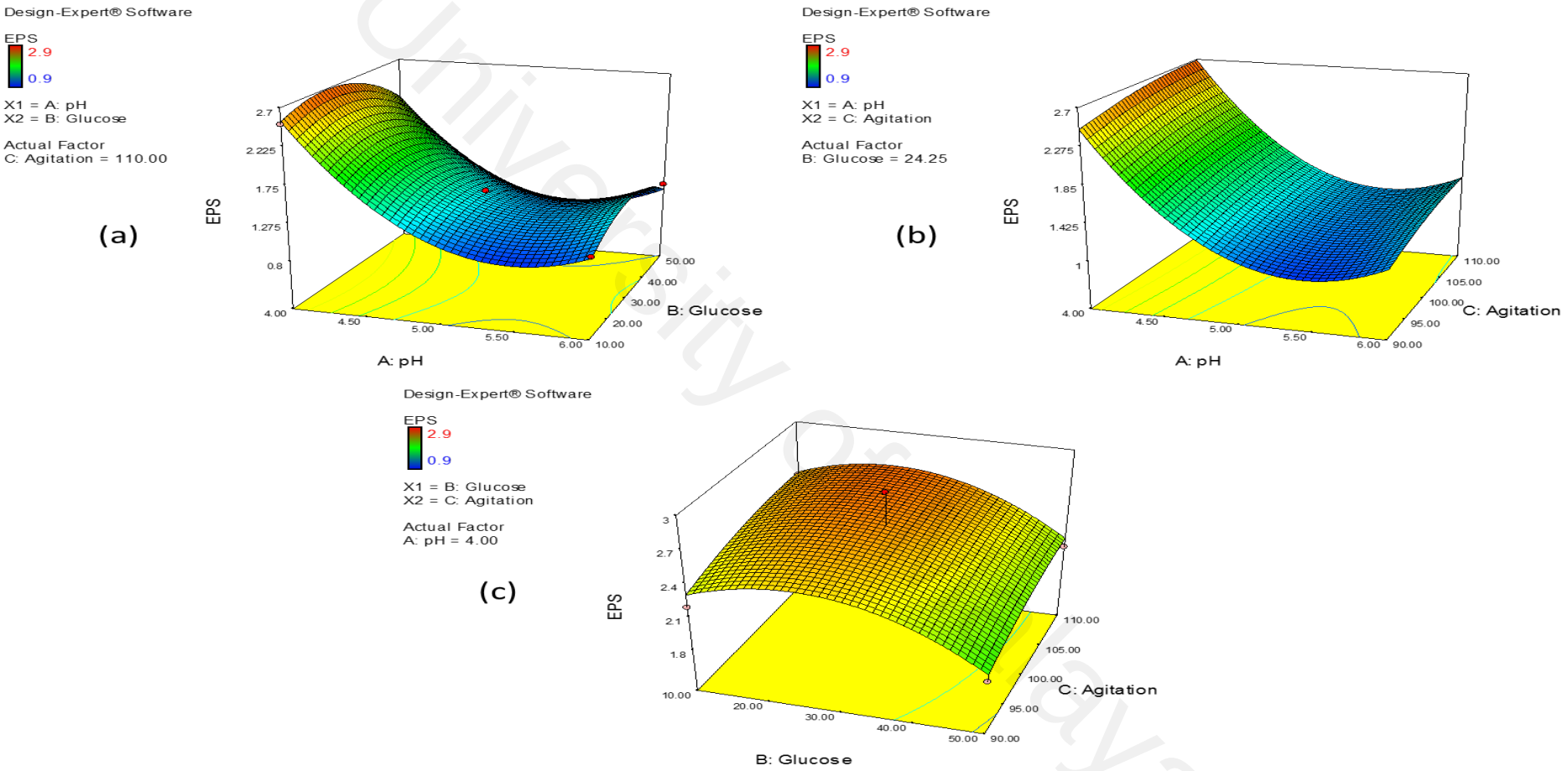

Figure 4.9: Response surface curve (3D plot) of EPS production from *G. lucidum* strain QRS 5120 showing the interaction between (a) pH and glucose, (b) pH and agitation, (c) Glucose and agitation

From the model, initial pH (A) shows the strongest effect ($p < 0.0001$) on EPS concentration meanwhile both quadratic terms of initial pH (AA) and initial pH and glucose (BB) shows significance effect at $p < 0.005$ and $p < 0.05$ respectively on the EPS production. However, negative effects are shown by glucose (B), agitation (C) and quadratic terms (C^2, AB, AC, and BC). Figure 4.9 shows the combined effect of initial pH, glucose concentration and agitation in three-dimensional plots (3D). One of the factors is at the optimum level, and the other two factors are within the experimental range. Figure 4.9A shows the effect of initial pH (A) and starting glucose concentration (B), Figure 4.9B shows the effect of A and agitation rate (C), and Figure 4.9C shows the effect of B and C on biomass production. From Figure 4.9A and 9B, it is obvious that increasing initial pH leads to a decrease in EPS production, agitation at all rate shows high EPS production and starting glucose concentration is normally distributed. The maximum EPS obtained was at initial pH 4, glucose concentration 24.25 g/L and at 110rpm. Figure 4.9C shows no significance effect of B and C for mycelium biomass production.

