

**OPTIMISATION OF BIOMASS AND
EXOPOLYSACCHARIDE PRODUCTION IN
WILD SERBIAN *Ganoderma lucidum* STRAIN
BGF4A1 USING RESPONSE SURFACE
METHODOLOGY**

NURUL AMIRAH BINTI HASSAN

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

**OPTIMISATION OF BIOMASS AND
EXOPOLYSACCHARIDE PRODUCTION IN
WILD SERBIAN *Ganoderma lucidum* STRAIN
BGF4A1 USING RESPONSE SURFACE
METHODOLOGY**

NURUL AMIRAH BINTI HASSAN

**THESIS SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTER OF
SCIENCE (BIOTECHNOLOGY)**

**INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

2019

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **NURUL AMIRAH HASSAN**

Matric No: **SOC 170005**

Name of Degree: **MASTER OF SCIENCE (BIOTECHNOLOGY)**

Title of Thesis (“this Work”): **OPTIMISATION OF BIOMASS AND EXOPOLYSACCHARIDE PRODUCTION IN WILD SERBIAN *Ganoderma lucidum* STRAIN BGF4A1 USING RESPONSE SURFACE METHODOLOGY** Field of Study: **FUNGAL BIOPROCESSING**

I do solemnly and sincerely declare that:

- (1) I am the sole author/writer of this Work;
- (2) This Work is original;
- (3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
- (4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
- (5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya (“UM”), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
- (6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate’s Signature

Date:

Subscribed and solemnly declared before,

Witness’s Signature

Date:

Name:

Designation:

**OPTIMISATION OF BIOMASS AND EXOPOLYSACCHARIDE
PRODUCTION IN WILD SERBIAN *Ganoderma lucidum* STRAIN BGF4A1
USING RESPONSE SURFACE METHODOLOGY**

ABSTRACT

A wild-Serbian medicinal mushroom *Ganoderma lucidum* strain BGF4A1 (GLSB) was isolated from Mount Avala, Serbia and morphologically identified based on its brown-liquorish cap and woody stipe. Molecularly, GLSB (642 bp) was sequenced and found to be 99% similar to the Serbian-originated *G. lucidum* strain BEOFB 434 and *G. lucidum* strain BEOFB 431. The isolate belongs to the *G. lucidum* species as the sequence dissimilarities (K_{nuc}) value between both sequences of the same fungal species was 0.001. In submerged-liquid fermentation, biomass and exopolysaccharide (EPS) production of GLSB was optimised using response surface methodology. The interactions between three variables: initial pH (4–6), temperature (20°C–30°C), and glucose concentration (10 g/L–50 g/L) were analysed using a central composite design. An analysis of variance revealed that the model was significant for all parameters investigated ($p < 0.05$). Temperature and glucose concentration were found to significantly influence mycelial biomass production, whereas for EPS production only glucose concentration had a significant effect. The model for biomass and EPS was validated by implementing the optimised conditions (pH 5.26, 50 g/L glucose, and 30°C) and was found to generate the highest biomass (3.12 g/L) and EPS (1.96 g/L). An efficient EPS-biomass production blueprint was thus established using optimised parameters for large-scale cultivation of Serbian *G. lucidum* strains.

Keywords: Serbian *Ganoderma lucidum*; exopolysaccharide; biomass; response surface methodology, submerged-liquid fermentation

**PENGOPTIMUMAN PENGHASILAN BIOMAS DAN POLISAKARIDA
LUARAN DALAM SERBIA STRAIN *Ganoderma lucidum* BGF4A1
MENGUNAKAN KAEDAH TINDAK BALAS PERMUKAAN**

ABSTRAK

Satu cendawan Serbia strain *Ganoderma lucidum* liar BGF4A1 (GLSB) telah diasingkan dari Mount Avala, Serbia dan dikenal pasti secara morfologi berdasarkan topi coklat dan kayu berkayu. Secara molekular, GLSB (642 bp) telah disusun dan didapati berkait rapat dengan strain *G. lucidum* BEOFB 434 dan *G. lucidum* strain BEOFB 431 dengan persamaan sebanyak 99%. Oleh itu, jarak evolusi (K_{nuc}) di antara urutan spesies kulat yang sama menunjukkan bahawa isolat tergolong dalam spesies *G. lucidum*. Dalam penapaian cecair-cecair (SLF), pengeluaran biomas dan polisakarida luaran (EPS) dari GLSB telah dioptimumkan menggunakan kaedah tindak balas permukaan (RSM). Interaksi antara tiga pemboleh ubah: pH awal (4 - 6), suhu (20°C - 30°C), dan kepekatan glukosa (10 g/L - 50 g/L) telah dianalisis menggunakan reka bentuk komposit pusat (CCD). Analisis varians (ANOVA) mendedahkan bahawa model itu penting bagi semua parameter yang dikaji ($p < 0.05$). Model untuk biomas dan EPS telah disahkan dengan menggunakan keadaan optimum statistik (pH 5.26, 50 g/L glukosa dan pada 30°C) dan didapati menjana biomas tertinggi (3.12 g/L) dan EPS (1.96 g/L). Bersama-sama, kaedah ini dapat menghasilkan pengeluaran biomass EPS yang efisien menggunakan parameter yang telah dioptimumkan untuk penghasilan *G. lucidum* skala tinggi untuk masa depan.

Kata kunci: *Ganoderma lucidum* Serbia; polisakarida luaran; biomas; metodologi tindak balas permukaan, penapaian cecair-cair

ACKNOWLEDGEMENTS

Alhamdulillah, all praise to Allah SWT for His greatness and for giving me the strength and courage to complete this thesis. First of all, I would like to use this opportunity to express my deepest and sincere gratitude to my supervisor, Dr. Wan Abd Al Qadr Imad Wan Mohtar for his support, motivation and for his phenomenal patience in dealing and guiding me throughout my research and thesis writing period. I could not imagine of having a better supervisor and mentor that would assist me the way that he did.

I also want to thank my fellow colleagues and laboratory staffs for the thought-provoking discussions, inspirational talks and exhilarating moments that we shared. Special thanks to my laboratory partner, Mr. Sugu, who has been guiding me on my lab work journey since day one, and most importantly, for spending his time teaching me the RSM software. I am also extending my gratitude to Mr. Azzimi, who is willing to help me a lot regardless of his own busy schedule.

Furthermore, I would also like to acknowledge my beloved parents, Mr. Hassan and Mrs. Azlina, my backbones, for their prayers and source of unfailing support, spiritually, mentally, and not to forget financially. I thank my siblings, Mrs. Syazwani, Ms. Wafa, Ms. Hana, Ms. Najwa, Mr. Amirul and my brother in law, Mr. Izhar for supporting me in any ways they could. Endless love to my niece and nephew, Maleesa and Iman who have made my life so colorful. Without my family, this accomplishment would not have been possible. Last but not least, I have to convey my very profound gratitude to my best friend, Ms. Anis and my fiancé Mr. Nik Faeiz who have always been supporting me during these emotional roller coaster rides throughout my years of study. You guys are very much appreciated. May God bless them with success and honour in their life.

TABLE OF CONTENTS

ORIGINAL LITERARY WORK DECLARATION	ii
ABSTRACT	iii
ABSTRAK	iv
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	ix
LIST OF TABLES	x
LIST OF SYMBOLS AND ABBREVIATIONS	xi
CHAPTER 1: INTRODUCTION	1
1.1 Problem statement.....	2
1.2 Objectives.....	3
CHAPTER 2: LITERATURE REVIEW	4
2.1 Fungi.....	4
2.2 Macrofungi.....	4
2.3 Taxonomy of <i>Ganoderma</i>	5
2.4 Taxonomy characters used to identify <i>Ganoderma</i> species.....	5
2.5 Medicinal mushroom from Serbian region.....	7
2.6 Common fermentation strategies.....	9
2.6.1 Solid state fermentation.....	9
2.6.2 Submerged liquid fermentation.....	10
2.7 Physicochemical factors that influence the SLF.....	10
2.7.1 Chemical factors.....	10
2.7.1.1 Carbon sources.....	11
2.7.1.2 Nitrogen sources.....	11

2.7.1.3	Culture pH	12
2.7.1.4	Aeration	12
2.7.2	Physical factors	12
2.7.2.1	Temperature	12
2.7.2.2	Agitation	13
2.7.2.3	Culture time	13
2.7.3	Biological factors	13
2.7.3.1	Inoculum	13
2.8	Bioactive compounds of <i>Ganoderma lucidum</i>	14
2.8.1	Polysaccharide	14
2.8.2	Triterpenes	15
2.9	Response surface methodology	15
2.9.1	Application of RSM in process optimisation	18
CHAPTER 3: MATERIALS AND METHODS		20
3.1	Molecular characterization	20
3.1.1	Mushroom mycelium	20
3.1.2	Preparation of mycelium for DNA extraction	22
3.1.3	gDNA extraction	22
3.1.4	PCR amplification	23
3.1.5	Purification and sequencing of PCR-amplified product	23
3.2	Data analysis	23
3.3	Phylogenetic analysis and plasmid matching	23
3.4	Batch fermentation	24
3.4.1	Optimisation of media using response surface methodology	25
3.4.2	Experimental validation of the optimized condition	26
3.5	Analytical methods	26

3.5.1	Mycelium biomass	26
3.5.2	Exopolysaccharide.....	27
CHAPTER 4: RESULTS AND DISCUSSION		28
4.1	Isolation and identification of <i>Ganoderma lucidum</i> strain BGF4A1 (GLSB)	28
4.1.1	Gel electrophoresis	28
4.1.2	Phylogenetic tree analysis	29
4.2	Morphological characteristics of an identified wild-Serbian <i>Ganoderma lucidum</i> strain BGF4A1 (GLSB) using macroscopic observations.....	32
4.3	Molecular characteristics of an identified wild-Serbian <i>Ganoderma lucidum</i> strain BGF4A1 (GLSB).....	33
4.4	Growth curve analysis	34
4.5	Optimisation using Response Surface Methodology.....	36
4.6	Optimisation of Mycelium Biomass Growth.....	39
4.7	Optimisation of EPS production.....	45
4.8	Optimized conditions verification	52
4.9	Comparison of the current study with the literature	53
CHAPTER 5: CONCLUSION.....		55
REFERENCES		56

LIST OF FIGURES

Figure 2.1	: A theoretical response model graph	17
Figure 2.2	: Central composite design for 2 and 3 factors	18
Figure 3.1	: Map of the type of locality and morphological identification of GLSB	21
Figure 4.1	: Amplified gene product isolated from GLSB on an agarose gel	28
Figure 4.2	: Phylogenetic tree of GLSB	30
Figure 4.3	: Alignment of blasting sequences of GLSB strain with <i>G. lucidum</i> in APE software	31
Figure 4.4	: Macroscopic observation of GLSB mycelium growth on PDA plate	32
Figure 4.5	: Biomass growth curve over time calculated by dry cell weight (DCW)	35
Figure 4.6	: EPS growth curve over time calculated by dry cell weight (DCW)	36
Figure 4.7	: Normal probability plot of the residuals for biomass	39
Figure 4.8	: Main effect graph plot of the mycelial biomass production	42
Figure 4.9	: Response surface (3D graph plot) of GLSB on mycelial biomass production	44
Figure 4.10	: Normal probability plot of the residuals for EPS	46
Figure 4.11	: Main effect graph plot showing the EPS production of GLSB	49
Figure 4.12	: Response surface (3D graph plot) of GLSB on EPS production	51

LIST OF TABLES

Table 2.1	: Reported research on medicinal mushroom reported from Serbian region	8
Table 2.2	: The optimisation applications of RSM in agriculture and sciences field	19
Table 3.1	: Experimental range and levels of independent variables	26
Table 4.1	: Information on species used in phylogenetic analysis	29
Table 4.2	: Experimental design using RSM with CCD and responses for mycelium biomass and EPS production of <i>G. lucidum</i>	37
Table 4.3	: Sequential model sum of squares (SMSS) analysis for biomass	38
Table 4.4	: Lack of fit test for biomass	38
Table 4.5	: Analysis of variance for the experimental results of the CCD quadratic model for mycelium biomass	41
Table 4.6	: Sequential model sum of squares (SMSS) analysis for EPS	45
Table 4.7	: Lack of fit test for EPS	45
Table 4.8	: Analysis of variance for the experimental results of the CCD quadratic model for EPS production	47
Table 4.9	: Validation of the model using optimized conditions	52
Table 4.10	: Comparison with published optimisation processes using <i>Ganoderma lucidum</i> in submerged-liquid fermentation	54

LIST OF SYMBOLS AND ABBREVIATIONS

BLAST	:	Basic Local Alignment Search Tool
EPS	:	Exopolysaccharide
g	:	Grams
Glc	:	Glucose
ITS	:	Internal transcribed spacer
L	:	Liters
µm	:	Micrometers
ml	:	Milliliter
NCBI	:	National Centre for Biotechnology Information
PDA	:	Potato dextrose agar
RPM	:	Revolutions per minute
RSM	:	Response surface methodology
SSF	:	Solid substrate fermentation
SLF	:	Submerged liquid fermentation
Temp	:	Temperature
v/v	:	Volume per volume
YE	:	Yeast extract

CHAPTER 1: INTRODUCTION

The well-known medicinal mushroom *Ganoderma lucidum* is considered staple food among Japanese (Rei-shi) and Chinese (Lingzhi) populations, and its use is prevalent throughout Asia where it has been used as a traditional medicine over two millennia (Shah & Modi, 2018), primarily to treat and prevent various diseases .

Ganoderma species have beneficial health effects attributable to various bioactive properties (Kashimoto et al., 2006; Wan-Mohtar et al., 2016b; Wan-Mohtar et al., 2016c). Nowadays, *Ganoderma* is widely cultivated and commercialised using submerged liquid fermentation (SLF) over solid-state fermentation (SSF) due to high demand in the global market (Liu et al., 2010). SSF can take several months to cultivate the fruiting body of *G. lucidum* and may be associated with complications arising from factors such as culture environment and quality (Supramani et al., 2019b; Tang et al., 2011). However, SLF appears to be more suitable for producing the exopolysaccharide (EPS) and requires a shorter fermentation time (Wan-Mohtar et al., 2016a).

Some studies have reported the optimisation of culture conditions in SLF of higher fungi to improve biomass and EPS yields (Ahmad et al., 2013; Supramani et al., 2019a). Utilisation of the conventional “one-factor-at-a-time” (OFAAT) method appears to be ineffective in the long term because of a shortage of specific information on interactions and correlations between independent variables (e.g., initial pH, agitation or glucose concentration), given that only variable is changed at a time while the others remain constant (Salehmin et al., 2015). Further, this method is time consuming and complicated as it requires multiple experimental trials. Response surface methodology (RSM) thus represents an alternative solution to OFAAT as it can examine several factors simultaneously, reducing cost, time, and labour requirements. According to Shah and Modi (2018), RSM can eliminate insignificant parameters and focus specifically on the

critical factors. In the current study, the optimal growth of mycelia was determined by evaluating several parameters such as temperature, initial pH, and glucose concentration.

Previous studies of *G. lucidum* from the Serbian region focused only on the fruiting bodies and bioactive composition following EPS production (Rašeta Milena et al., 2017; Stojkovic et al., 2014). Thus, the present study aimed to optimise the culture conditions of identified Serbian *G. lucidum* in shake flasks to obtain a high yield of mycelial biomass and EPS using RSM. The optimised parameters may be used for future studies of large-scale submerged cultivation of Serbian *G. lucidum*.

1.1 Problem statement

Nowadays, the latest trend of cultivation technique presents in industry especially for *Ganoderma lucidum* production, is submerged-liquid fermentation (SLF) over conventional solid-state fermentation (SSF). This is particularly, in generating quicker, and large-scale production of mycelial biomass and its metabolites (Wan-Mohtar et al., 2016b). There are number of studies conducted by Serbian Mycologists were mainly focusing on discoveries from fruiting bodies of Serbian *Ganoderma lucidum* due to their limited numbers in the wild. And yet, there are no studies has been carried out in SLF using wild Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB) and most of their bioactive exopolysaccharides are extracted from fruiting bodies and none from the mycelium. By cultivating GLSB in liquid culture, we can produce the desired compounds in bulk, cheap, safely and to a consistent quality compared to solid culture. Besides, the optimisation of culture response conditions for this present study is enhanced by response surface methodology (RSM). This method is seemed to be more efficient than the “one-factor-at-a-time” (OFAAT) method where the RSM exhibited the correlations among multiple variables used. To date, there are no study that has been reported to study the optimisation of culture conditions for maximum production of mycelial biomass and EPS

by GLSB using RSM. Hence, this study can be used as a blueprint to screen the optimum culture condition for an upscale bioreactor application of *Ganoderma lucidum* in Serbian wild forest.

1.2 Objectives

- 1) To identify the wild *Ganoderma* species isolated from Serbian mountain of Avala.
- 2) To optimize selected growth parameters for biomass and exopolysaccharide production in the identified species using response surface methodology.