4.4.3 Optimisation of IPS production

The analysis of variance (ANOVA) of IPS production is as shown in Table 4.6. The predicted coefficient determination indicates 96.88 % (R^2 = 0.9688) of the variability in the response can be explained using this model. The model is highly significant (p < 0.0001). The adjusted coefficient determination value (Adj. R^2 = 0.9407) implies the significance of the model and is within reasonable agreement with the predicted R^2 value. By considering the significant terms, the model in term of actual variables of biomass was regressed and is expressed by Eq. 4.3.

$$\begin{aligned}\text{IPS} = & \\ & -6.393409091 - 1.127954545 \times \text{pH} + 0.027068182 \times \text{Glucose} \\ & + 0.221659091 \times \text{Agitation} + 0.104545455 \times \text{pH}^2 \\ & - 0.000113636 \times \text{Glucose}^2 - 0.000954545 \times \text{Agitation}^2 \\ & - 0.00125 \times \text{pH} \times \text{Glucose} - 0.005 \times \text{pH} \times \text{Agitation} \\ & - 0.000125 \times \text{Glucose} \times \text{Agitation}\end{aligned} \quad (4.3)$$

Table 4.6: Analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for IPS production the mycelium of *G. lucidum* strain QRS 5120.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	3.935181818	9	0.437242424	34.47789725	< 0.0001
A: pH	3.844	1	3.844	303.1111111	< 0.0001
B: Glucose	0.009	1	0.009	0.709677419	0.4192
C: Agitation	0.004	1	0.004	0.315412186	0.5867
A^2	0.030056818	1	0.030056818	2.370071685	0.1547
B^2	0.005681818	1	0.005681818	0.448028674	0.5184
C^2	0.025056818	1	0.025056818	1.975806452	0.1901
AB	0.005	1	0.005	0.394265233	0.5441
AC	0.02	1	0.02	1.577060932	0.2377
BC	0.005	1	0.005	0.394265233	0.5441
Residual	0.126818182	10	0.012681818		
Lack of Fit	0.098484848	5	0.01969697	3.475935829	0.0989
Pure Error	0.028333333	5	0.005666667		
Cor Total	4.062	19			
Std. Dev. = 0.11261357902943		**R^2 = 0.96877937424466**			
Mean = 0.83		**Adjusted R^2 = 0.94068081106486**			

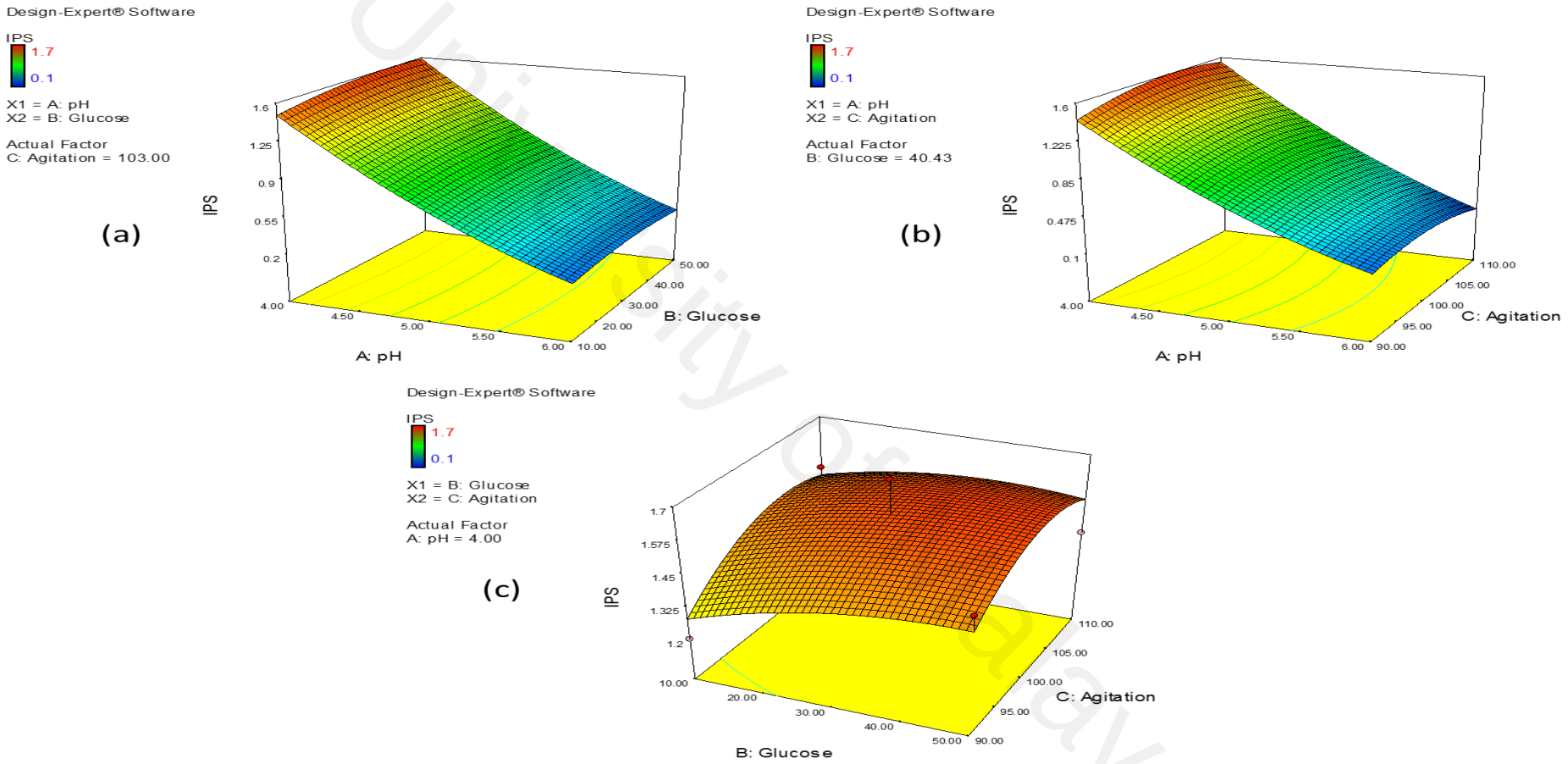

Figure 4.10: Response surface curve (3D plot) of IPS production from *G. lucidum* strain QRS 5120 showing the interaction between (a) pH and glucose, (b) pH and agitation, (c) Glucose and agitation

From the model, initial pH (A) shows the strongest effect ($p < 0.0001$) on IPS concentration. However, negative effects are shown by glucose (B), agitation (C) and quadratic terms (A^2, B^2, C^2, AB, AC, and BC). Figure 4.10 shows the combined effect of initial pH, glucose concentration and agitation in three-dimensional plots (3D). One of the factors is at the optimum level, and the other two factors are within the experimental range. Figure 4.10A shows the effect of initial pH (A) and starting glucose concentration (B), Figure 4.10B shows the effect of A and agitation rate (C), and Figure 4.10C shows the effect of B and C on biomass production. From Figure 4.10A and 4.10B, it is obvious that increasing initial pH leads to decrease of IPS production, agitation at all rate shows high IPS production and at all concentration of starting glucose gives high IPS concentration. By this, it is observed that there is no interaction between the factors. The maximum IPS obtained was at initial pH 4, glucose concentration 40.45 g/L and at 103 rpm. From Figure 4.10C, shows no significant effect of B and C for IPS production as well.

4.4.4 Verification of Optimised Conditions

Table 4.7: Validation of the model with optimised conditions.

Run	Variables			Response		
	pH	Glucose	Agitation	Biomass (g/L)	EPS (g/L)	IPS (g/L)
Biomass	4.01	32.09	102.45	5.12 ± 0.5	-	-
EPS	4	24.25	110	-	2.49 ± 0.8	-
IPS	4	40.43	103	-	-	1.52 ± 0.4
Biomass + EPS	4	24.75	107.58	5.11 ± 0.4	2.57 ± 0.7	-
Biomass + IPS	4	40.45	102.95	5.13 ± 0.5	-	1.57 ± 0.3
EPS + IPS	4	26.5	105.92	-	2.62 ± 0.4	1.52 ± 0.6
Biomass + EPS + IPS	4	26.52	102.9	5.19 ± 0.6	2.64 ± 0.6	1.52 ± 0.4

Table 4.7 shows the optimised conditions, which were applied to verify mycelium biomass, EPS and IPS production statistical model. To verify the predictive ability of the model, Eq. 4.1, 4.2 and 4.3, experiments were done. The mycelium biomass (5.12 g/L), EPS (2.49 g/L), and IPS (1.52 g/L) were produced under optimized condition, which agrees with the predicted value (5.25 g/L), (2.69 g/L) and (1.59 g/L) respectively. These showed that models (Eq.4.1), (Eq.4.2) and (Eq.4.3) are valid for mycelium biomass, EPS and IPS production, respectively.

Table 4.8: Comparison of Ganoderma lucidum optimisation using submerged-liquid fermentation with the literature.

Optimisation method	Cultivation mode	Initial pH	Glucose concentration	Agitation	EPS	IPS	Biomass	Reference
Response surface methodology	Shake Flask	4	26.5	100	2.64	1.52	5.19	(Supramani et al., 2019)
Taguchi's orthogonal array	Shake Flask	6.5	12.1	160	0.42	NA*	18.7	(Chang et al., 2006)
Orthogonal matrix	Shake Flask	NA*	50	150	1.723	NA*	7.235	(Yuan et al., 2012)

*NA = Not available, EPS = Exopolysaccharide, IPS = Intracellular polysaccharide

Supramani, S., Ahmad, R., Ilham, Z., Annuar, M. S. M., Klaus, A., & Wan-Mohtar, W. A. A. Q. I. (2019). Optimisation of biomass, exopolysaccharide and intracellular polysaccharide production from the mycelium of an identified *Ganoderma lucidum* strain QRS 5120 using response surface methodology. *AIMS Microbiology, 5*(1), 19-38.

Chang, M. Y., Tsai, G. J., & Houng, J. Y. (2006). Optimization of the medium composition for the submerged culture of *Ganoderma lucidum* by Taguchi array design and steepest ascent method. *Enzyme and Microbial Technology, 38*(3-4), 407-414.

Yuan, B. J., Chi, X. Y., & Zhang, R. J. (2012). Optimization of Exopolysaccharides Production from a Novel Strain of *Ganoderma lucidum* Cau5501 in Submerged Culture. *Brazilian Journal of Microbiology, 43*(2), 490-497.

A current statistical optimisation approach to determine the preeminent parameters for obtaining efficient biomass, EPS, and IPS production using *Ganoderma lucidum* in a controlled shake-flask fermentation are shown in Table 4.8. As reported, only two previous studies utilizing *G. lucidum* for this purpose were reported, which only utilized EPS and biomass production. Chang et al. (2006) stated that the mycelium formation and polysaccharide production was markedly improved by cultivating in the optimal medium under the optimal operating conditions. On the other hand, Yuan et al. (2012) also supported by stating that optimal media increases the EPS production and mycelium formation remarkably. The current study was more efficient in producing biomass and EPS concentration compared to Yuan et al. (2012) and Chang et al. (2006), with the addition of IPS. The optimised key parameters reported in this study are the latest for *G. lucidum* using RSM on initial pH, glucose concentration and agitation in enhancing the production of biomass, EPS and IPS. The current optimised method can be applied to achieve a combination of lower biomass but higher EPS and IPS in specialised bioreactors.

4.5 Determination of the productive type of pellet morphology for exo (EPS)- and intracellular (IPS) – polysaccharide production in submerged fermentation (SmF) of *Ganoderma lucidum* QRS 5120

The pellet morphology influences the production of EPS and IPS due to the branching out hyphae (Wan-Mohtar et al., 2016a). Therefore, the optimised pellet morphology for highest production of EPS and IPS was investigated.

4.5.1 Morphological analysis

The morphological analysis was performed macroscopically (Section 4.5.1.1) and microscopically (Section 4.5.1.2)