University of Malaya

CHAPTER 2: LITERATURE REVIEW

2.1 Fungi

In general, fungi are classified based on their morphology which are filamentous or yeast. The filamentous fungi are saprophytic microorganisms, usually recognized by having tubular cells, a mass of branching and thread-like hyphae which formed mycelium (Yang et al., 2009). Typically, in wild, fungi ingested nutrients from the environment (woods) through the mycelium by discharging catalysts into the rotting log or any other substrates and these proteins will breakdown natural polymers into smaller units, for instance, monomers. At that point, the mycelium assimilates these monomers, utilizing a blend of facilitated diffusion and active transport (Yang et al., 2004).

According to Agudelo-Escobar et al. (2017), a variation of cultivation methods can simply affect the morphological characteristics of fungi. For instance, in submerged cultivation, the mycelium grows in a form of pellets while in solid substrate, the mycelium grows over the solid surface and their growth can cover over a large region.

2.2 Macrofungi

In recent decades, there are extensive classification studies have been conducted to classify the fungi because of the variation of morphological characters occurred due to the evolution (Badalyan et al., 2012). As for now, Kingdom Mycetae comprises of Blastocladiomycota, Chytridiomycota, Glomeromycota, Entomophthoromycota, Neocallimastigomycota, Kickxellomycota, Mucoromycota, Basidiomycota and Ascomycota phyla (Raghukumar, 2017). Both Ascomycota and Basidiomycota are known as higher fungi (Fazenda et al., 2008). Macrofungi or higher fungi is well-known as filamentous fungi and characterized based on their clear, visible carpophores structures (Hilszczańska, 2012) and their hyphae organization which having septate hyphae (Fazenda et al., 2008).

2.3 Taxonomy of *Ganoderma*

In many countries in Asia including China, Japan and Korea, wild mushrooms are traditionally cultivated not just to be consumed as food but also for medicinal purposes mainly because of its texture, therapeutic and nutritional value (Klaus et al., 2011). Wild mushrooms have been claimed to many researchers that they have high level of antioxidant compounds which can help lessen the cell damage caused by oxidation and hence promoting health. One of them is *Ganoderma lucidum* which comes from the family of Ganodermataceae. The species of *Ganoderma* usually can be found in subtropical and tropical regions and can live under both hot and humid environments (Moncalvo, 2000). The distinct of the *G. lucidum* morphological characteristics such as the size, colour and shape of fruit bodies can be influenced by the various environmental conditions (Moncalvo, 2000). Some other studies reported that the growth habitat plays as one of the important factors to the profile, bioactive properties (Heleno et al., 2013) and chemical properties of wild mushrooms. Furthermore, a study reported by Klaus et al. (2015) demonstrated that the biological properties of wild mushrooms are also affected by their morphology and the polysaccharides structure. *G. lucidum* is generally characterized based on their pileus, stipe shape and its colors and spores' dimensions (Ćilerdžić et al., 2018) and have varied in colors, mainly from orange-red to brown but the red is most common one (Nasreen et al., 2005). The fruiting body of *G. lucidum* usually grows on hardwoods, are stipitate, more or less imbricate pileal surface (Szedlay et al., 1996), have smaller (Xing et al., 2016) and numerous, narrowly ellipsoid with inter-wall pillars and “smooth” walls of basidiospores. Several authors however, define distinct spore dimensions (Szedlay et al., 1996).

2.4 Taxonomy characters used to identify *Ganoderma* species

The taxonomy identification of basidiomycetes was conventionally based mainly on the morphological features of the fruiting bodies. However, some issues will probably

arise such as the absence of basidiocarps in certain periods of the year, their morphological plasticity and the existence of cryptic species when the identification is merely based on fruiting body (Gottlieb & Wright, 1999a; Gottlieb & Wright, 1999b; Moncalvo & Ryvarden, 1997). Therefore, for these reasons many taxonomists implemented advanced biochemical and molecular techniques to distinguish this *Ganoderma* species such as sexual compatibility tests, isozyme analysis and DNA based techniques (PCR-RFLPs and DNA sequence analysis).

Sexual compatibility tests are often used for determining whether two isolates belong to the same biological species, and/or to identify an unknown isolate. Two viable isolates which success in exchanging their nuclei are deemed to be conspecific. Through sexual compatibility testing, Adaskaveg and Gilbertson (1986) discovered that homokaryons of *G. tsugae* were not infertile with any homokaryons of *G. lucidum*, meaning that both were separated species. Meanwhile, for homokaryons *G. lucidum* from North America and homokaryons *G. resinaceum* from Europe, both were completely infertile and concluded that both were synonymous.

Isozyme analysis have commonly used to tackle taxonomic issues in fungi, targeting plasticity or overlapping morphological characteristics within a genus or species (Micales et al., 1992). This analysis includes the separation of total protein extracts by gel electrophoresis where variations in electrophoretic profiles imply genetic variability. For instance, similar profiles demonstrate isolates of the same species while isolates of distinct species generate distinct profiles (Bonde et al., 1993). This approach was effectively used by Smith and Sivasithamparam (2000b) for identification study of Australian species of *Ganoderma* by using cellulose acetate gel electrophoresis (CAGE) and polyacrylamide gel electrophoresis (PAGE). It was found that CAGE is a simpler

tool to use for *Ganoderma* identification as it used small number of isozyme loci compared to PAGE which encountered more complex banding patterns.

Next is DNA-based methods including PCR-RFLPs (Bruns & Szaro, 1992; Bruns et al., 1991) and DNA sequence analysis of the internal transcribed spacers (Moncalvo et al., 1995a; Moncalvo et al., 1995b; Park et al., 2012; Smith & Sivasithamparam, 2000a; Xing et al., 2016) and the mitochondrial small-subunit mtSSU (Hong & Jung, 2004) rDNA regions. These methods are far more advance compared to other previous methods in taxonomy studies as they were proven to lessen the risk of misidentification of complex species. For instance, previous study by Adaskaveg and Gilbertson (1986) revealed that *G. lucidum* in North America was conspecific with European *G. resinaceum*. However, both species were found to be distinct with both ITS (Moncalvo et al., 1995a; Moncalvo et al., 1995b) and mtSSU (Hong & Jung, 2004). The best approach, however, is to incorporate multiple available methods since relying only on morphological and cultural characteristics are not always useful especially in classification of closely related species (Bishop et al., 2015).

2.5 Medicinal mushroom from Serbian region

Several studies have been conducted which mainly focusing on the biological properties of wild mushroom species in Serbian region such as basidiomycete, *Schizophyllum commune*, *Laetiporus sulphureus*, *Grifola frondosa*, *Piptoporus betulinus* and including *Ganoderma lucidum*.

Table 2.1: Reported research on medicinal mushroom reported from Serbian region.

Species	Locality	Study details	References
Basidiomycete, <i>Schizophyllum commune</i>	Mountain Avala Belgrade, Republic of Serbia	Examined antioxidant activities and chemical characterization	Klaus et al. (2011)
<i>Piptoporus betulinus</i> (Bulliard: Fries) Karst	Ust. Kut district of Irkutsk	Isolation and structural characterization of a dominant polysaccharide of fruiting bodies.	Olennikov et al. (2012)
<i>Laetiporus sulphureus</i> (Bulliard: Fries)	Belgrade, Republic of Serbia	Examined antioxidant activities	Klaus et al. (2013)
<i>Morchella esculenta</i> (L.) Pers	Braganca (Northeast Portugal) and Jabučki rid (Northern Serbia)	Comparative study on chemical and bioactive properties	Heleno et al. (2013)
<i>Ganoderma lucidum</i> (Curtis) P. Karst	Bojčinska forest, Belgrade, Serbia and China	Comparative study on chemical and bioactive properties	Stojkovic et al. (2014)
<i>Ganoderma lucidum</i> BEOFB 431	Bojein Forest near Belgrade, Serbia	Comparative study on biological activities between two cultivated substrate types	Ćilerdžić et al. (2014)

Table 2.1, continued.

<i>Grifola frondosa</i> (Dickson: Fries)	Belgrade, Republic of Serbia	Examined antimicrobial, antioxidant and cytotoxic activity	Klaus et al. (2015)
<i>Ganoderma lucidum</i> (1200715), <i>Ganoderma lucidum</i> (1200724)	Morovičke šume, Serbia & Donji, Lapac, Croatia	Comparative study on antioxidant capacities of two <i>G. lucidum</i> of different geographical origins	Rašeta Milena et al. (2017)

2.6 Common fermentation strategies

2.6.1 Solid state fermentation

Basically, solid state fermentation (SSF) is a traditional cultivation technique of microorganisms on a solid substrate that require almost no water presence to produce the desired products of interest (Mienda et al., 2011). According to Subramaniyam and Vimala (2012), the utilized solid substrates like bran, bagasse and paper pulp are usually contain high amount of nutrition, easy to get but depending on the availability of the substrates at that region or country and can be recycled easily. However, by utilizing these substrates, the fermentation process is quite slow and steady but can be used for long periods. Although this process is a bit time consuming, but it is great for fungi and other microorganisms that can live suitably in less moisture condition. Some of the SSF popular products are “Koji” and “Tempeh” (Mienda et al., 2011) which involve the fermentation of the filamentous fungi. Basically, “Koji” is the fermentation of soy sauce by the growth

of *Aspergillus oryzae* on wheat bran and soybean while “Tempeh” is the fermented soybeans with the growth presence of *Rhizopus oligosporus*.

Despite its advantages, SSF is a process that have a greater difficulty of handling, controlling and monitoring those fermentation parameters such as temperature, pH, oxygen, products hygiene and microbial growth rate compared to liquid submerged fermentation (SLF) (Steinkraus, 1984).

2.6.2 Submerged liquid fermentation

Recently, in mushroom cultivation, the agro-industrial has been switched to submerged liquid fermentation (SLF) to produce the desired bioactive compounds that mainly secreted into the fermentation broth. SLF utilizes free flowing liquid substrate rapidly (fast process) and has a better performance than SSF (Subramaniam & Vimala, 2012). However, substrates media needs to be replaced with fresh media or constantly supplanted/supplemented by nutrients. This fermentation method is most appropriate for microorganisms, for instance, bacteria that require high dampness content. Furthermore, the cultivation of *Ganoderma* in SLF only takes weeks to harvest while for SSF, it takes several months for industrial scale resulting a lower production cost (Hsu & Cheng, 2018). Other advantages of SLF are high quality and productivity of desired product (Wan-Mohtar et al., 2016b), less occurrence of contamination because of high safety standard and the cultivation process is easy to deal with as of controlling and monitoring.

2.7 Physicochemical factors that influence the SLF

2.7.1 Chemical factors

The chemical factors like carbon sources, nitrogen sources, pH index and oxygen intake are vital in affecting the growth of mycelium to the highest level (Petre, 2015).

2.7.1.1 Carbon sources

There are several types of carbon sources including glucose, maltose, xylose, mannitol, xylan, starch, molasses and bagasse. However, glucose was the most favourable carbon sources utilized by tropical edible macrofungi (Chandra & Purkayastha, 1977). This was supported by some of the published works supporting this claim which were Songulashvili et al. (2008) and Petre et al. (2010), indicating that the glucose was one of the best carbon sources for mycelia growth of *G. lucidum* as it consists of a single monosaccharide sugar. This correlates to the ability of the fungi to generate cellular energy efficiently by fast metabolization of glucose (Garraway & Evans, 1984; Jonathan & Fasidi, 2001).

2.7.1.2 Nitrogen sources

For higher fungi, most Basidiomycetes prefer complex organic nitrogen sources (urea, yeast extract, peptone) compared to inorganic sources (nitrite, nitrate and ammonium salts) because the organic sources have better performance in synthesizing those essential amino acids in submerged culture (Papaspyridi et al., 2010). One particular study done by Shah and Modi (2018), indicated that out of six nitrogen sources (peptone, yeast extract, ammonium sulphate, ammonium chloride, sodium nitrate, potassium nitrate) that were tested, the yeast extract yielded the best biomass production of *G. lucidum*. Similar observations were made by other researchers that working on Basidiomycetes fungal species including *G. lucidum*, *Lentinula edodes*, *Pleurotus ostreatus* and *Psathyrella atroumbonata* also confirmed that the yeast extract was one of the most efficient nitrogen sources for mycelia growth and fungal biomass yield (Jonathan & Fasidi, 2001; Petre et al., 2010). Generally, this proved that organic nitrogen was effective in enhancing the mycelial growth than inorganic nitrogen sources (Jayasinghe et al., 2008; Jonathan & Fasidi, 2001; Petre et al., 2010).

2.7.1.3 Culture pH

The culture pH is one of the main variables contributing to fungal growth, metabolite production, cell morphology and broth rheology of edible mushrooms in the range of 3.0 and 7.5 pH (Petre, 2015). For *G. lucidum*, the suitable pH range that has been recorded for some authors was at pH 5 ~ pH 9 and pH 3 ~ pH 7 (Jayasinghe et al., 2008; Kim et al., 2006). Some studies proved that lower pH resulted an optimum mycelial growth while higher pH index resulted high yield of EPS formation (Lee et al., 1999; Wagner et al., 2003). Those finding showed that mushrooms have a wide pH variety for their beneficial production of mycelia.

2.7.1.4 Aeration

Fungi are strictly aerobic microorganisms and therefore, aeration is a key factor in metabolic process development. A comparative research with Yang and Liao (1998) evaluated the impact of aeration on biomass and product formation by *G. lucidum* at distinct aeration rates in a bioreactor. However, the dissolved oxygen concentration was not initially measured and therefore, making it hard to assess the independent impact of the aeration. As the effectiveness of oxygen transfer also relying on the agitation technique and speed and not only on its design and operation. In addition, the broth conditions can also alter the performance of the oxygen transfer during fermentation.

2.7.2 Physical factors

2.7.2.1 Temperature

According to Wagner et al. (2003), fermentation with *G. lucidum* have been done at 25°C to 35°C with most established at 30°C. Furthermore, there was no mycelial growth was recorded at 15°C and 35°C as this might be due to some of the significant enzymes which catalyze metabolic processes of tested mushrooms are denatured and inactivated.

At 20°C, the mycelia showed a considerable growth, but the optimal mycelial growth temperature was at 25°C to 30°C.

2.7.2.2 Agitation

When the improper stirring regimes are introduced in submerged fermentation, this could cause to significant cellular metabolic modifications, as some regions may not be supplied with enough nutrients as shear forces are produced (Petre, 2015). Those shear forces generated can bring damage to the mycelium structure in several ways including cell disruption, inducing morphological change, lead to various changes in growth rate and formation of bioproducts. Therefore, an optimum agitation is essential to ensure the appropriate transfer of oxygen into the medium while deterring shear stress (Wagner et al., 2003). Fazenda et al. (2008) reported that the mycelial growth of *G. lucidum* has been studied between the range of agitation speeds of 50 – 250 rpm in shake flasks. The maximum growth is at 100 rpm and has started to decline when the rpm over 100 because of the shear tension on the mycelium (Yang & Liao, 1998).

2.7.2.3 Culture time

The quality of the product of interest such as biomass, exo and endo-polysaccharides are determined by the harvest times of the mushroom species in SLF (Fazenda et al., 2008). Hence, for this experiment, the biomass and EPS growth rate are determined to identify when is the best time for maximum biomass and EPS production.

2.7.3 Biological factors

2.7.3.1 Inoculum

Apart from glucose concentrations and agitation regime, another factor that has a major impact on the pellet size distribution is the inoculum density which resulted some implications for product outcomes. For instance, the cell content of IPS in smaller pellets is greater while in bigger pellets, the ganoderic acid (GA) content is higher (Wagner et

al., 2003). At day 8, 68.3% of the pellets had a diameter less than 1.2 mm with the inoculum density of $\rho(X) = 670$ mg/L while compared to the same day, with inoculum density $\rho(X) = 70$ mg/L, 91.0% of the pellets had a diameter greater than 1.6 mm (Fang & Zhong, 2002b).

2.8 Bioactive compounds of *Ganoderma lucidum*

2.8.1 Polysaccharide

One of the main bioactive components that can be obtained from *Ganoderma* is polysaccharide. The polysaccharide is known as the long-chain carbohydrate which is comprised of small sugar or monomer units known as monosaccharides. Hence, the hydrolysis process of polysaccharides gives a vast number monomer unit which are linked together by a glycosidic linkage. The general chemical formula of the polysaccharide is $(C_6H_{10}O_5)_n$, where the n is a number that more than 40. Polysaccharide extraction usually used to detect bioactive activities which display distinct patterns of a beta-linked glucose backbone and degrees of branching (Villares et al., 2012). Generally, there are two forms of polysaccharides which called intracellular polysaccharides (IPS) and exopolysaccharides (EPS). EPS can be harvested from the culture broth which is outside the mycelium (Hsu et al., 2017) while IPS can isolated from the inside of mycelium (Fang & Zhong, 2002a). EPS extraction from fruiting bodies is far more complicated than from mycelia. Because of that, the EPS production has been relying on submerged cultivation lately (Lee et al., 2007). Polysaccharides has therapeutic potential that mainly focusing on the functions of (1,3)- β -D-glucan (G) produced by EPS extraction (Wan-Mohtar et al., 2016b). A study by Xu et al. (2008) also proved that bioactive polysaccharides from mycelium of *G. lucidum* exerted some pharmacological effects such as antitumor, antioxidant and immunomodulation activities. Other recent study performed by Ai-lati et al. (2017), isolated and purified the EPS of *G. lucidum* to investigate the antitumor activities and proved that *G. lucidum* polysaccharides could treat cancer.