4.5.1.1 Macroscopic

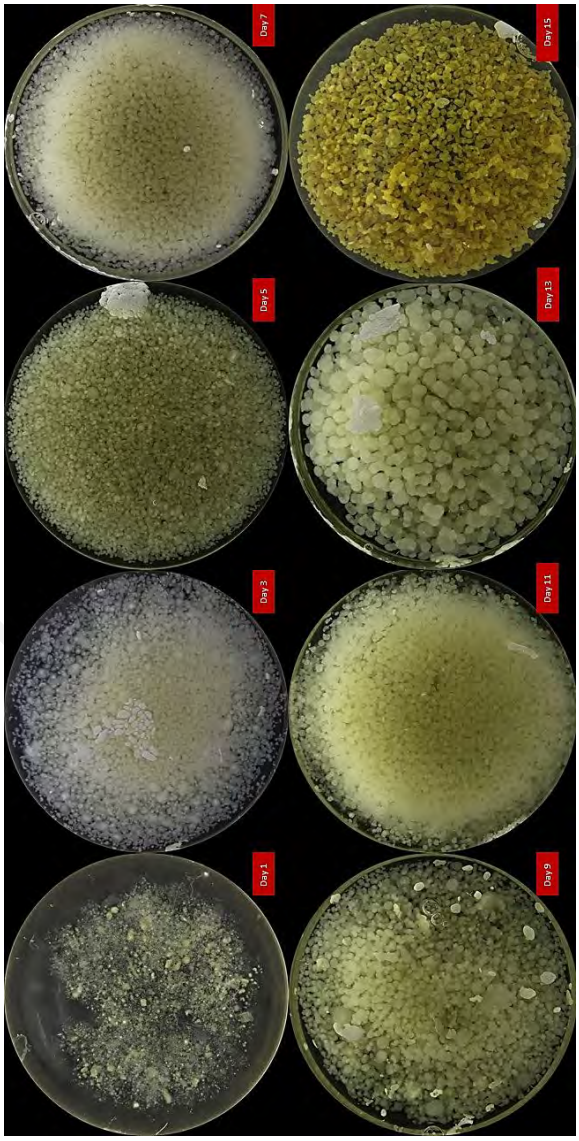

Figure 4.11: Macroscopic images of *G. lucidum* strain QRS 5120 pellets in submerged fermentation (SmF)

4.5.1.2 Microscopic

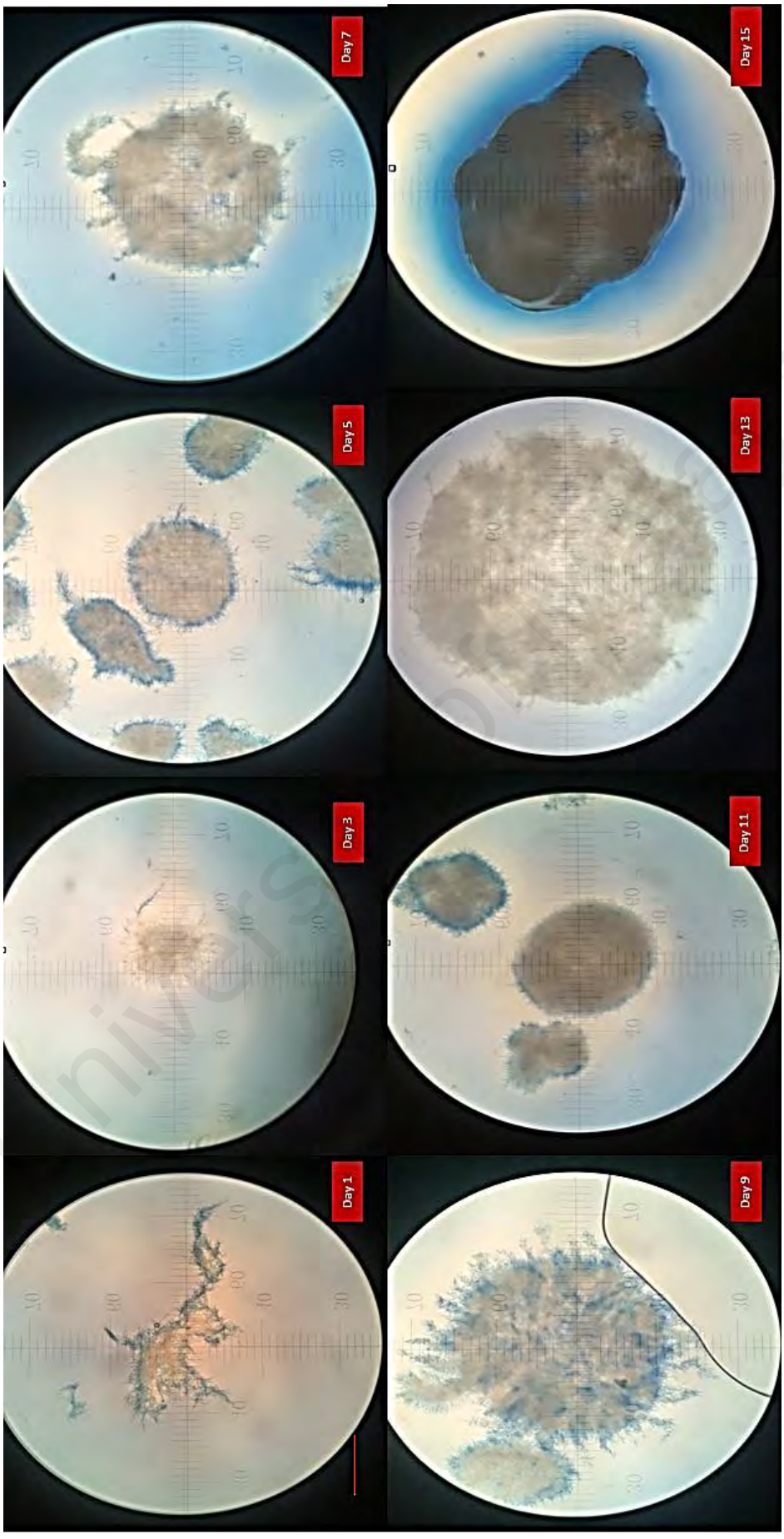

Figure 4.12: Microscopic images of *G. lucidum* strain QRS 5120 pellets in submerged fermentation (SmF). Bar = 100µm

To understand how does morphology of the pellets interact with each phase of growth, morphological analysis was performed up to 15th day. *G. lucidum* QRS 5120 undergoes death phase at day 15. Figure 4.11 shows the macroscopic morphology analysis and Figure 4.12 shows the microscopic analysis of *G. lucidum* QRS 5120. We can observe at day 1, the mycelium was in a dispersed manner, and slowly they clump up together to form ovoid pellets. At day 11, the pellets were ovoid-hairy pellets (Figure 4.12, day 11). According to Wan-Mohtar et al. (2016a), starburst-like pellets gave the ideal EPS production. At day 13, the pellets were a bigger and almost smooth surface. The bigger size leads to cell death, due to oxygen transfer limitation. The inner part of the pellet gets deprived of oxygen and becomes hollow (Figure 4.12, day 13). This is the reason why larger pellet produces more intracellular polysaccharide. The hallow part of the pellets gets filled with the polysaccharide. This was supported by a study conducted by Espinosa-Ortiz et al. (2016). At day 15, the death phase by referring to a growth profile, it tallies with the morphology. The pellets shrunk in size and turned dark yellowish-brown, which indicated autolysis had occurred.

4.5.2 Large scale fermentation

Figure 4.13: Process of large-scale biomass production of *G. lucidum* in Bioreactor 2-L (Sartorius Biostat A plus). Image A shows Bioreactor 2-L (Sartorius Biostat A plus) without inoculation. Image B shows Bioreactor 2-L (Sartorius Biostat A plus) inoculated with *G. lucidum.* (Day 3) [Working volume = 1.5-L] and image C shows Bioreactor 2-L (Sartorius Biostat A plus) inoculated with *G. lucidum.* (Day 10) [Working volume = 1.5-L]

After completing the growth profile, *G. lucidum* QRS 5120 was subjected to the 2-L bioreactor, as shown in Figure 4.13. Using the optimised condition and growth profiling, the yield rate was calculated in the bioreactor. In the 2-L stirred-tank bioreactor (STR), 1.9-fold higher biomass (9.75 g/L: smooth pellets) and 2.1-fold higher EPS (5.43 g/L: ovoid-hairy pellets) were generated compared to shake flask. By this, we have maximised the biomass and EPS production in a shorter period.

4.6 Characterisation of polysaccharide from *G. lucidum* QRS 5120

4.6.1 FTIR spectroscopy

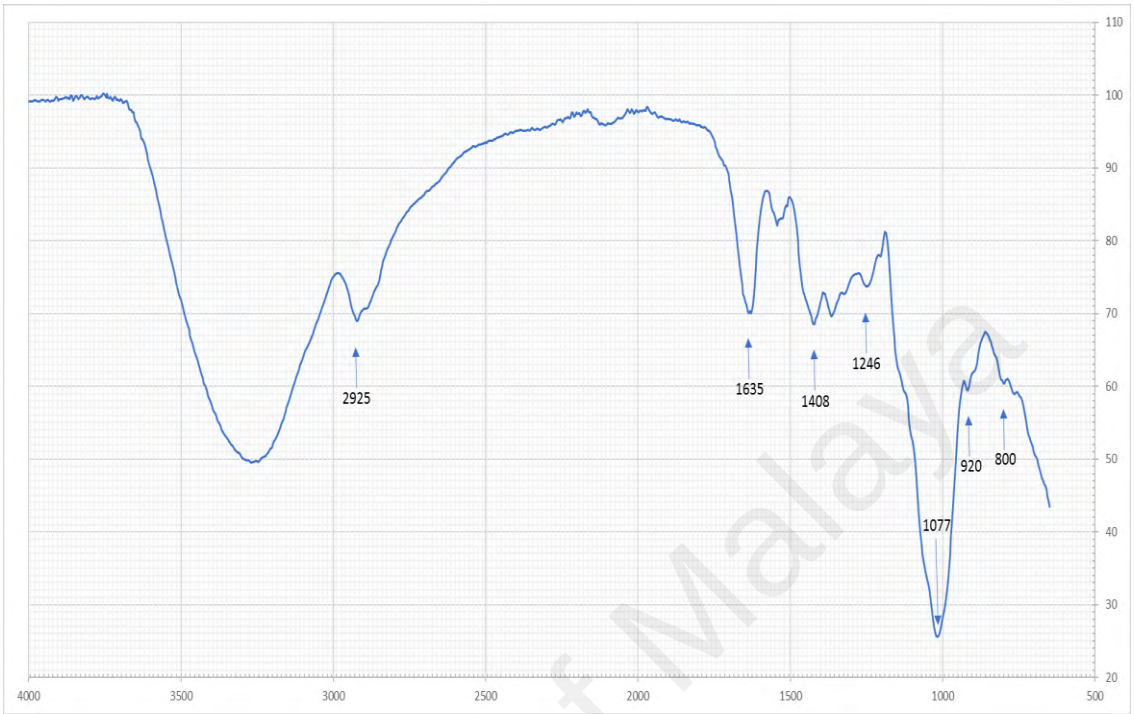

Figure 4.14: FTIR spectroscopy of crude EPS from *G. lucidum* strain QRS 5120

FTIR spectroscopy was used to identify the functional groups characteristic of exopolysaccharide from *G. lucidum* QRS 5120 (Figure 4.14). The broad-stretched peak from 3500 cm^{-1} to 3000cm^{-1} indicated stretching vibration of OH group in the sugar residue (Zhou et al., 2014). The weak peak at 2925 cm^{-1} is associated with stretching vibration of C-H in the sugar ring. C=O stretching vibration falls in between 1600 cm^{-1} – 1650cm^{-1}. In Figure 4.14, we can observe a peak at 1635cm^{-1}, which indicates the presence of C=O. The absorbance at 1077 cm^{-1} shows the present of C-O-C and -OH in pyran structure (β - glucans), and the absorbance at 920 $cm^{-1}$and 800cm^{-1} indicates the α - linked glycosyl (Kozarski et al., 2012; Osińska-Jaroszuk et al., 2014). These structural confirmations indicate the EPS from *G. lucidum* QRS 5120 contains β - glucans and α - linked glycosyl.