2.8.2 Triterpenes

Apart from polysaccharides, triterpenes are proven to be one of the most significant bioactive metabolites that exert some medicinal properties. Triterpenes consist of six units of isoprene, a subtype of terpenes. Isoprene can form linear chains or ring-like structures in triterpenes. Basically, ganoderic acids (GA) are a subtype of triterpenes which consist of four cyclic and two linear isoprenes (Liu et al., 2012). According to Yue et al. (2010), there were over 140 *G. lucidum* GAs which have been successfully identified. Through direct interaction with molecular receptors, triterpenes can target cellular activities like apoptosis, regulation of the cell cycle and angiogenesis due to their low molecular weight compounds (CFR Ferreira et al., 2010). One of the health benefits of triterpenes in *G. lucidum* is to have anti-cancer activity with immediate suppression of cell proliferation (cancer-specific cell cycle arrest and apoptosis) mechanisms (Fukuzawa et al., 2008; Wu et al., 2012). Besides, both studies by Thyagarajan et al. (2010) and De Silva et al. (2012) demonstrated that *G. lucidum* triterpene extracts gave significant results in inhibiting and reducing colon tumour development through animal testing.

2.9 Response surface methodology

For optimisation process, there are two basic methods which comprise of conventional (mechanistic) method and statistical method as to exhibit the most appropriate solutions for the domain of the process model (Box et al., 1978; Khuri, 2017). The conventional method often called as “one-factor-at-a-time” method where in experiment, one variable is changed while the others remain on a fixed level. This method is easy to apply, yet it has issues with identifying and handling those interactions and correlations between the variables involved which can greatly affect the outcomes. So, that is where a statistical method exists and began to be utilized broadly in many fields of industrial applications including food industry, physical and engineering-based industry (Myers et al., 1989). Myers et al. (1995), state that RSM “is a collection of statistical and mathematical

techniques useful for developing, improving, and optimizing process. It also has important applications in the design, development, and formulation of new products, as well as in the improvement of existing product designs". It is vital to understand that since it includes a statistical approach the outcomes are to be viewed as an approximation.

Based on the response surface methodology book by Myers et al. (1995), the input parameters are often called as independent variables while the response (output) is usually interpreted as quality characteristic or the measurement of the performance. Example of variables can be temperature, agitation and time with vary individual units. For responses, can be biomass yield, viscosity and others. However, the coded variables (X_1 , X_2 , X_3) often applied as they are dimensionless with the mean of zero. This is for instance, if the variable X_1 is the coded variable for the agitation and the response is biomass yield, at that point, the opposite (negative) response for the X_1 value would mean the low yield of biomass as the increasing of agitation. In this case, the coded factors for high value of X_1 will be +1 while for a low value will be -1 and the mean will be zero.

By considering all the distinctive factors, their correlations and interactions between these factors RSM is fit for figuring out which of the factors that is progressively imperative for the needed reaction (having a bigger effect) and how they influence one another by visualizing them in 3D-surface plotted graph as shown in Figure 2.1. RSM can not only used for optimizing the parameters to get an optimum response, but it also can be applied to accomplish certain standards of client's specification while minimizing variation in the response and able to handle robust structured procedures.

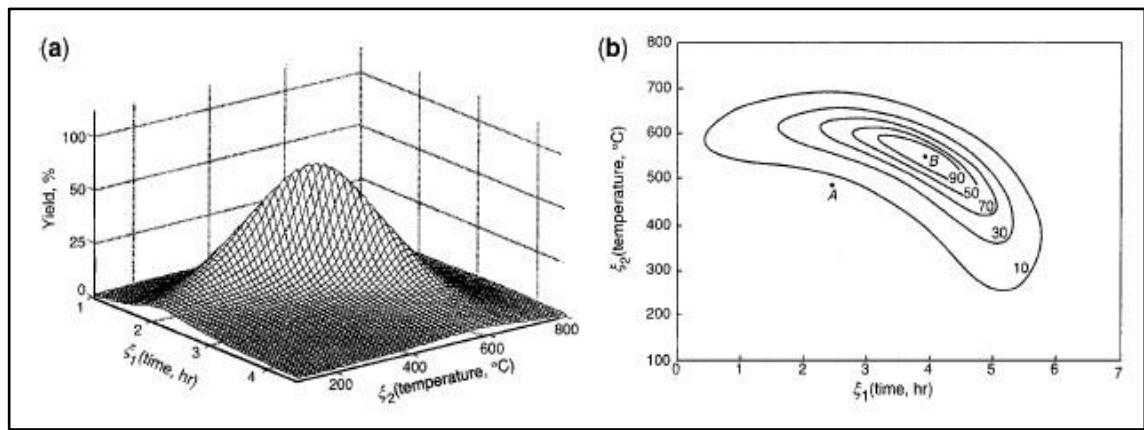


Figure 2.1: (a) A response model graph illustrating the interaction between yield of a chemical process and the process parameters reaction time (ξ_1) and temperature (ξ_2); (b): the contour plot.

Generally, a polynomial equation is used to define the correlation between the input parameters and output responses. There are first-order models which involve no interaction between variables and second-order models that usually rely on factorial designs. Therefore, there should have some interactions occurred. The first-order model equation is regressed in Eq. 2.1 while the second-order in Eq. 3.1 (*Chapter 3*).

$$\eta = \beta^0 + \beta_i X_i + \beta_{ii} X_{ii} \dots + \beta_k X_k \quad (2.1)$$

A least square method is a method that capable of reducing the residual error calculated by the total amount of square deviations between the actual and the estimated responses as to determine the optimal polynomial equation for the RSM model. Once done, the statistical significance procedure needs to be done by using analysis of variance approach (ANOVA). This is where the regression model significance tests, significance of individual model coefficient and lack of fit are performed.

The most well-known design for second-order surface model is the central composite design (CCD). Figure 2.2a shows a general CCD with two variables which made up a two-dimensional. Figure 2.2b is the central composite face-centered design (CCFD) with three variables which made up a three-dimensional design.

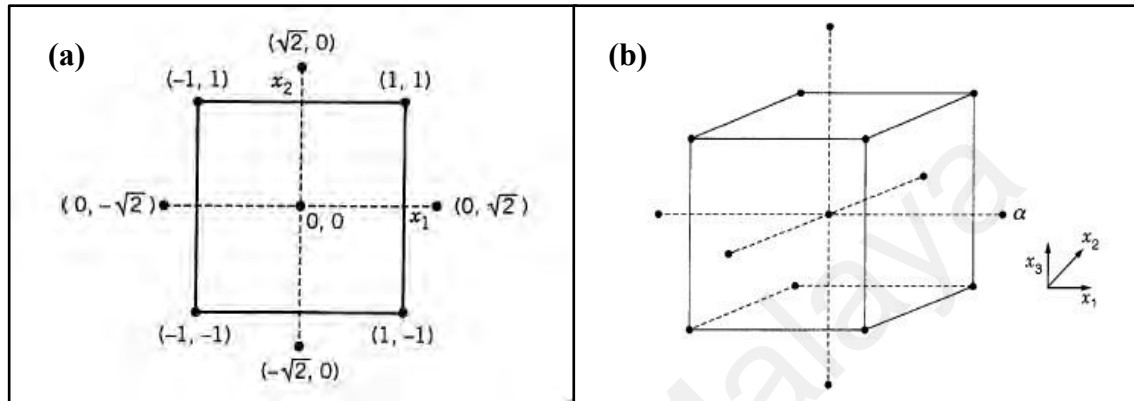


Figure 2.2: (a) Central composite design for $k = 2$ and $\alpha = \sqrt{2}$; (b) Central composite design for $k = 3$ and $\alpha = \sqrt{3}$.

2.9.1 Application of RSM in process optimisation

The application of RSM in optimisation has been implemented in diverse fields ranging from electronic technology to food products since early 1970s (Khuri, 2017). In recent years, RSM concepts in biological research studies, which focused on agriculture and food sciences, have been extensively explored. Table 2.2 represents some of the studies which have implemented the RSM for their research area.

Table 2.2: The optimisation applications of RSM in agriculture and sciences field.

Author / Year	Area of research	Design method / Statistical software used
Diniz and Martin (1996)	Studied the influence of pH, temperature and enzyme substrate ratio (E/S) on the of hydrolysis of dogfish muscle protein.	Box-Behnken Design (BBD)
Broudiscou et al. (1999)	Evaluated the impacts of several mineral compounds on feed degradation and microbial productivity in a continuous culture system.	Franquart and Central Composite Design (CCD)
Deshpande et al. (2008)	Study the optimisation of a chocolate-flavoured, peanut-soy beverage.	STATISTICA™, Version 6.0
Tian et al. (2013)	Studied the optimisation of pomegranate seed oil extraction by ultrasound.	Box-Behnken Design (BBD)
Samaram et al. (2015)	Studied the optimisation of papaya seed oil extraction by ultrasound.	Central Composite Design (CCD)

CHAPTER 3: MATERIALS AND METHODS

3.1 Molecular characterization

3.1.1 Mushroom mycelium

Wild-Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB) stock culture was obtained from a mycologist (Prof. Dr. Anita Klaus, University of Belgrade, Serbia). The fruiting body (Figure 3.1b) was found at the base of wild oak (*Quercus robur* L.) from the mountain of Avala which is 511m above the sea level as shown in Figure 3.1a. It is located 16 km south-east of downtown Belgrade, of which the entire area of the mountain are belongs to the Belgrade City area. The mountain has been protected since 1859 as a natural monument and in 1936, it was declared as a national park. Upon collection, the carpophores (Figure 3.1b) and basidiospores structure (Figure 3.1c) were analyzed to confirm correct species (Phillips, 1981). The specimens and mycelial cultures were deposited at the herbarium of the Department of Industrial Microbiology (University of Belgrade – Faculty of Agriculture) for culture collections and future stock requirements.

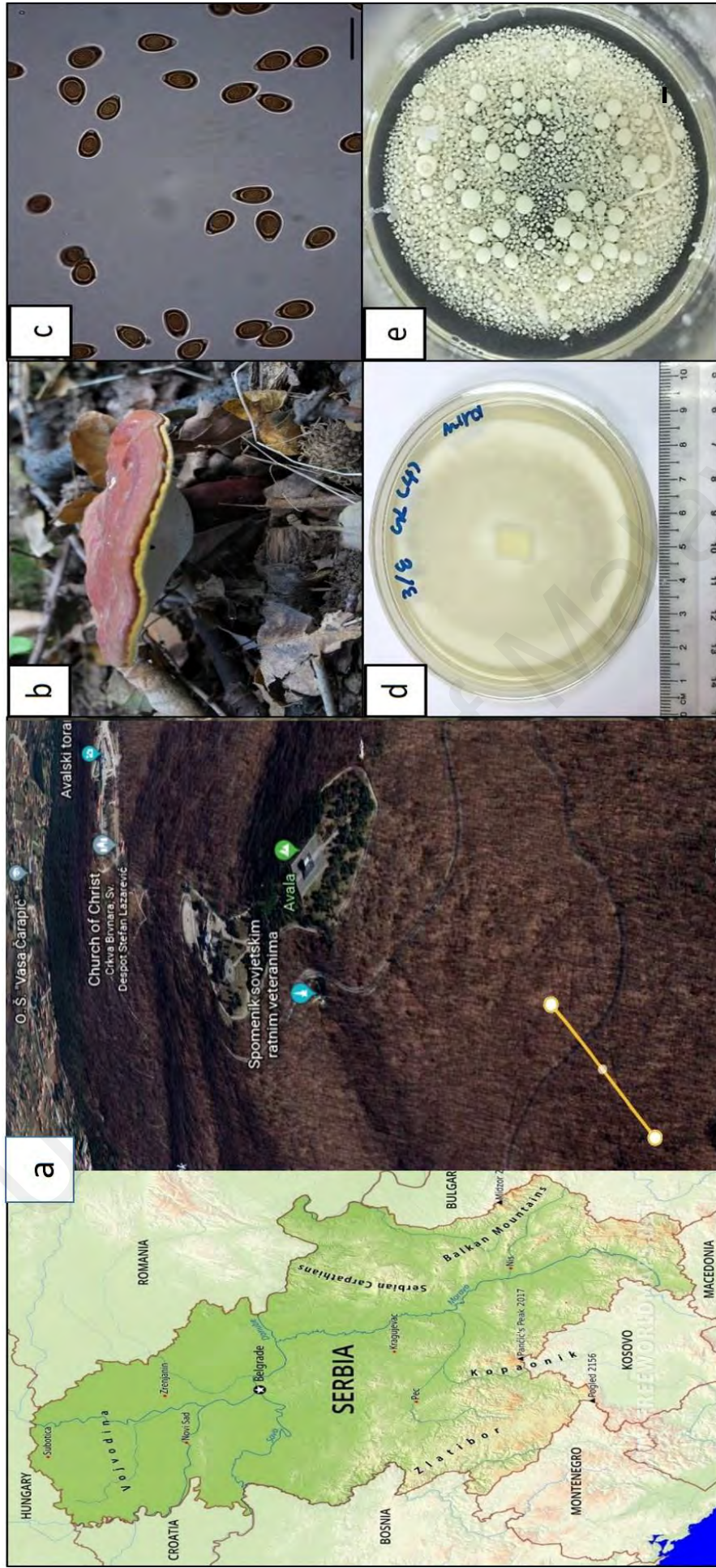


Figure 3.1: (a) Map of the type of locality of wild-Serbian GLSB found at the mountain of Avala (yellow bar indicates 165-m distance at the coordinates 44°41'25''N 20°30'51''E). Source: Google Maps, 2018; (b): fruiting body of wild-Serbian *G. lucidum* BGF4A1 (GLSB); (c): double-walled basidiospores of wild-Serbian GLSB under a microscope (100× under oil immersion) (bar = 10 μm); (d): wild-Serbian GLSB grown on potato dextrose agar; (e): wild-Serbian GLSB grown in a shake flask at day 10 of second seed culture (bar = 1 mm).

3.1.2 Preparation of mycelium for DNA extraction

Upon preparation of mycelium for DNA extraction, the cultures were first subcultured onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK) and incubated for 7 days at 26°C. The cultures were stored at 4°C to prevent any contamination and to maintain the viability of the sample cultures. After that, the cultured mycelium was finely ground in liquid nitrogen using a set of mortar and pestle and stored at -20°C.

3.1.3 gDNA extraction

Genomic DNA (gDNA) extraction of resuspended freeze-dried mycelium (30 mg) was performed in a tube containing lysis buffer (500 µL). The suspension was pipetted repeatedly until the consistency of the mixture became frothy. RNase A was added to the solution, then incubated for 5 min at 37°C. Next, NaCl solution (165 µL, 5 mol/L) was added to encourage precipitation of the cell debris, polysaccharide, and protein and the solution was mixed by vortexing before being centrifuged (13,000 rpm) for 20 min at 4°C. The supernatant was removed and transferred to 1.5 mL tube containing 400 µL of chloroform:phenol and mixed by inversion until the solution turned cloudy. After centrifugation (20 min, 4°C), the aqueous phase of the mixture was collected and separated with an equivalent volume of chloroform. Two volumes of 95% ethanol were added to precipitate the DNA, and the solution was resuspended in 500 µL of lysis buffer and mixed by pipetting to separate the DNA from polysaccharide. Next, 165 µL of 5 mol/L NaCl was added and the sample was mixed by inversion. Then, 2 volumes of chloroform were added, and the sample was centrifuged (13,000 rpm) for 10 min at 4°C to produce a DNA pellet. Ethanol (95%) was used to precipitate the DNA and the pellet was washed with ethanol (70%) before air-drying. The pellet was dissolved in Tris-EDTA buffer (50 µL) to form purified fungal gDNA, which was stored at -20°C (Zhou et al., 2007).

3.1.4 PCR amplification

The PCR procedure was as described by Liu et al. (2000) and Tamura et al. (2013) with slight modifications. Two common fungal internal transcribed spacer primers (ITS1 and ITS4) were used for the amplification of the PCR product. PCR reactions contained each primer (0.5 pmol), dNTP mix (200 μ M, Promega), PCR buffer (ThermoFisher Scientific, Waltham, USA), DNA polymerase (0.5 U, Promega, Madison, USA), and water. Amplification of the targeted fragments was performed using an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 98°C for 120 s (1 cycle); 25 cycles of annealing and extension (98°C for 15 s; 60°C for 30 s; 72°C for 30 s), and 1 cycle for final extension at 72°C for 10 min.

3.1.5 Purification and sequencing of PCR-amplified product

Purification and sequencing were performed according to the method of Supramani et al. (2019a). Targeted PCR yields were subjected to 1% agarose gel PCR Purification Tool (Tiangen Biotech Co., China) analysis for 1 hour at 80 V, and sequenced using a BigDye® Terminator v3.1 sequencer (Applied Biosystems Co., USA).

3.2 Data analysis

Clustal Omega (Sievers & Higgins, 2018) was used to align the sequences. The sequences were compared with related sequences of fungal species using BLAST software (NCBI) (adapted from <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for basic identification and information on previous taxonomic and phylogenetic studies. Fungal sequences were deposited in NCBI GeneBank.

3.3 Phylogenetic analysis and plasmid matching

The evolutionary distance (K_{nuc}) from the neighbour joining (NJ) analysis was constructed using the Maximum Composite Likelihood method (Tamura et al., 2004).

The analysis was performed in the Molecular Evolutionary Genetics Analysis (MEGA-X) (Kumar et al., 2018) which involved 11 nucleotide sequences. Next, a phylogenetic tree was constructed and the closest K_{nuc} of the isolated commercial fungus was categorised as similar species. The GLSB strain was verified using A plasmid Editor (Capelja et al., 2014) software (v2.0.55, May, 2018; adapted from <http://jorgensen.biology.utah.edu/wayned/ape/>) and is attached as supplementary data.

3.4 Batch fermentation

The inoculum preparation followed Wan-Mohtar et al. (2016b) which involved the preparation of two stages of seed culture. Both stages were cultivated for 10 to 15 days respectively following response surface methodology (RSM) conditions given (Table 3.1). In the first seed culture, four *G. lucidum* mycelial agar squares (5mm×5mm) from a 10-day-old culture plate were inoculated into a 250 mL Erlenmeyer flask containing 100 mL of optimized media solution. For the second seed inoculation, the mycelium obtained from the previous seed culture was homogenized aseptically using a hand blender for 10 seconds to yield more growing hyphae. This was performed using a sterile beaker in a laminar flow before the 20% (20 mL) of the homogenized inoculum was transferred into a 500 mL Erlenmeyer flask, consisting of 200 mL medium. Throughout the process, the inoculum was inoculated into fresh medium around day 9 to 11 (late exponential phase) in which the cells were at their most agile and vital physiological state. Thus, the entire process of the inoculum preparation took approximately less than 30 days. Meanwhile, EPS fermentation was performed in a 500 mL Erlenmeyer shake flask with 200 mL working volume.

The composition of the medium used in all stages of fermentation was as follows (g/L): [KH₂PO₄ (mono-potassium phosphate) 0.5, K₂HPO₄ (dipotassium phosphate) 0.5, yeast extract (YE) 1, NH₄Cl (ammonium chloride) 4, and MgSO₄ (magnesium sulphate) 0.5].

There were 20 distinct sets of culture conditions were set for optimisation in RSM as followed in Table 4.2.

3.4.1 Optimisation of media using response surface methodology

A central composite design (CCD) was chosen for optimisation purpose, testing three independent variables as shown in Table 3.1. Meanwhile, the selected responses were mycelial biomass (g/L) and EPS (g/L). A complete factorial CCD design in actual factors and the levels of each variables, and both responses, are presented in Table 4.2. All analytical runs were conducted in triplicates to ensure reproducibility. The optimal values of the operation parameters were statistically determined by analysis of variance (ANOVA) and plotted the three-dimensional (3D) response surface graphs using Design-Expert Software (version 7.0; © Stat-Ease, Inc. 2019). The interactions obtained were analysed by second-order polynomial equation as shown in Eq. 3.1:

$$Y = \beta^0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i < j}^k \beta_{ij} X_i X_j \quad (3.1)$$

Where Y is the predicted response, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and $X_i X_j$ are the coded values of independent factors. Any variables with $p < 0.0500$ was considered as significant at 95% level of confidence.

Table 3.1: Experimental range and levels of independent variables.

Independent variables	Range and levels		
	-1	0	1
Initial pH	4	5	6
Glucose (g/L)	10	30	50
Temperature (°C)	20	25	30

3.4.2 Experimental validation of the optimized condition

The generated optimisation model from the analysis of the response surface was validated by carrying out the tests in triplicates under the optimized condition. The mycelial biomass from the harvested *G. lucidum* cultivation process was determined by filtering a 5 mL sample for every 2 days from a second seed culture through a pre-dried and weighed GF/C filter (Whatman Ltd., U.K.) followed by repeated washing of the mycelial biomass with distilled water. The mycelial filter cakes were dried overnight in a food dehydrator (Morgan Food Dehydrator MFD-A8) at 35°C and then placed in a desiccator (24 hours) prior to weighing. The weight of the mycelial biomass was calculated by subtracting the pre-weighed filter mass from the mass with the filtrate, then multiplied by the dilution factor to get the biomass (g/L). All values were means of at least three replicates.

3.5 Analytical methods

3.5.1 Mycelium biomass

The mycelial biomass from the harvested *G. lucidum* fermentation process was measured by filtering a 5 mL sample for every 2 days from a second seed culture through a pre-dried and weighed GF/C filter (Whatman Ltd., U.K.) followed by repeated washing

of the mycelial biomass with distilled water. The mycelial or pellets filter cake were dried overnight in a food dehydrator (Morgan Food Dehydrator MFD-A8) at 35°C and then placed in a desiccator for 24 hours prior to weighing. The weight calculation of the mycelial biomass was by subtracting the pre-weighed filter mass from the mass with the filtrate and multiplied by the dilution factor to get the biomass in g/L. All values were means of at least three replicates.

3.5.2 Exopolysaccharide

The exopolysaccharide (EPS) was extracted from the supernatants of the harvested fermentation broth from *Section 3.5.1* by adding four volumes of ethanol (95% (v/v)) and left overnight at 4°C to facilitate the precipitation of crude EPS. The precipitate was then centrifuged twice at 8000 rpm for 10 min. Then, the crude EPS pellet was filtered through a pre-dried and pre-weighed GF/C glass microfiber filter paper and washed twice with 5 mL of ethanol (95% (v/v)) before kept till dry in the food dehydrator until constant weight (Morgan Food Dehydrator MFD-A8), and the weight of crude EPS was then determined (Wan et al., 2018). All measurements were carried out in triplicates.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Isolation and identification of *Ganoderma lucidum* strain BGF4A1 (GLSB)

4.1.1 Gel electrophoresis

According to Moncalvo et al. (1995a), the rDNA region and distinct variation found in the ITS rDNA region can be used to determine the species during molecular identification of wild *Ganoderma*. Figure 4 shows an agarose gel (1%) with a 642 bp ITS fragment produced for the wild *Ganoderma* sample.

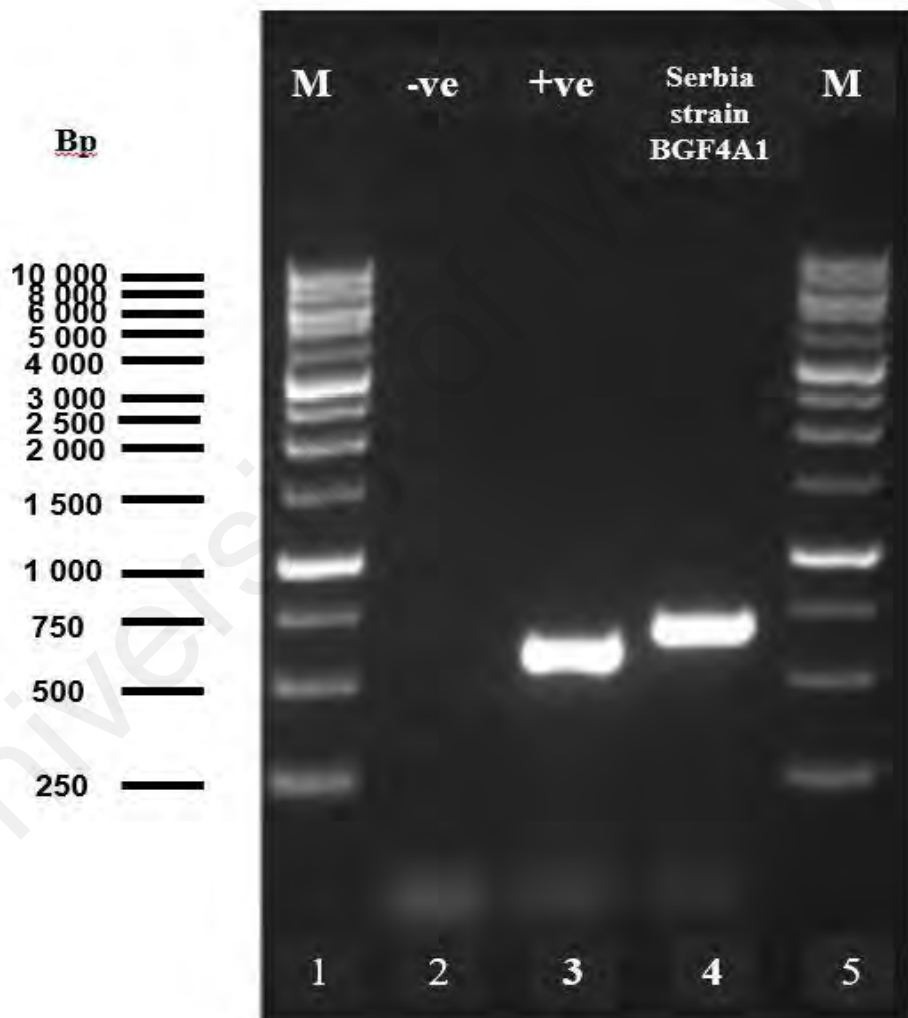


Figure 4.1: Amplified gene product isolated from wild-Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB) on an agarose gel, with a target band of 642 bp in size. DNA marker (Lane 1 and 5); PCR no template control (Lane 2); positive control fungal gDNA, 10 ng (Lane 3); PCR BGF4A1 product (Lane 4).

4.1.2 Phylogenetic tree analysis

Table 4.1: Information on species used in phylogenetic analysis.

No	Species/Isolates	Reference	Gene Bank Accession Numbers
1	<i>Ganoderma lucidum</i> strain BEOFB 431	Ćilerdžić et al. (2018)	KX371594.1
2	<i>Ganoderma lucidum</i> strain NM20 (TENN)	Unpublished	MF755277.1
3	<i>Ganoderma lucidum</i> strain BEOFB 434	Ćilerdžić et al. (2018)	KX371596.1
4	<i>Ganoderma lucidum</i> OE-234	Unpublished	AY636059.1
5	<i>Ganoderma lucidum</i> isolate HSBU200898	Unpublished	KT343316.1
6	<i>Ganoderma lucidum</i> strain ATCC 64251	Park et al. (2012)	JQ520187.1
7	<i>Ganoderma weberianum</i> strain CBS 219.36	Vu et al. (2019)	MH855780.1
8	<i>Ganoderma</i> sp. CDM-2007a strain DMC 513	Douanla-Meli and Langer (2009)	EU089970.1
9	<i>Ganoderma sichuanense</i> voucher HMAS42798	Cao et al. (2012)	JQ781877.1
10	<i>Ganoderma lucidum</i> / <i>Ganoderma lucidum</i> fusant isolate SMCC170.01.61	Unpublished	FJ501561.1
11	<i>Ganoderma lucidum</i> strain BGF4A1	This study	-

The studied sequence was analyzed and compared with other top-10 related reference taxa as shown in Table 4.1 which were retrieved from the NCBI Gene Bank. A detailed phylogenetic analysis diagram (Figure 4.2), showed the evolutionary distance (K_{nuc}) values. The tree was generated based on the Maximum Composite Likelihood method by following the method of Ab Kadir et al. (2016).

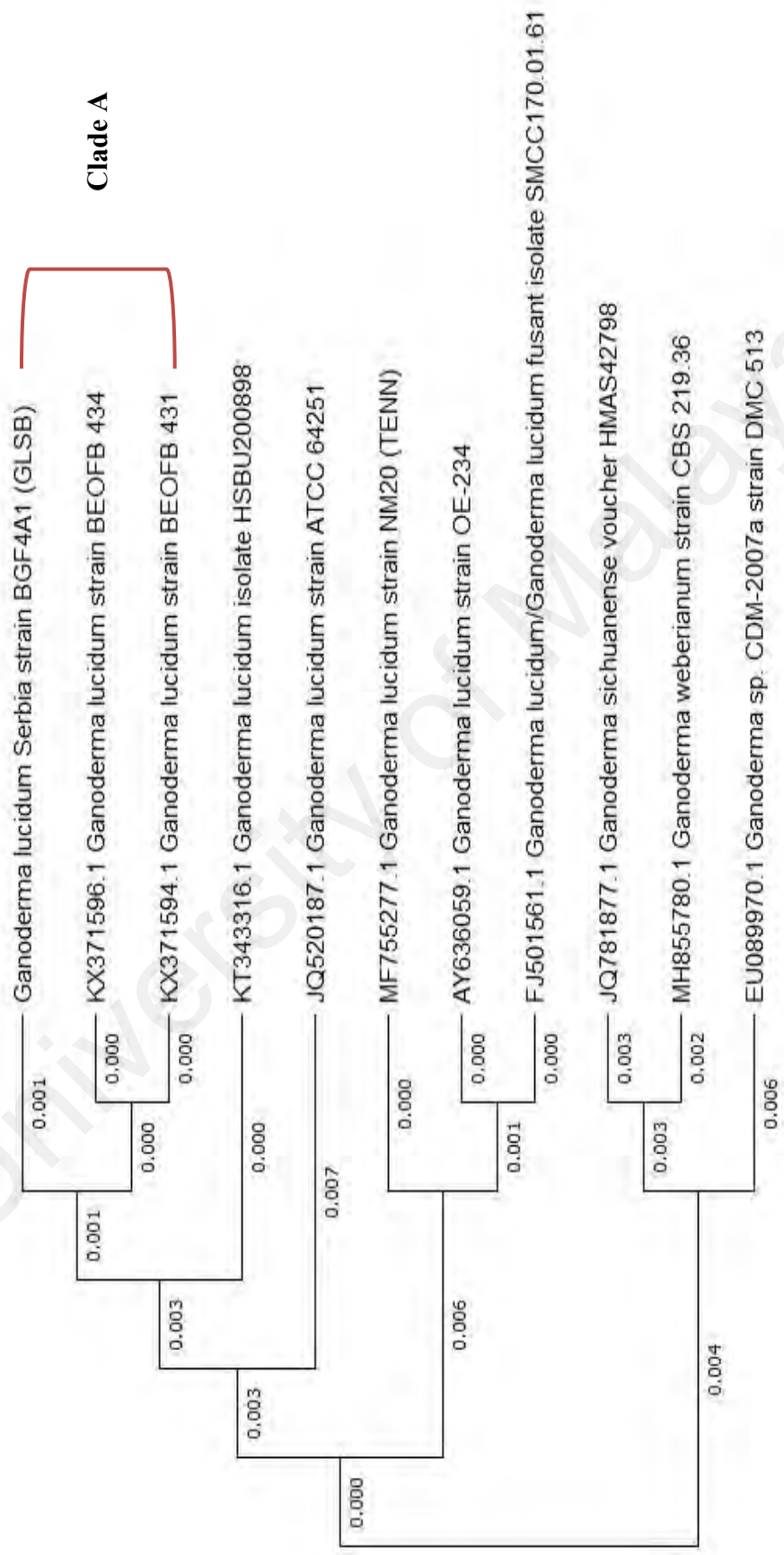


Figure 4.2: Phylogenetic tree of *Ganoderma lucidum* strain BGF4A1 (GLSB) generated by neighbour-joining (NJ) with evolutionary distance.