4.6.2 ^{1}H – NMR spectroscopy

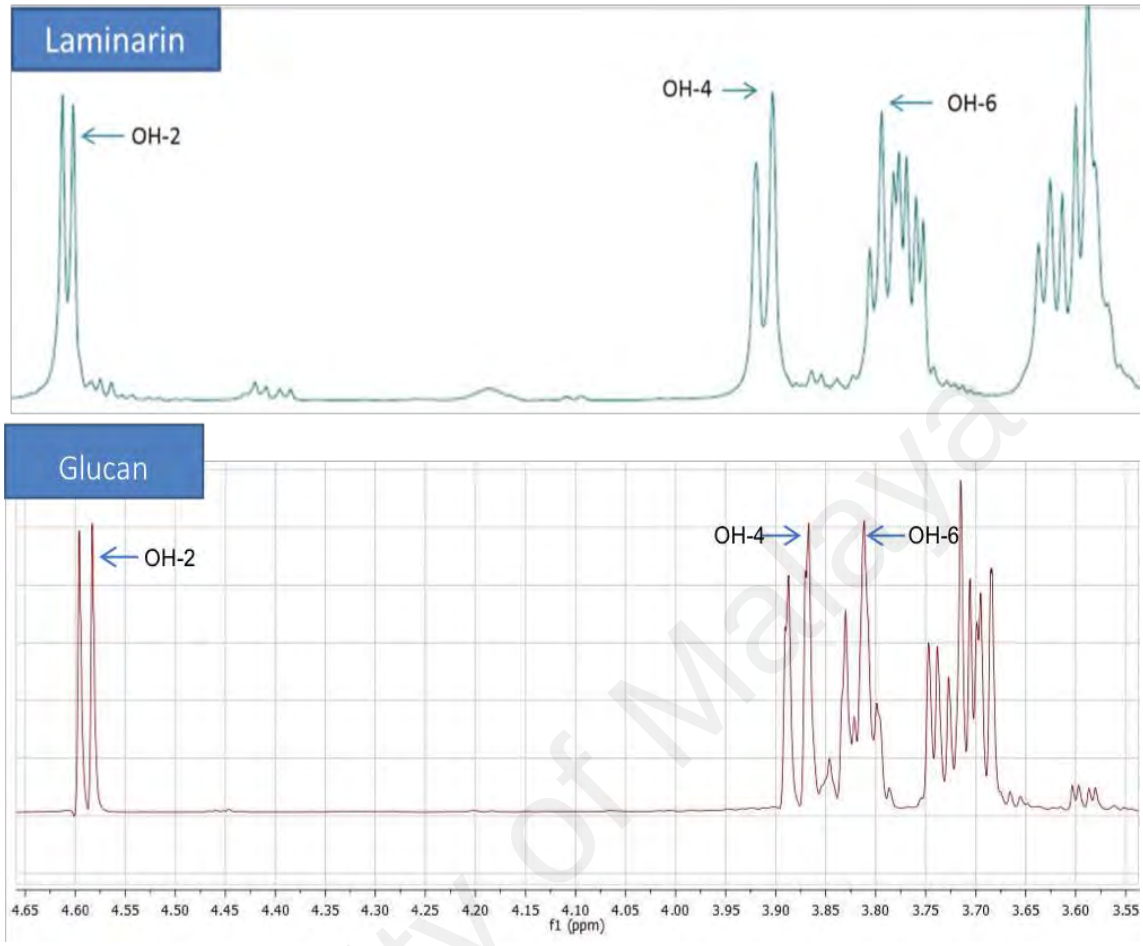

Figure 4.15: ^{1}H - NMR spectroscopy of glucan (G) obtained from *G. lucidum* strain QRS 5120 compared with standard (Laminarin)

Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) was used to study the chemical structure of G obtained from *G. lucidum* strain QRS 5120. ^{1}H-NMR spectrum for crude EPS of *G. lucidum* QRS 5120 was obtained at room temperature (25℃) using $D_2$0-*d* as a solvent. ^{1}H-NMR spectra of G were compared with laminarin (standard β-1,3-D-glucan) from *L. digitata*. The signals at δ 4.58, 3.87 and 3.81 were assigned to OH-2, OH-4, and OH-6, respectively comparing to laminarin (Wan-Mohtar et al., 2016c). From the FTIR (Figure 4.14) result and ^{1}H-NMR (Figure 4.15) result, it is possible to conclude that G was composed of (1-3)-β-D-linkages.

4.7 Screening potential bioactivity of glucan sulphate (GS) extracted from *G. lucidum* strain QRS 5120

4.7.1 Antimicrobial test

Table 4.8: Zone of inhibition by GS.

No./G	Bacteria	GS			Mean	Positive Control
		The diameter of Zone of Inhibition (mm)				Vancomycin (30 µg)
		89 mg/ml				
		Plate 1	Plate 2	Plate 3		
1 (-ve)	*Escherichia coli*	33.26	33.35	33.35	33.32 ± 0.05	13.5 ± 0.3
2 (-ve)	*Serratia marcescens*	24	26.5	23.25	24.58 ± 1.7	0 (no inhibition)
3 (+ve)	*Staphylococcus epidermidis*	43.65	35.75	38.3	39.23 ± 4	13.4 ± 0.4
4 (+ve)	*Staphylococcus aureus*	35.2	34.8	35.9	35.3 ± 0.6	12.4 ± 0.6