Thu Jan 24, 2019 20:44 CST
 GL SERBIA_642 bp.seq from 1 to 650
 Alignment to
 MG911000.1 GL.txt from 1 to 644

Matches (|): 620
 Mismatches (#): 22
 Gaps (): 10
 Unattempted (.): 0

```

1  TTCCGTAGGTGAACCTGCGAAGGATCATTATCGAGTTTGAAGCGGCTCTTACCGAGGATGTSCACACCCCTGCTCATCCACTCTACACCT 100
   |||||
1  TTCCGTAGGTGAACCTGCGAAGGATCATTATCGAGTTTGAAGCGGCTCTTACCGAGGATGTSCACACCCCTGCTCATCCACTCTACACCT 100
   |||||
101 GTGCACCTTACTGTGGGTTCCAGACGTTGTGAAGCGGCTCTTACCGAGCTT-GTAAAGCGGCTGCTGTGCTGCGGTTATCACAAACTCTATAAAGT 199
   |||||
101 GTGCACCTTACTGTGGGTTCCAGACGTTGTGAAGCGGCTCTTACCGAGCTT-GTAAAGCGGCTGCTGTGCTGCGGTTATCACAAACTCTATAAAGT 196
   |||||
*
200 ATTAGAATGTGTAATGCGATGTAACGCATATATACAACCTTACGAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCGAAATGCGATAAGT 299
   |||||
197 ATTAGAATGTGTAATGCGATGTAACGCATATATACAACCTTACGAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCGAAATGCGATAAGT 296
   |||||
*
300 AATGTGAATGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGGCTCCCTTGGTATCCGAGGAGCATGCTGTTGAGTGCATGAAATCTT 399
   |||||
297 AATGTGAATGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGGCTCCCTTGGTATCCGAGGAGCATGCTGTTGAGTGCATGAAATCTT 396
   |||||
*
400 CAACTTACAGACCCCTTTGGGGTTGTAGGCTTGGACTTTGGAGGCTTGTGCGCCATGTTTGGTGGCTCCCTCTAAATGTATTAGCTTGATTCCTTGGG 499
   |||||
397 CAACCTACAGGCCCTTGGC-GGTTTGTAGGCTTGGACTT-GGAGGCTTGTGCGCCCTTGTTCGGTCCCTCTTAAATGCAATAGCTTGATTCCTTGGG 494
   |||||
*
500 GATCGGCTCTCGGTGTGATAATGTCTACGCGCGCAGCCGTGAAGCGGTTTGGGAGCTTCTAACCCGTC-TCGTTTGTGAGACAGCTTTATGACCTCTGACC 598
   |||||
495 GATCGGCTCTCGGTGTGATAATGTCTACGCGCGCAGCCGTGAAGCGGTTTGGGAGCTTCTAACCCGTC-TCGTTTGTGAGACAGCTTTATGACCTCTGACC 592
   |||||
*
599 TCAAAATCAGGTAGGACTACCCGCTGAACCTAAGCATATCAATAAGCGGAGGA 650
   |||||
593 TCAAAATCAGGTAGGACTACCCGCTGAACCTAAGCATATCAATAAGCGGAGGA 644
   |||||

```

Figure 4.3: Alignment of GLSB strain with *G. lucidum* (Accession no: MG911000.1). Source: A plasmid editor (Capelja et al., 2014) (v2.0.55, May 4, 2018).

4.2 Morphological characteristics of an identified wild-Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB) using macroscopic observations.

The characteristic of wild-Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB) mycelium was observed as shown in Figure 4.4. On the 3rd day of inoculation (Figure 4.4a), the mycelium started to form a small and white circular growth from the center of the plate. The white mat of fungus spread densely on the 10th day of growing (Figure 4.4b).

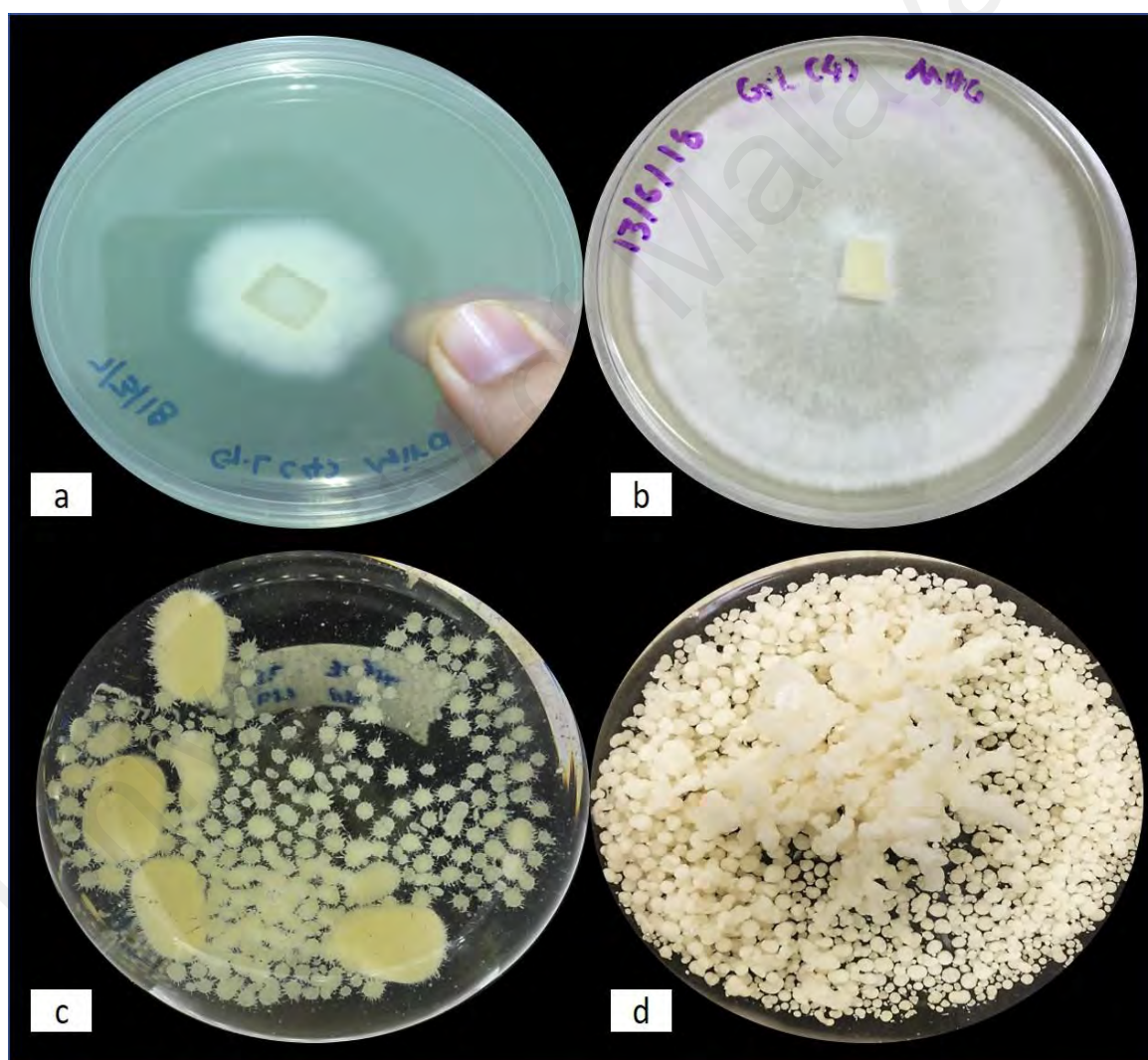


Figure 4.4: Macroscopic observation of wild-Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB) mycelium growth on PDA plate at (a) day 3 and (b) day 10; GLSB mycelium cultured in a shake flask at (c) day 10 of first seed culture and (d) day 10 of second seed culture.

Meanwhile, during shake-flask culture, GLSB strain formed a dispersed mycelium with filamentous pellets (Figure 4.4c). Basically, the morphology of mycelial pellets was depending on their growing stages and culture conditions (e.g. initial pH, temperature, glucose concentration and agitation rate).

4.3 Molecular characteristics of an identified wild-Serbian *Ganoderma lucidum* strain BGF4A1 (GLSB)

Based on the Maximum Composite Likelihood method phylogenetic tree that has been constructed (Figure 4.2), it can be observed that studied strain was placed in the same clade (Clade A) with two other *G. lucidum* strains (BEOFB 434 and BEOFB 431), which originated from Serbia and the sequence was found to be similar to both strains with 99% similarity in the Blast database. This proved that the studied sample belongs to *Ganoderma lucidum* species as those two strains, BEOFB 434 and BEOFB 431 were considered as *G. lucidum* sensu stricto (Ćilerdžić et al., 2018).

However, the studied strain has a longer branch length with a genetic distance of 0.001 compared to the other two strains, BEOFB 434 and BEOFB 431 ($K_{nuc} = 0.000$) even they could be the same species because they were taken from distinct places or region in Serbia. BEOFB 434 and BEOFB 431 were samples taken from Bojcin forest (Ćilerdžić et al., 2018) while the studied sample was taken from Mount of Avala. According to Chong et al. (2013), the locality of the samples taken can influence the genetic diversity of a species that can result to a morphological variation among similar species. This is because different localities have different climatic conditions such as humidity, rainfall and altitude.

Other than that, based on the NCBI Genebank database, the other nearest species with 99% maximum identity was a *Ganoderma lucidum* isolate HSBU200898 [Accession number of KT3433161; unpublished data]. Meanwhile, *Ganoderma lucidum* strain

ATCC 64251 was a sister clade to BEOFB 434, BEOFB 431, HSBU200898 and studied organism, with a maximum identity of 98%. This *Ganoderma lucidum* strain originated from Taiwan (Park et al., 2012). This justified that according to Adaskaveg and Gilbertson (1986), that the *Ganoderma lucidum* 'collections' assembled from many distinct regions especially *G. lucidum* from Europe were not conspecific with *G. lucidum* in North America and in Asia. This might arise because of the vicariance events (Smith & Sivasithamparam, 2000a).

Phylogenetically, the clustering within the bottom clade consisting of *Ganoderma sichuanense*, *Ganoderma weberianum*, and *Ganoderma* sp. CDM-2007a showed that they were distinct species from the other *Ganoderma lucidum* sp. with 96% maximum identity in the NCBI Genebank respectively.

In addition, the species confirmation was supported by the alignment of blasting sequences of GLSB strain with other *G. lucidum* strain in plasmid editor (Capelja et al., 2014) software (Figure 4.3) which had a 100% of similarity in BLAST software. This software detected 620 matches and low number of nucleotides mismatched by 22 mismatches when aligned with *G. lucidum* (accession number: MG91100.1), which confirmed the GLSB strain as a *G. lucidum* species.

4.4 Growth curve analysis

The study of the growth of filamentous fungi could be vital in many fields including clinical analysis and agriculture. For instance, in clinical analysis, Meletiadis et al. (2001) used the growth curve study to optimize the methodology for antifungal susceptibility testing in different types of media. Meanwhile, in agriculture, Wan-Mohtar et al. (2016a) used the growth curve to study the morphology and the optimisation of repeated batch fermentation (RBF) while to enhance the EPS production. In this study, growth curve analysis was performed for biomass and exopolysaccharide of *Ganoderma lucidum* strain

BGF4A1 (GLSB) under selected optimized culture condition (Table 4.2) which at 25°C, 50 g/L of glucose, and initial pH 5. This is to determine the best day of fungi cultivation to extract the specific product including biomass and EPS.

Figure 4.5 shows the normal pattern of fungus growth curve, recorded for the dry weight of mycelia cultured over distinct period. Apparently, the growth curve shows that the fungi adapt quickly with culture medium as it used up the nutrient quickly from Day 0 to Day 2. The highest value of biomass achieved was at Day 10 (3.41g/L), where the transition phase took place. Transition phase is when there is high variation and rapid changes. Meanwhile, the death phase was at Day 14.

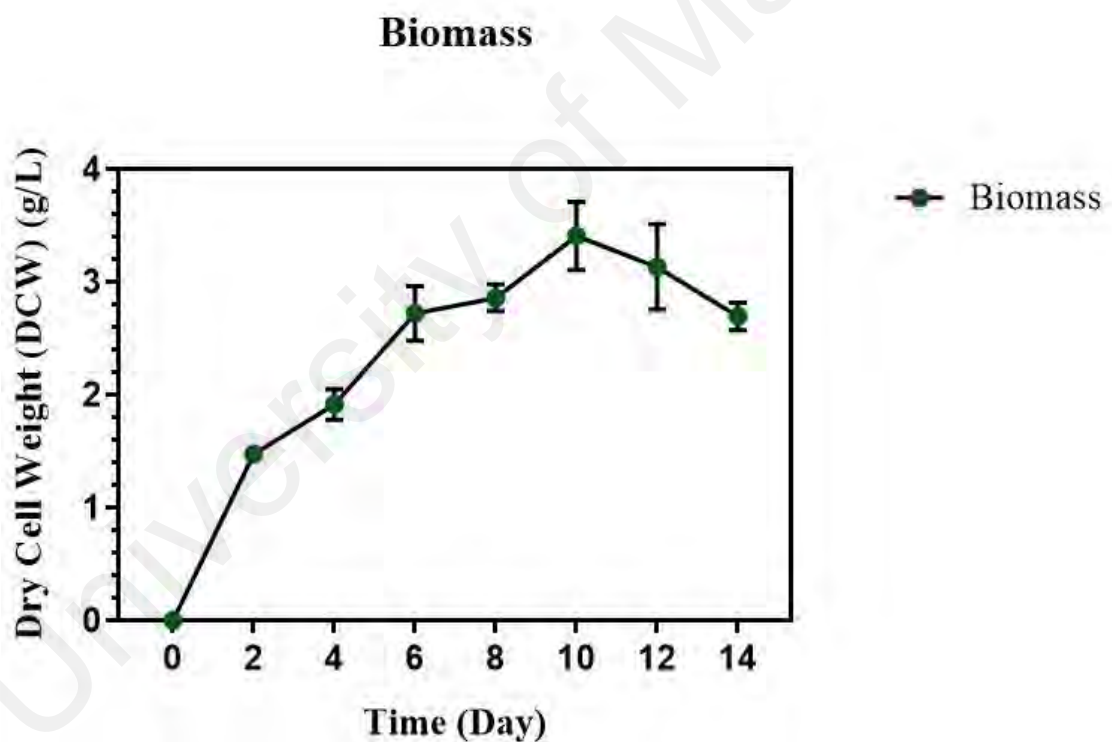


Figure 4.5: Biomass growth curve over time calculated by dry cell weight (DCW)

Exopolysaccharide (EPS)

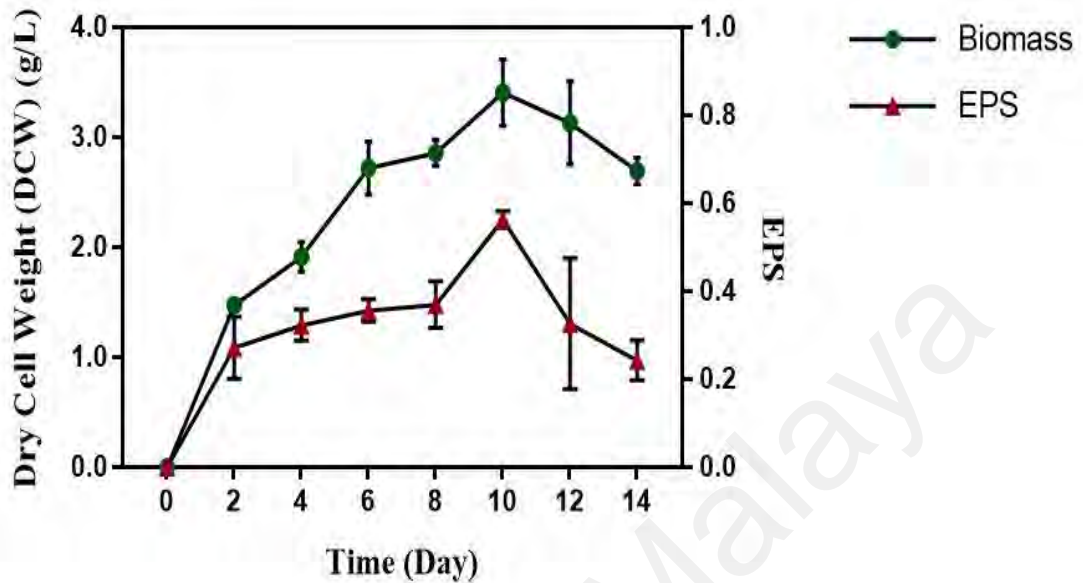


Figure 4.6: EPS growth curve over time calculated by dry cell weight (DCW)

The time profile for the highest level of EPS was done in Figure 4.6. The maximum EPS production achieved at Day 10 (2.25g/L) which is the same day with biomass.

4.5 Optimisation using Response Surface Methodology

The effects of temperature, initial pH, and glucose concentration on biomass and EPS production from *G. lucidum* mycelium were evaluated. Twenty distinct sets of culture conditions were used for optimisation in RSM as tabulated in Table 4.

Table 4.2: Experimental design using RSM with CCD and responses for mycelium biomass and EPS production of *G. lucidum*.

Run Order	Variables			Responses	
	Initial pH	Temperature °C	Glucose (g/L)	Mycelium biomass (g/L)	EPS (g/L)
				Actual value	Actual value
1	5	25	30	1.38	1.03
2	5	30	30	2.92	1.50
3	4	30	10	1.90	0.73
4	5	25	30	1.81	1.61
5	5	25	30	1.40	1.41
6	4	20	10	1.14	0.63
7	6	30	10	2.48	0.69
8	6	20	10	1.81	0.48
9	5	25	30	2.29	1.62
10	5	25	10	1.91	0.50
11	6	20	50	1.83	0.93
12	5	25	30	2.46	1.38
13	5	20	30	1.96	1.55
14	4	30	50	2.97	0.88
15	6	25	30	1.42	0.91
16	6	30	50	2.45	1.84
17	5	25	50	3.41	2.25
18	5	25	30	2.19	1.76
19	4	20	50	1.95	1.51
20	4	25	30	0.92	0.58

The sum of squares sequential model (SMSS) and lack of fit test were carried out as shown in Table 4.3 and Table 4.4. This was to determine the appropriate polynomial

equation to represent the correlations between the input parameters and the output response (biomass).