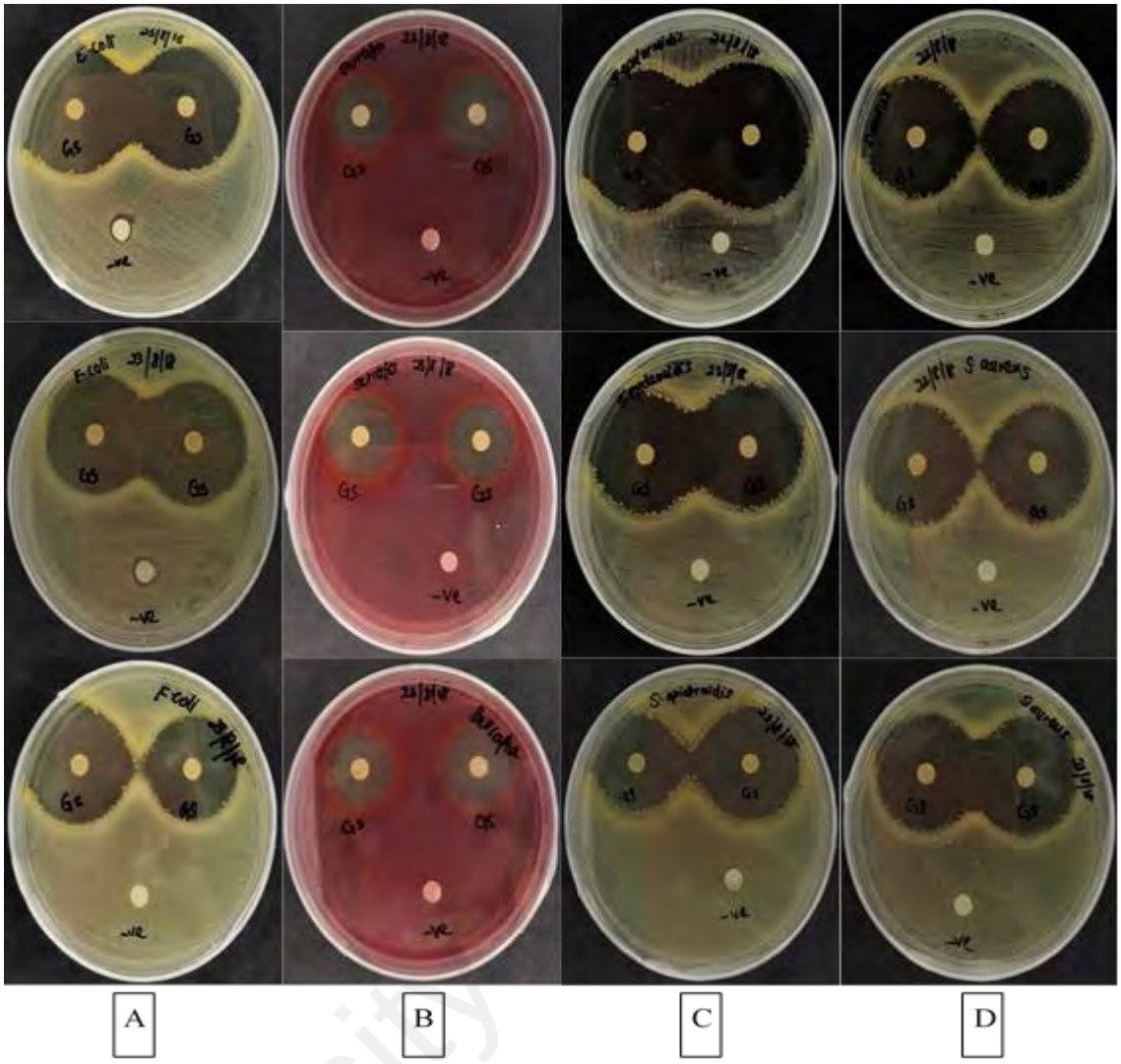

Figure 4.16: Zone of inhibition by Glucan sulphate (GS) against common pathogenic bacteria. (A) GS against *Escherichia coli*, (B) GS against *Serratia marcescens*, (C) GS against *Staphylococcus aureus*, and (D) GS against *Staphylococcus epidermidis*

Antimicrobial effect of GS from *Ganoderma lucidum* QRS 5120 was tested against four species of common pathogenic bacteria, two gram-positive (*S. epidermidis* and *S. aureus*) and two gram-negative (*E. coli* and *S. marcescens*) are shown in Figure 4.16. The strength was measured quantitatively by the absence or presence of inhibition and zone diameters, as shown in Table 4.9. The zone of inhibition showed GS possessed antimicrobial against *E. coli* (33.32 ± 0.05 mm), *S. marcescens* (24.58 ± 1.7 mm), *S. epidermidis* (39.23 ± 4 mm), *S. aureus* (35.3 ± 0.6 mm). GS exhibited strongest antimicrobial activity against *S. epidermidis* (39.23 ± 4 mm). According to Skalicka-Wozniak et al. (2012) and Wan-Mohtar et al. (2016c), a polysaccharide from *G. lucidum*

has significant antibacterial activity towards many bacterial species. There are no significant changes in the strength of antibacterial activity between different strains of *G. lucidum*. Many other studies have also concluded that *G. lucidum* possesses high antimicrobial activity (Ergun, 2017; Heleno et al., 2013; Nasim et al., 2011; Vazirian et al., 2014). Other than the extract, the mycelium of *G. lucidum* was also reported to have antimicrobial activity, a study conducted by Kamble et al. (2011).

4.7.2 Antifungal – demelanizing test

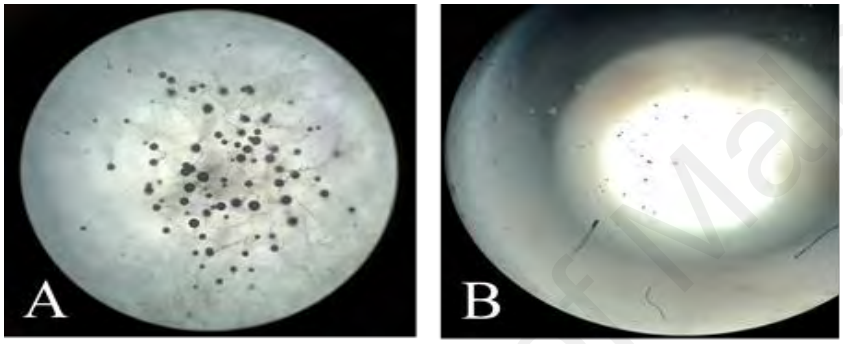

Figure 4.17: Antifungal control results. (A) shows positive control (50 µl dH2O + 50 µl spores) and (B) shows negative control (50 µl DMSO + 50 µl spores)

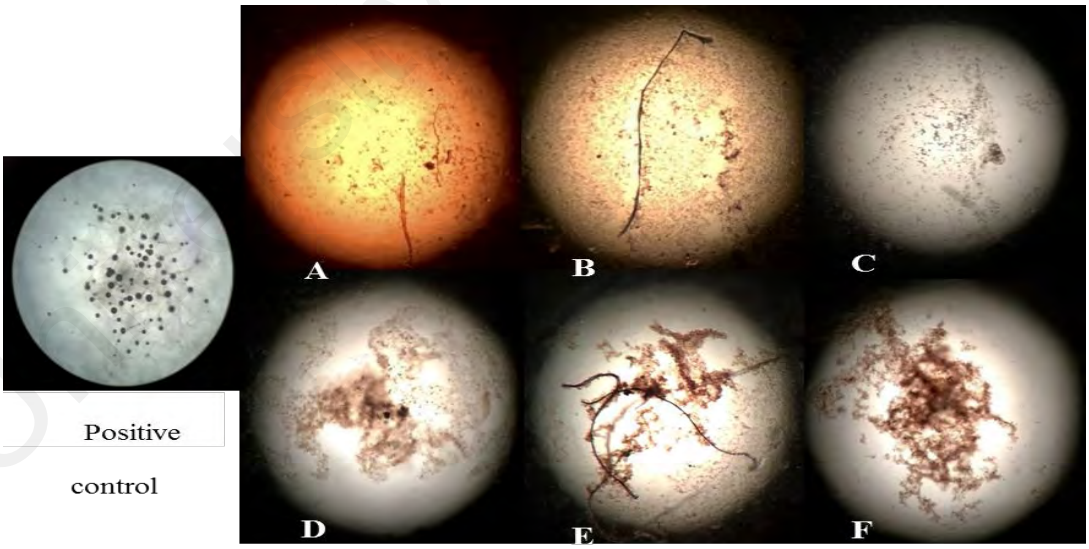

Figure 4.18: Antifungal-demelanizing effect from glucan sulphate (GS) on *A. niger*. (A) concentration at 89 mg/ml ;(B) concentration at 44.5 mg/ml ;(C) concentration at 22.25 mg/ml ;(D) concentration at 11.125 mg/ml ;(E) concentration at 5.56 mg/ml and (F) concentration at 2.78 mg/ml

The antifungal-demelanizing activity of GS was tested against *A. niger*. Figure 4.17 shows the positive control and the effect of DMSO (negative control) on *A. niger*. Figure 4.18 shows the morphology changes of *A.niger* when treated at different concentration of GS. At all concentration of GS, mycelium was present but not conidiophores by comparing to a positive control (Figure 4.18A). Depigmentation occurs to *A .niger* when subjected to GS, and this shows there was variation in the conidiophores melanin. Melanin plays a major role to project the fungus from harmful toxins. By disrupting the melanin, the fungal loses its protection from the toxins and eventually the growth of fungi (*A.niger*) would be suppressed. According to Wan-Mohtar et al. (2017) and Heleno et al. (2013), GS possess the demelanizing activity and able to suppress the growth of *A. fumigatus, A. versicolor, A. ochraceus, A.s niger, T. viride, P. funiculosum, P. ochrochloron, and P. verrucosum*. These microfungi are the most common pathogenic microfungi.

CHAPTER 5: CONCLUSION

This study conducted in this thesis is focused into the morphological of *G. lucidum* QRS 5120 and its optimisation of culture condition for high production of exopolysaccharide (EPS) in submerged fermentation (SmF) using batch fermentation technique. A specific statistical software called Response Surface Methodology (RSM) was used for the optimisation of media condition for production of biomass, EPS and intracellular-polysaccharide (IPS), where the software provides the conditions that need to be tested and the results will be calculated using Analysis of Variance (ANOVA) for each factor for its significant effect on the production. Then, the structure of EPS was analysed using Fourier Transform Infrared Spectroscopy (FTIR) and Proton Nuclear Magnetic Resonance (^{1}H-NMR). Finally, the EPS was tested for its bioactivity characteristics, where it was tested for antimicrobial and anti-fungal activity.

Using these optimised conditions, the production of EPS was increased drastically with lower biomass production usage, and the morphology of pellets were studied extensively. The structure analysis of EPS showed EPS was made of a chain of β-glucans linked by α-glycosyl and the EPS was found to have bioactivity towards common pathogenic bacteria and fungus. As a conclusion, this study demonstrated, EPS, which contains β-glucans, can be used as nutritional supplements and food additives.

5.1 Future works

Future studies for *G. lucidum* strain QRS 5120 could address different fermentation strategies, such as repeated-batch fermentation (RBF). Other than that, more bioactivity test can be done, and the structure EPS can be further analysed using carbon NMR. Moreover, a strategy can be explored for the prevention of mycelium clinging on to the wall of shake-flasks and bioreactors for even distribution of nutrients.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS:

1. Wan-Mohtar, W A A Q I., Mahmud, N., **Supramani, S.**, Ahmad, R., Zain, N A M., Hassan, N A., Peryasamy, J., Halim-Lim, S A. Fruiting-body-base flour from an oyster mushroom—a waste source of antioxidative flour for developing potential functional cookies and steamed-bun. *AIMS Agriculture and Food*, 2018, 3(4), 481-492. (ISI indexed)

2. **Supramani, S.**, Ahmad, R., Ilham, Z., Annuar, M. S. M., Klaus, A., & Wan-Mohtar, W. A. A. Q. I (2019). Optimisation of biomass, exopolysaccharide and intracellular polysaccharide production from the mycelium of an identified *Ganoderma lucidum* strain QRS 5120 using response surface methodology. *AIMS Microbiology*, *5*(1), 19-38. (ISI indexed)

3. **Supramani, S.**, Jailani, N., Ramarao, K., Zain, N. A. M., Klaus, A., Ahmad, R., & Wan-Mohtar, W. A. A. Q. I. (2019). Pellet diameter and morphology of European *Ganoderma pfeifferi* in a repeated-batch fermentation for exopolysaccharide production. *Biocatalysis and Agricultural Biotechnology*, *19*, 101118. (ISI indexed)

PAPER PRESENTED:

1. **Supramani, S.**, Ahmad, R., Ilham, Z., Annuar, M. S. M., Klaus, A., & Wan-Mohtar, W. A. A. Q. I (2018). *Optimisation of biomass, exopolysaccharide and intracellular polysaccharide production from the mycelium of an identified Ganoderma lucidum strain QRS 5120 using response surface methodology*. Paper presented at the International Conference of Beneficial Microbes, 30th August – 1st July 2018, Kuching, Sarawak, Malaysia.

2. **Supramani, S.**, Ahmad, R., Ilham, Z., Annuar, M. S. M., Klaus, A., & Wan-Mohtar, W. A. A. Q. I (2018). *The medicinal mushroom Ganoderma lucidum QRS 5120: Optimization of biomass, EPS and IPS using RSM from liquid mycelial cultures*. Paper presented at the Biological Sciences Graduate Congress, 18th – 20th December 2018, Bangkok, Thailand.