Table 4.3: Sequential model sum of squares (SMSS) analysis for biomass.

Sequential Model Sum of Squares						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	82.39364	1	82.39364			
Linear vs Mean	2.871435	3	0.957145	3.225485	0.0506	Aliased
2FI vs Linear	0.485777	3	0.161926	0.493892	0.6927	Aliased
Quadratic vs 2FI	2.628503	3	0.876168	5.363314	0.0185	Suggested
Cubic vs Quadratic	0.503761	4	0.12594	0.668787	0.6369	Aliased
Residual	1.12987	6	0.188312			
Total	90.01299	20	4.500649			

Table 4.4: Lack of fit test for biomass.

Lack of Fit Tests						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	3.674372	11	0.334034	1.55576	0.3274	Aliased
2FI	3.188595	8	0.398574	1.856357	0.2568	Aliased
Quadratic	0.560092	5	0.112018	0.521725	0.7538	Suggested
Cubic	0.05633	1	0.05633	0.262358	0.6303	Aliased
Pure Error	1.073539	5	0.214708			

Both analyses suggested that the relationship between input parameters and biomass can be modelled using quadratic equations.

The normal probability plot of the residuals in Figure 4.7 demonstrates the distinction between the predicted and experimental data for all response variables of biomass production where the underlying initial pH, temperature and glucose concentration are framing a straight line. The information data on both sides of the line are normally

distributed which tells that both test information and the proposed quadratic model are in great concession to represent the variable responses of mycelial biomass production.

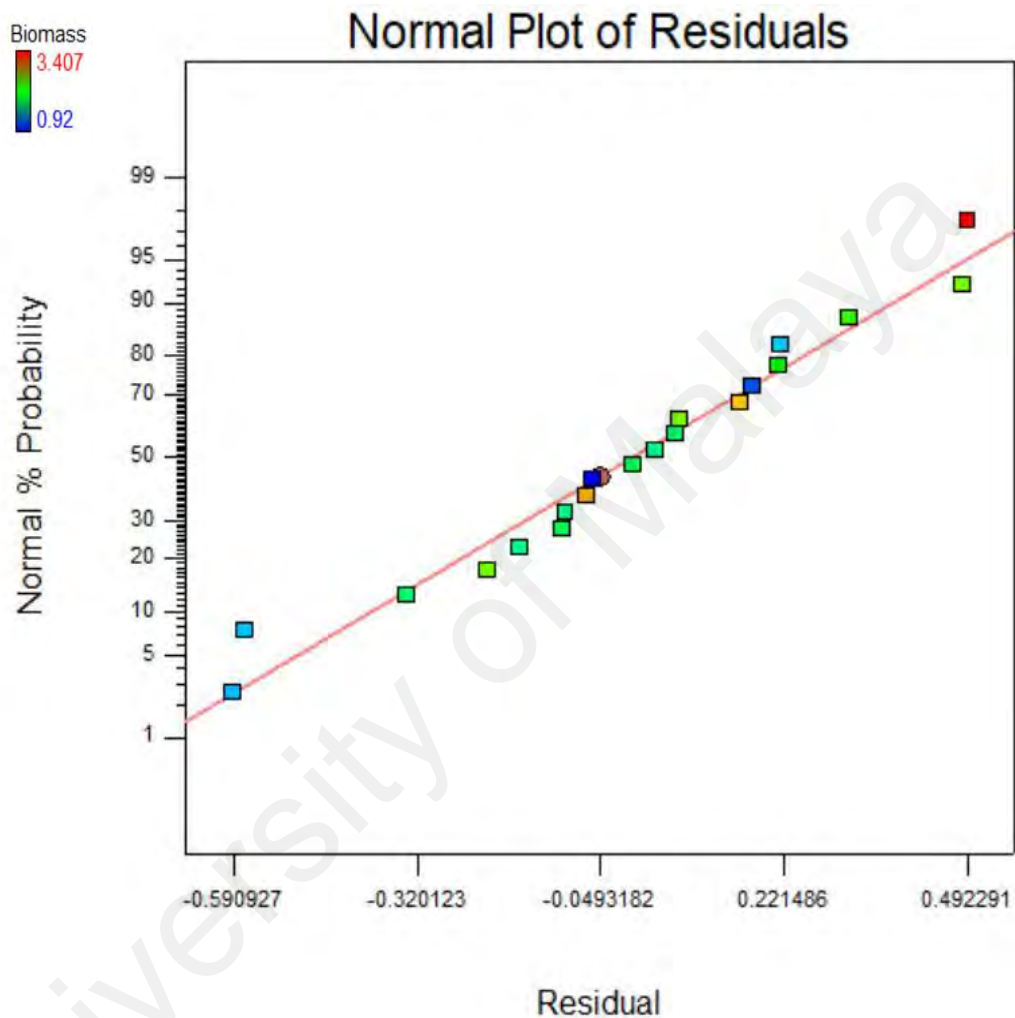


Figure 4.7: Normal probability plot of the residuals for biomass

4.6 Optimisation of Mycelium Biomass Growth

ANOVA for mycelium biomass production is shown in Table 4.5. The model was significant, as the value of “Prob > F” was 0.0196 (<0.05). This showed that the response variable of the quadratic model was significant at a 95% confidence level. The coefficient determination ($R^2 = 0.7856$) stipulated that 78.56% of the variability in the response can

be explained by the model, while the remaining variability was not. The model for biomass yield was regressed by considering the actual variables and is expressed in Eq. 4.1:

$$\begin{aligned}
 \mathbf{Biomass} = & -15.17172 + 9.59432 \times \text{pH} - 0.64477 \times \text{temperature} - \\
 & 0.021022 \times \text{glucose} - 0.012175 \times \text{pH} \times \text{temperature} - 0.011869 \times \text{pH} \times \\
 & \text{glucose} + 0.00025875 \times \text{temperature} \times \text{glucose} - 0.88232 \times \text{pH}^2 + \\
 & 0.015567 \times \text{temperature}^2 + 0.0015117 \times \text{glucose}^2 \quad (4.1)
 \end{aligned}$$

The main effect graphs for biomass production were plotted as shown in Figure 4.8. Those graphs illustrated the influence of selected parameters (initial pH, temperature and glucose) on the production of biomass. Figure 4.8a shows the initial pH main factor curve which indicates that as the most optimal pH level for mycelial production was at pH 5 compared with other pH level involved especially pH 4 and pH 6. Figure 4.8b suggested that an increase in temperature from 20°C to 30°C resulted in an increase of biomass yield. This significant increment is support by temperature (B) p-value is less than 0.05 in ANOVA analysis (Table 4.5). Reported studies by Boddy (1983); Cartwright and Findlay (1934), suggested that the optimal temperature for basidiomycetes growth ranges from 20°C to 30°C. Meanwhile, Figure 4.8c shows a glucose factor influencing the biomass production as the glucose concentration increased, the mycelial growth increased. The ANOVA analysis (Table 4.5) supported that glucose concentration (C) was statistically significant ($p < 0.05$). From the model, among the three variables, temperature exhibited the strongest effect ($p = 0.0104$), followed by glucose concentration ($p = 0.0252$) and pH ($p = 0.4067$). Both quadratic terms of initial pH (AA) and glucose concentration (CC) also showed a significant effect ($p < 0.05$) on mycelium

biomass yield. However, negative effects were shown by initial pH (A) and quadratic terms (AB, AC, BC, and B²).

Table 4.5: Analysis of variance for the experimental results of the CCD quadratic model for mycelium biomass.

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	5.985715066	9	0.665079452	4.071172726	0.0196	Significant
A: pH	0.1225449	1	0.1225449	0.750138128	0.4067	
B: Temperature	1.6192576	1	1.6192576	9.912014813	0.0104	Significant
C: Glucose	1.1296321	1	1.1296321	6.914854133	0.0252	Significant
AB	0.029646125	1	0.029646125	0.1814738	0.6791	
AC	0.450775125	1	0.450775125	2.759344601	0.1277	
BC	0.005356125	1	0.005356125	0.032786624	0.8599	
A ²	2.140834778	1	2.140834778	13.10476235	0.0047	Significant
B ²	0.416521841	1	0.416521841	2.549668846	0.1414	
C ²	1.005510278	1	1.005510278	6.155063144	0.0325	Significant
Residual	1.633631134	10	0.163363113			
Lack of fit	0.560091801	5	0.11201836	0.521724527	0.7538	Not significant
Pure error	1.073539333	5	0.214707867			
Cor total	7.6193462	19				
Std. dev. = 0.40418203			Mean = 2.0297			
R ² = 0.785594316			Adj R ² = 0.5926292			
Adequate precision = 7.255077177						

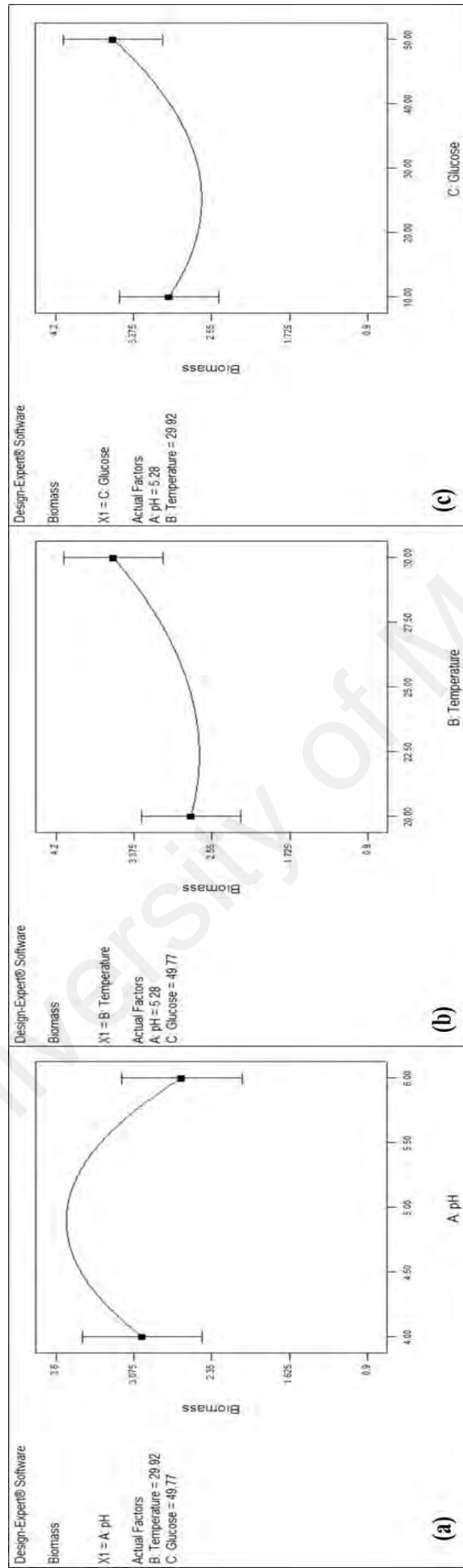


Figure 4.8: (a) Main effect graph plot showing the mycelial biomass production in response to variation of parameters: X1 = A: initial pH with actual factors B: temperature = 29.92°C and C: glucose = 49.77 g/L; (b) X1 = B: temperature with actual factors A: initial pH = 5.28 and C: glucose = 49.77 g/L and (c) X1 = C: glucose with actual factors A: initial pH = 5.28 and B: temperature = 29.92°C of wild-Serbian *G. lucidum* BGF4A1

The quadratic models were represented as response surface 3D graphs (Figure 4.9), with the combination effect of initial pH, temperature, and glucose concentration. One factor was maintained at a constant value corresponding to the other two factors that were varied within the experimental range. Figure 4.9a shows the effect of initial pH (A) and temperature (B), Figure 4.9b shows the effect of initial pH (A) and glucose concentration (C), and Figure 4.9c shows the effect of temperature (B) and glucose concentration (C) on mycelial biomass production. Both figure 4.9a and 4.9b shows that at highest temperature and glucose concentration, changes in pH from low to high point resulted in curved response, which signifies some significance due to (A^2) term in full model is significant while (A) term alone is not significant. However, at any given pH, the effects of temperature and glucose concentration are stronger and the suitable initial pH appeared to be a pH of 5.00. Meanwhile, Figure 4.9c shows that the effects of glucose and temperature on production of mycelial biomass were more important than that of initial pH. The maximum yield of biomass exhibited by the sample was observed at 30°C, initial pH of 5.26, and 50 g/L glucose concentration.

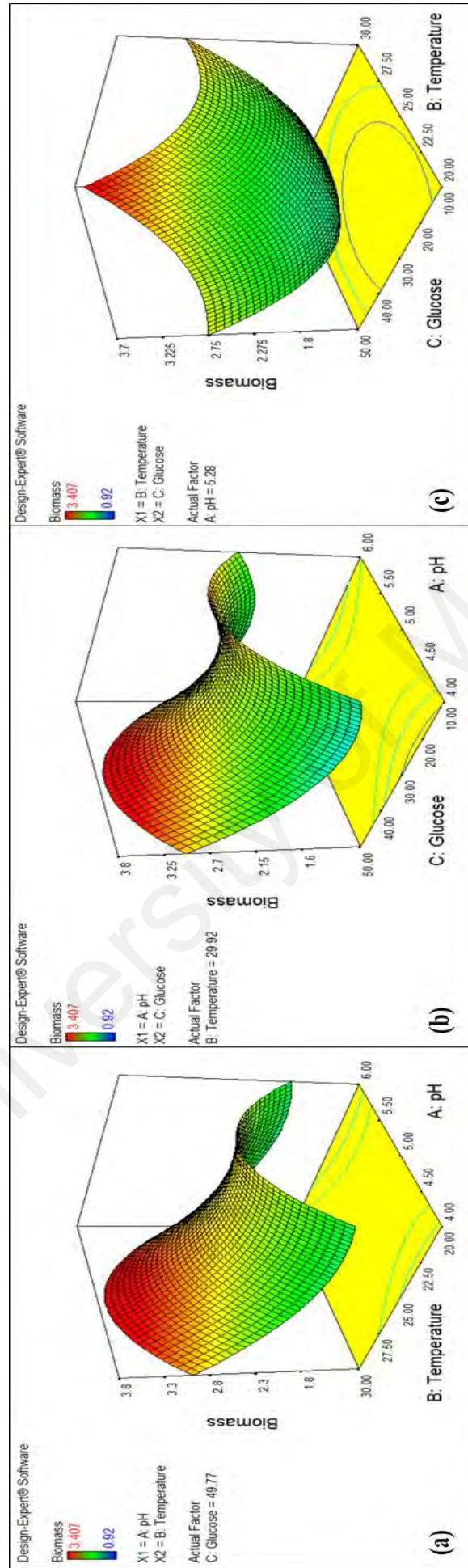


Figure 4.9: Response surface (3D graph plot) showing the combined effects of (a) X1 = A: initial pH and X2 = B: temperature with actual factor C: glucose = 49.77 g/L; (b) X1= A: initial pH and X2= C: glucose concentration with actual factor B: temperature = 29.92°C and (c) X1= B: temperature and X2= C: glucose concentration with actual factor A: initial pH = 5.28 of wild-Serbian *G. lucidum* BGF4A1 on mycelial biomass production.

4.7 Optimisation of EPS production

The determination of suitable polynomial equation was carried out by the sum of squares sequential model (SMSS) (Table 4.6) and lack of fit test (Table 4.7). This was to depict the correlations between the input parameters and the output response (EPS).

Table 4.6: Sequential model sum of squares (SMSS) analysis for EPS

Sequential Model Sum of Squares						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	28.32676	1	28.32676			Suggested
Linear vs Mean	1.980527	3	0.660176	3.436155	0.0423	Aliased
2FI vs Linear	0.376194	3	0.125398	0.604255	0.6238	Aliased
Quadratic vs 2FI	1.55028	3	0.51676	4.503176	0.0303	Suggested
Cubic vs Quadratic	0.784711	4	0.196178	3.24408	0.0965	Aliased
Residual	0.362835	6	0.060473			
Total	33.38131	20	1.669065			

Table 4.7: Lack of fit test for EPS

Lack of Fit Test						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	2.738467	11	0.248952	3.709568	0.0796	Aliased
2FI	2.362273	8	0.295284	4.399958	0.0597	Aliased
Quadratic	0.811993	5	0.162399	2.419862	0.1772	Suggested
Cubic	0.027282	1	0.027282	0.40652	0.5518	Aliased
Pure Error	0.335553	5	0.067111			

Both analyses suggested that the relationship between input parameters and EPS can be modelled using quadratic equations.

The normal probability plot of the residuals in Figure 4.10 shows the difference between the predicted and experimental data for all response variables of EPS production where the initial pH, temperature and glucose concentration data points are approximately linear. The information data on both sides of the line are normally distributed which tells that both test information and the proposed quadratic model are in great concession to represent the variable responses of EPS production.

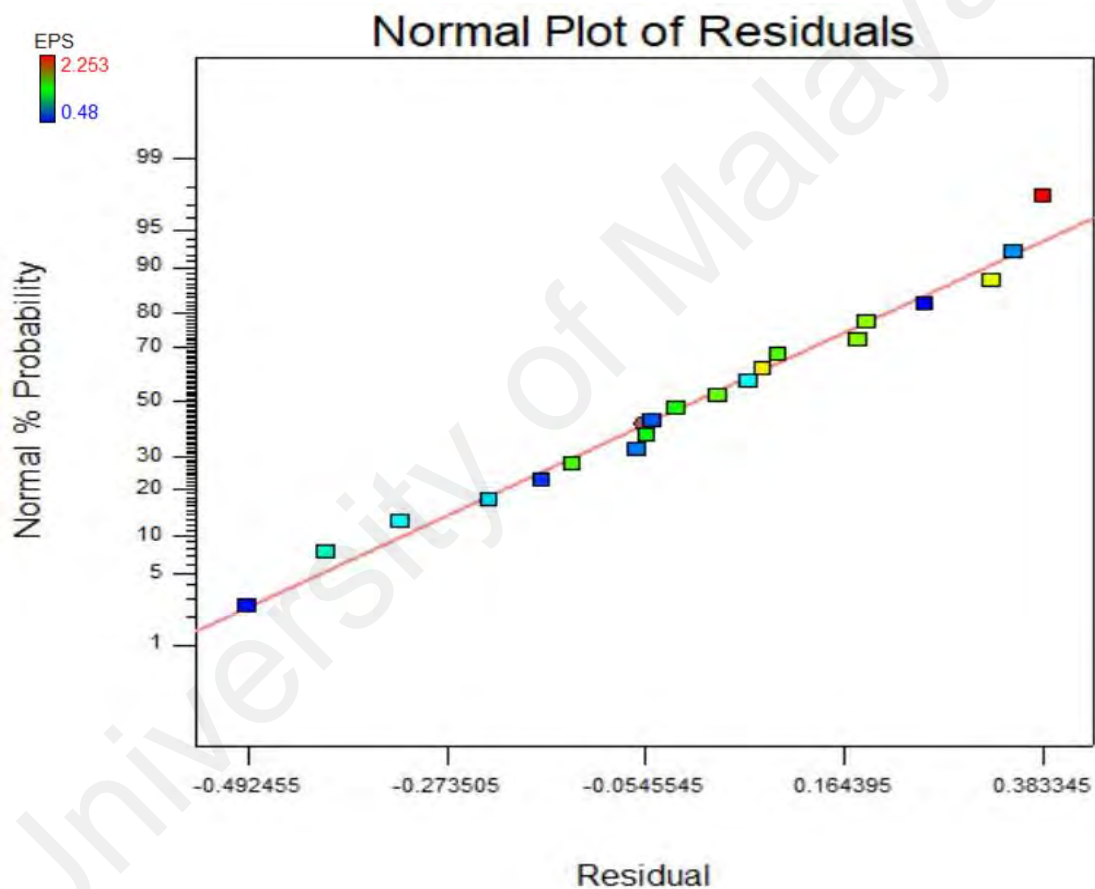


Figure 4.10: Normal probability plot of the residuals for EPS

ANOVA for EPS production is shown in Table 4.8. The predicted coefficient determination indicates that 77.30% ($R^2 = 0.7730$) of the variability in the response can be explained using this model. The model was significant for further analysis ($p < 0.05$). The model for EPS production was regressed by considering the actual variables and is expressed in Eq. 4.2:

$$\begin{aligned}
 \text{EPS} = & -6.23644 + 5.24125 \times \text{pH} - 0.48852 \times \text{temperature} + 0.00515386 \times \\
 & \text{glucose} + 0.040875 \times \text{pH} \times \text{temperature} + 0.00361875 \times \text{pH} \times \text{glucose} - \\
 & 0.0000412500 \times \text{temperature} \times \text{glucose} - 0.63191 \times \text{pH}^2 + 0.00592364 \times \\
 & \text{temperature}^2 - 0.00000477273 \times \text{glucose}^2
 \end{aligned} \tag{4.2}$$

The main effect graphs for EPS production were plotted as shown in Figure 4.11. Those graphs illustrated the influence of selected parameters (initial pH, temperature and glucose) on the production of EPS. Figure 4.11a shows the initial pH (A) main factor curve which indicates that as the most optimal pH level for mycelial production was at pH 5. A study by Kim et al. (2006), found that the optimum pH level for EPS production of *G. lucidum* was at pH 5. Meanwhile, the increase in temperature from 20°C to 30°C in Figure 4.11b, resulted in no significant difference on biomass production. Figure 4.11c shows a glucose factor influencing the biomass production as the glucose concentration increased, the mycelial growth increased. From the ANOVA analysis model, EPS production was dependent only on glucose concentration (C). The other variables, temperature and initial pH, were found to be insignificant for EPS production with $p > 0.05$. Among the studied variables, glucose concentration demonstrated the strongest effect ($p = 0.0022$), followed by temperature ($p = 0.6251$) and pH ($p = 0.6340$). The quadratic terms of initial pH (AA) also showed a significant effect at $p < 0.05$ on the yield of EPS production. However, negative effects were shown by initial pH (A), temperature (B), and quadratic terms (AB, AC, BC, B² and C²).

Table 4.8: Analysis of variance for the experimental results of the CCD quadratic model for EPS production.

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	3.907001848	9	0.434111316	3.782953663	0.0249	Significant
A: pH	0.0276676	1	0.0276676	0.241102327	0.6340	

Table 4.8, continued.

B: temperature	0.02916	1	0.02916	0.254107471	0.6251	
C: glucose	1.9236996	1	1.9236996	16.76359536	0.0022	Significant
AB	0.334153125	1	0.334153125	2.911893196	0.1187	
AC	0.041905125	1	0.041905125	0.365171651	0.5591	
BC	0.000136125	1	0.000136125	0.001186227	0.9732	
A ²	1.098100023	1	1.098100023	9.569115908	0.0114	Significant
B ²	0.060310023	1	0.060310023	0.525556494	0.4851	
C ²	1.00227E-05	1	1.00227E-05	8.73405E-05	0.9927	
Residual	1.147545952	10	0.114754595			
Lack of fit	0.811992619	5	0.162398524	2.419861579	0.1772	Not significant
Pure error	0.335553333	5	0.067110667			
Cor total	5.0545478	19				
Std. dev. = 0.338754476			Mean = 1.1901			
R ² = 0.772967633			Adj R ² = 0.568638502			
Adequate precision = 6.860330024						

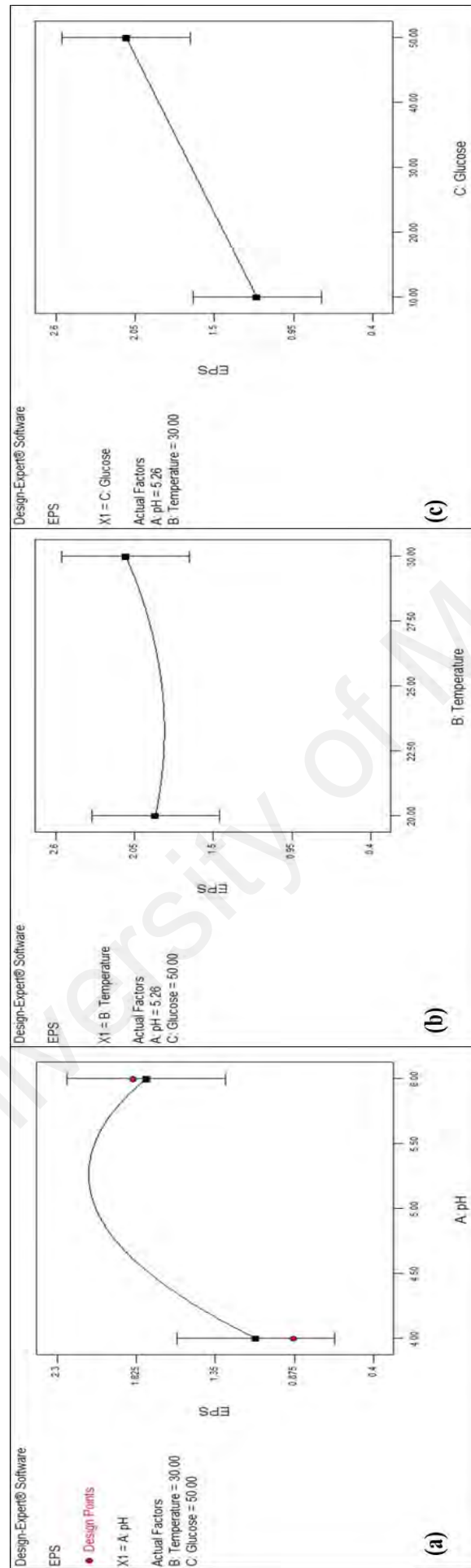


Figure 4.11: Main effect graph plot showing the EPS production in response to variation of parameters: (a) X1 = A: initial pH with actual factors B: temperature = 30°C and C: glucose = 50 g/L; (b) X1 = B: temperature with actual factors A: initial pH = 5.26 and C: glucose = 50 g/L and (c) X1 = C: glucose with actual factors A: initial pH = 5.26 and B: temperature = 30°C of wild-Serbian *G. lucidum* BGF4A1.

Figure 4.12a shows the 3D plot of initial pH (A) and temperature (B), which both had a negative effect on EPS production at the quadratic level. The maximum yield of EPS production was observed at an initial pH of 5.0. Meanwhile, Figure 4.12b shows that EPS production was affected by glucose concentration (C), as the maximum value of EPS (2.253 g/L) was at high glucose concentration (50 g/L). Figure 4.12c also shows a positive coefficient of glucose concentration (C), indicating a linear effect with increased glucose concentration (C), and resulting in increased EPS production while at low glucose concentration (C), the increase in temperature (B) had minimal effect on EPS production. The results of this experiment supported those of previous study, reported by Hsieh et al. (2006), showing that glucose had a positive effect on EPS production. The optimum conditions for maximum yield of EPS production were therefore determined to be initial pH of 5.26, 50 g/L of glucose concentration, and temperature of 30°C.

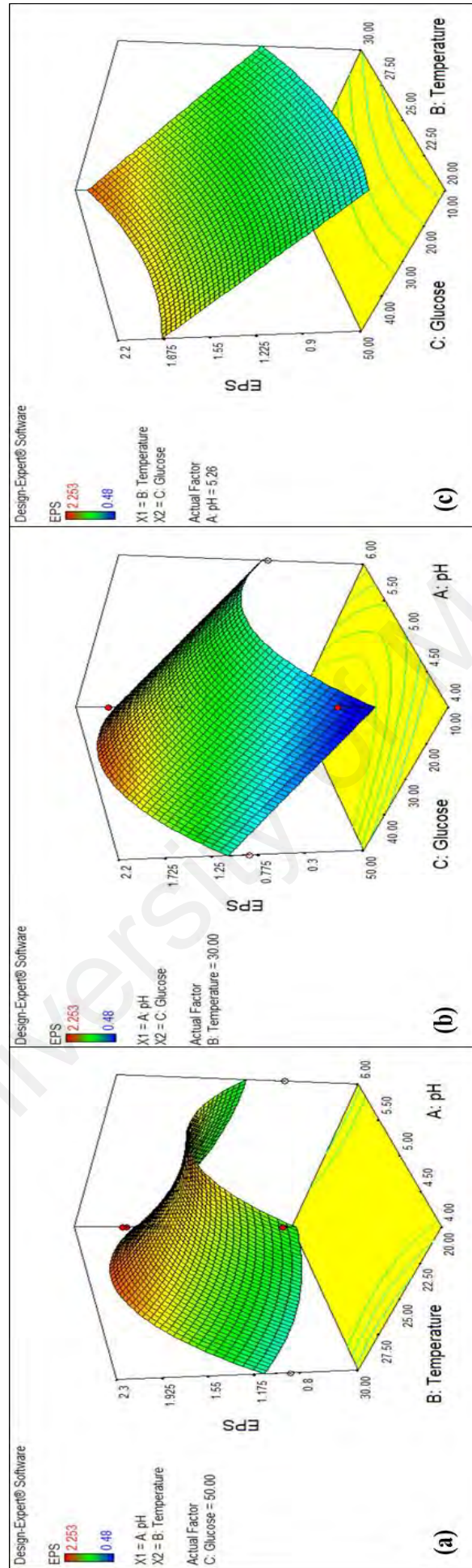


Figure 4.12: Response surface (3D graph plot) showing the combined effect of (a): X1 = A: initial pH and X2 = B: temperature with actual factor C: glucose = 50 g/L (b) X1 = A: initial pH and X2 = C: glucose concentration with actual factor B: temperature = 30°C and (c) X1 = B: temperature and X2 = C: glucose concentration with actual factor A: initial pH = 5.26 of wild-Serbian *G. lucidum* BGF4A1 on EPS production.

4.8 Optimized conditions verification

To verify the effectiveness of the model, the biomass and EPS yields were measured and compared with the predicted values of responses under the statistically optimal conditions. The validation experiment was performed in triplicate. The predicted values for mycelial biomass and EPS production were 3.45 g/L and 2.11 g/L, respectively, which were in line with the experimental values of 3.09 g/L and 1.98 g/L (10.43% and 6.16% difference, respectively, between the values). Thus, the validity of the model under Eq. 4.1 and Eq. 4.2 was justified for biomass and EPS production as the average error of deviation was <15% (Milkey et al., 2014).

Table 4.9: Validation of the model using optimized conditions.

Run	Variables			Response	
	pH	Temperature	Glucose	Biomass (g/L)	EPS (g/L)
Biomass	5.28	29.92	47.99	3.09 ± 0.1	-
EPS	5.26	30.00	50.00	-	1.98 ± 0.3
Biomass + EPS	5.26	30.00	50.00	3.12 ± 0.3	1.96 ± 0.4

4.9 Comparison of the current study with the literature

Recent statistical optimisation approaches to determine suitable parameters for efficient mycelial biomass and EPS production using *Ganoderma lucidum* in controlled shake-flask fermentation are shown in Table 4.10. As reported, only two previous studies applied distinct statistical approaches other than RSM by presenting the mycelial biomass and EPS production as responses. According to Chang et al. (2006) and Baojing et al. (2012), both studies stated that under optimal conditions of medium prepared, the yield of biomass and EPS were significantly increased. Meanwhile, both current study and study by Supramani et al. (2019a) demonstrated a high EPS yield but low mycelial biomass using the same optimisation technique. However, the present study therefore demonstrated higher efficiency in producing EPS compared with previous studies based on the data shown in Table 4.10. The production of EPS relative to its biomass by Serbian *Ganoderma* is 12%, 61%, and 39% higher than that of Malaysian, Taiwan and China *Ganoderma*, respectively. Hence, RSM represents an effective statistical optimisation approach for improving the biomass and EPS yields from *G. lucidum*.

Table 4.10: Comparison with published optimization processes using *Ganoderma lucidum* in submerged-liquid fermentation.

Origin	Optimisation approach	Cultivation mode	Initial pH	Glucose (g/L)	Temperature (°C)	Biomass (g/L)	EPS (g/L)	$Y_{EPS/X}$	References
Serbia	Response surface methodology	Shake flask	5.26	50	30	3.12	1.96	0.63	<i>Current study</i>
Malaysia	Response surface methodology	Shake flask	4	26.5	-	5.19	2.64	0.51	Supramani et al. (2019a)
Taiwan	Taguchi's orthogonal array	Shake flask	6.5	12.1	34	18.7	0.42	0.02	Chang et al. (2006)
China	Orthogonal matrix	Shake flask	-	50	30	7.235	1.723	0.24	Baojing et al. (2012)

CHAPTER 5: CONCLUSION

This present study evinced that the studied strain, BGF4A1 (GLSB) is morphologically, molecularly and phylogenetically identified as *G. lucidum* species. The RSM with CCD was applied for statistical optimisation of mycelial biomass and EPS production of *G. lucidum* by SLF. Both temperature and glucose concentration ($p < 0.05$) were found to be significant variables for higher yield of mycelial biomass production by 3.12 g/L, while only glucose concentration showed a significant effect by producing 1.98 g/L of EPS. From the quadratic model, optimal growth conditions generated for maximum mycelial biomass and EPS production were identified at a temperature of 30°C, initial pH of 5.26, and 50 g/L of glucose concentration.

REFERENCES

- Ab Kadir, S., Wan, W. A. A. Q. I., Mohammad, R., Lim, S. A. H., Mohammed, A. S., & Saari, N. (2016). Evaluation of commercial soy sauce koji strains of *Aspergillus oryzae* for γ -aminobutyric acid (GABA) production. *Journal of industrial microbiology & biotechnology*, 43(10), 1387-1395.
- Adaskaveg, J. E., & Gilbertson, R. L. (1986). Cultural studies and genetics of sexuality of *Ganoderma lucidum* and *G. tsugae* in relation to the taxonomy of the *G. lucidum* complex. *Mycologia*, 78(5), 694-705.
- Agudelo-Escobar, L. M., Gutiérrez-López, Y., & Urrego-Restrepo, S. (2017). Effects of aeration, agitation and pH on the production of mycelial biomass and exopolysaccharide from the filamentous fungus *Ganoderma lucidum*. *Dyna*, 84(200), 72-79.
- Ahmad, R., Al-Shorgani, N. K. N., Hamid, A. A., Yusoff, W. M. W., & Daud, F. (2013). Optimization of medium components using response surface methodology (RSM) for mycelium biomass and exopolysaccharide production by *Lentinus squarrosulus*. *Advances in Bioscience and Biotechnology*, 4(12), 1079.
- Ai-lati, A., Liu, S., Ji, Z., Zhang, H., & Mao, J. (2017). Structure and bioactivities of a polysaccharide isolated from *Ganoderma lucidum* in submerged fermentation. *Bioengineered*, 8(5), 565-571.
- Badalyan, S., Gharibyan, N., Iotti, M., & Zambonelli, A. (2012). *Morphological and genetic characteristics of different collections of Ganoderma P. Karst. species*. Paper presented at the The 18th Congress of the International Society for Mushroom Science.
- Baojing, Y., Xiaoyan, C., & Rijun, Z. (2012). Optimization of exopolysaccharides production from a novel strain of *Ganoderma lucidum* CAU5501 in submerged culture. *Brazilian Journal of Microbiology*, 43(2), 490-497.
- Bishop, K. S., Kao, C. H., Xu, Y., Glucina, M. P., Paterson, R. R. M., & Ferguson, L. R. (2015). From 2000 years of *Ganoderma lucidum* to recent developments in nutraceuticals. *Phytochemistry*, 114, 56-65.
- Boddy, L. (1983). Effect of temperature and water potential on growth rate of wood-rotting basidiomycetes. *Transactions of the British Mycological Society*, 80(1), 141-149.
- Bonde, M. R., Micales, J. A., & Peterson, G. L. (1993). The use of isozyme analysis for identification of plant-pathogenic fungi. *Plant Disease*(10), 961-968.
- Box, G. E., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters*.
- Broudiscou, L. P., Papon, Y., & Broudiscou, A. F. (1999). Effects of minerals on feed degradation and protein synthesis by rumen micro-organisms in a dual effluent fermenter. *Reproduction Nutrition Development*, 39(2), 255-268.

- Bruns, T. D., & Szaro, T. M. (1992). Rate and mode differences between nuclear and mitochondrial small-subunit rRNA genes in mushrooms. *Molecular Biology and Evolution*, 9(5), 836-855.
- Bruns, T. D., White, T. J., & Taylor, J. W. (1991). Fungal molecular systematics. *Annual Review of Ecology and Systematics*, 22(1), 525-564.
- Cao, Y., Wu, S.-H., & Dai, Y.-C. (2012). Species clarification of the prize medicinal *Ganoderma* mushroom “Lingzhi”. *Fungal Diversity*, 56(1), 49-62.
- Capelja, E., Stevic, N., Galovic, V., Novakovic, M., & Karaman, M. (2014). RDNA based analysis of autochthonous fungal species from Serbia. *Genetika*, 46(1), 33-42.
- Cartwright, K., & Findlay, W. (1934). Studies in the physiology of wood-destroying fungi II. Temperature and rate of growth. *Annals of Botany*, 48(2), 481-495.
- CFR Ferreira, I., A Vaz, J., Vasconcelos, M. H., & Martins, A. (2010). Compounds from wild mushrooms with antitumor potential. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, 10(5), 424-436.
- Chandra, A., & Purkayastha, R. (1977). Physiological studies on Indian edible mushrooms. *Transactions of the British Mycological Society*, 69(1), 63-70.
- Chang, M.-Y., Tsai, G.-J., & Houn, 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.
- Chong, K.-P., Abdullah, S., & Tong-Leong, N. (2013). Molecular fingerprint of *Ganoderma* spp. from Sabah, Malaysia. *International Journal of Agriculture and Biology*, 15(6), 1112-1118.
- Ćilerdžić, J., Vukojević, J., Stajić, M., Stanojković, T., & Glamočlija, J. (2014). Biological activity of *Ganoderma lucidum* basidiocarps cultivated on alternative and commercial substrate. *Journal of ethnopharmacology*, 155(1), 312-319.
- Ćilerdžić, J. L., Vukojević, J. B., Klaus, A. S., Ivanović, Ž. S., Blagojević, J. D., & Stajić, M. M. (2018). Wheat straw – a promising substrate for *Ganoderma lucidum* cultivation. *Acta Scientiarum Polonorum, Hortorum Cultus*, 17(1), 13-22.
- De Silva, D. D., Rapior, S., Fons, F., Bahkali, A. H., & Hyde, K. D. (2012). Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. *Fungal Diversity*, 55(1), 1-35.
- Deshpande, R. P., Chinnan, M. S., & McWatters, K. H. (2008). Optimization of a chocolate-flavored, peanut-soy beverage using response surface methodology (RSM) as applied to consumer acceptability data. *LWT - Food Science and Technology*, 41(8), 1485-1492.
- Diniz, F. M., & Martin, A. M. (1996). Use of response surface methodology to describe the combined effects of pH, temperature and E/S ratio on the hydrolysis of dogfish

(*Squalus acanthias*) muscle. *International Journal of Food Science & Technology*, 31(5), 419-426.

- Douanla-Meli, C., & Langer, E. (2009). *Ganoderma carocalcareus* sp. nov., with crumbly-friable context parasite to saprobe on *Anthocleista nobilis* and its phylogenetic relationship in *G. resinaceum* group. *Mycological Progress*, 8(2), 145-155.
- Fang, Q.-H., & Zhong, J.-J. (2002a). Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. *Process Biochemistry*, 37(7), 769-774.
- Fang, Q. H., & Zhong, J. J. (2002b). Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*. *Biotechnology Progress*, 18(1), 51-54.
- Fazenda, M. L., Seviour, R., McNeil, B., & Harvey, L. M. (2008). Submerged culture fermentation of "Higher fungi": The macrofungi. *Advances in Applied Microbiology*, 63, 33-103.
- Fukuzawa, M., Yamaguchi, R., Hide, I., Chen, Z., Hirai, Y., Sugimoto, A., . . . Nakata, Y. (2008). Possible involvement of long chain fatty acids in the spores of *Ganoderma lucidum* (Reishi Houshi) to its anti-tumor activity. *Biological and Pharmaceutical Bulletin*, 31(10), 1933-1937.
- Garraway, M. O., & Evans, R. C. (1984). *Fungal nutrition and physiology*: Wiley-Interscience.
- Gottlieb, A., & Wright, J. (1999a). Taxonomy of *Ganoderma* from southern South America: subgenus *Elfvingia*. *Mycological Research*, 103(10), 1289-1298.
- Gottlieb, A. M., & Wright, J. E. (1999b). Taxonomy of *Ganoderma* from southern South America: subgenus *Ganoderma*. *Mycological Research*, 103(6), 661-673.
- Heleno, S. A., Stojković, D., Barros, L., Glamočlija, J., Soković, M., Martins, A., . . . Ferreira, I. C. F. R. (2013). A comparative study of chemical composition, antioxidant and antimicrobial properties of *Morchella esculenta* (L.) Pers. from Portugal and Serbia. *Food Research International*, 51(1), 236-243.
- Hilszczańska, D. (2012). Medicinal properties of macrofungi. *Forest Research Papers*, 73(4), 347-353. doi:10.2478/v10111-012-0033-1
- Hong, S. G., & Jung, H. S. (2004). Phylogenetic analysis of *Ganoderma* based on nearly complete mitochondrial small-subunit ribosomal DNA sequences. *Mycologia*, 96(4), 742-755.
- Hsieh, C., Tseng, M.-H., & Liu, C.-J. (2006). Production of polysaccharides from *Ganoderma lucidum* (CCRC 36041) under limitations of nutrients. *Enzyme and Microbial Technology*, 38(1-2), 109-117.

- Hsu, K.-D., & Cheng, K.-C. (2018). From nutraceutical to clinical trial: frontiers in *Ganoderma* development. *Applied Microbiology and Biotechnology*, 102(21), 9037-9051.
- Hsu, K.-D., Wu, S.-P., Lin, S.-P., Lum, C.-C., & Cheng, K.-C. (2017). Enhanced active extracellular polysaccharide production from *Ganoderma formosanum* using computational modeling. *Journal of Food and Drug Analysis*, 25(4), 804-811.
- Jayasinghe, C., Imtiaj, A., Hur, H., Lee, G. W., Lee, T. S., & Lee, U. Y. (2008). Favorable culture conditions for mycelial growth of Korean wild strains in *Ganoderma lucidum*. *Mycobiology*, 36(1), 28-33.
- Jonathan, S., & Fasidi, I. (2001). Effect of carbon, nitrogen and mineral sources on growth of *Psathyrella atroumbonata* (Pegler), a Nigerian edible mushroom. *Food Chemistry*, 72(4), 479-483.
- Kashimoto, N., Hayama, M., Kamiya, K., & Watanabe, H. (2006). Inhibitory effect of a water-soluble extract from the culture medium of *Ganoderma lucidum* (Rei-shi) mycelia on the development of pulmonary adenocarcinoma induced by N-nitrosobis (2-hydroxypropyl) amine in Wistar rats. *Oncology Reports*, 16(6), 1181-1187.
- Khuri, A. I. (2017). Response surface methodology and its applications in agricultural and food sciences. *Biometrics & Biostatistics International Journal*, 5(5), 1-11.
- Kim, H. M., Park, M. K., & Yun, J. W. (2006). Culture pH affects exopolysaccharide production in submerged mycelial culture of *Ganoderma lucidum*. *Applied biochemistry and biotechnology*, 134(3), 249-262.
- Klaus, A., Kozarski, M., Niksic, M., Jakovljevic, D., Todorovic, N., & Griensven, v. L. J. L. D. (2011). Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. *Food Science and Technology = Lebensmittel-Wissenschaft und Technologie*, 44(10), 2005-2011.
- Klaus, A., Kozarski, M., Niksic, M., Jakovljevic, D., Todorovic, N., Stefanoska, I., & Van Griensven, L. J. L. D. (2013). The edible mushroom *Laetiporus sulphureus* as potential source of natural antioxidants. *International Journal of Food Sciences and Nutrition*, 64(5), 599-610.
- Klaus, A., Kozarski, M., Vunduk, J., Todorovic, N., Jakovljevic, D., Zizak, Z., . . . Van Griensven, L. J. L. D. (2015). Biological potential of extracts of the wild edible Basidiomycete mushroom *Grifola frondosa*. *Food Research International*, 67, 272-283.
- Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), 1547-1549.
- Lee, K. M., Lee, S. Y., & Lee, H. Y. (1999). Bistage control of pH for improving exopolysaccharide production from mycelia of *Ganoderma lucidum* in an air-lift fermentor. *Journal of bioscience and bioengineering*, 88(6), 646-650.

- Lee, W. Y., Park, Y., Ahn, J. K., Ka, K. H., & Park, S. Y. (2007). Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*. *Enzyme and Microbial Technology*, 40(2), 249-254.
- Liu, J., Kurashiki, K., Fukuta, A., Kaneko, S., Suimi, Y., Shimizu, K., & Kondo, R. (2012). Quantitative determination of the representative triterpenoids in the extracts of *Ganoderma lucidum* with different growth stages using high-performance liquid chromatography for evaluation of their 5 α -reductase inhibitory properties. *Food Chemistry*, 133(3), 1034-1038.
- Liu, W., Wang, H., Pang, X., Yao, W., & Gao, X. (2010). Characterization and antioxidant activity of two low molecular weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, 46(4), 451-457.
- Liu, Y., Vidanes, G., Lin, Y. C., Mori, S., & Siede, W. (2000). Characterization of a *Saccharomyces cerevisiae* homologue of *Schizosaccharomyces pombe* Chk1 involved in DNA-damage-induced M-phase arrest. *Molecular and General Genetics MGG*, 262(6), 1132-1146.
- Meletiadis, J., Meis, J. F. G. M., Mouton, J. W., & Verweij, P. E. (2001). Analysis of growth characteristics of filamentous fungi in different nutrient media. *Journal of Clinical Microbiology*, 39(2), 478.
- Micales, J., Bonde, M., & Peterson, G. (1992). Isozyme analysis in fungal taxonomy and molecular genetics. *Handbook of applied mycology*, 4, 57-79.
- Mienda, B. S., Idi, A., & Umar, A. (2011). Microbiological features of solid state fermentation and its applications - An overview. *Research in Biotechnology*, 2(6), 21-26.
- Milkey, K., Samsudin, A., Dubey, A., & Kidd, P. (2014). Comparison between taguchi method and response surface methodology (RSM) in modelling CO₂ laser machining. *Jordan Journal of Mechanical & Industrial Engineering*, 8(1), 35-42.
- Moncalvo, J.-M., & Ryvarden, L. (1997). *A nomenclatural study of the Ganodermataceae Donk: Fungiflora*.
- Moncalvo, J.-M., Wang, H.-F., & Hseu, R.-S. (1995a). Gene phylogeny of the *Ganoderma lucidum* complex based on ribosomal DNA sequences. Comparison with traditional taxonomic characters. *Mycological Research*, 99(12), 1489-1499.
- Moncalvo, J.-M., Wang, H.-H., & Hseu, R.-S. (1995b). Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia*, 87(2), 223-238.
- Moncalvo, J. (2000). *Systematics of Ganoderma*. J. Flood, P. D. Bridge and M. Holderness, eds.): CAB International, Wallingford.
- Myers, R. H., Khuri, A. I., & Carter, W. H. (1989). Response surface methodology: 1966–1988. *Technometrics*, 31(2), 137-157.

- Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M. (1995). Response surface methodology: process and product optimization using designed experiments", John Willey & Sons. Inc., New York, NY, 134-174.
- Nasreen, Z., Kausar, T., Nadeem, M., & Bajwa, R. (2005). Study of different growth parameters in *Ganoderma lucidum*. *Micologia Aplicada International*, 17(1), 5-8.
- Olennikov, D., Agafonova, S., Rokhin, A., Penzina, T., & Borovskii, G. (2012). Branched glucan from the fruiting bodies of *Piptoporus betulinus* (Bull.: Fr) Karst. *Applied biochemistry and microbiology*, 48(1), 65-70.
- Papaspyridi, L.-M., Katapodis, P., Gonou-Zagou, Z., Kapsanaki-Gotsi, E., & Christakopoulos, P. (2010). Optimization of biomass production with enhanced glucan and dietary fibres content by *Pleurotus ostreatus* ATHUM 4438 under submerged culture. *Biochemical engineering journal*, 50(3), 131-138.
- Park, Y., Kwon, O. C., Son, E., Yoon, D., Han, W., Nam, J., . . . Lee, C. S. (2012). Genetic diversity analysis of *Ganoderma* species and development of a specific marker for identification of medicinal mushroom *Ganoderma lucidum*. *African Journal of Microbiology Research*, 6(25), 5417-5425.
- Petre, M. (2015). *Mushroom biotechnology: developments and applications*: Academic Press.
- Petre, M., Teodorescu, A., Tuluca, E., Bejan, C., & Andronescu, A. (2010). Biotechnology of mushroom pellets producing by controlled submerged fermentation. *Romanian Biotechnological Letters*, 15(2), 50-55.
- Phillips, R. (1981). Mushrooms and other fungi of Great Britain & Europe: the most comprehensively illustrated book on the subject this century. *Italy: Pan Books*.
- Raghukumar, S. (2017). *Fungi in coastal and oceanic marine ecosystems: marine fungi*. Cham: Cham: Springer International Publishing.
- Rašeta Milena, J., Vrbaški Sara, N., Bošković Eleonora, V., Popović Mira, R., Mimica-Dukić Neda, M., & Karaman Maja, A. (2017). Comparison of antioxidant capacities of two *Ganoderma lucidum* strains of different geographical origins. *Zbornik Matice Srpske za Prirodne Nauke*, 2017(133), 209-219.
- Salehmin, M. N. I., Annuar, M. S. M., & Ilham, Z. (2015). Gas-liquid and liquid-liquid mass transfers in simulated and actual high cell density fermentations. *Chemical Engineering Communications*, 202(10-12), 1628-1634.
- Samaram, S., Mirhosseini, H., Tan, C. P., Ghazali, H. M., Bordbar, S., & Serjouie, A. (2015). Optimisation of ultrasound-assisted extraction of oil from papaya seed by response surface methodology: oil recovery, radical scavenging antioxidant activity, and oxidation stability. *Food Chemistry*, 172, 7-17.
- Shah, P., & Modi, H. (2018). Statistical optimization of culture conditions for enhanced mycelial biomass production using *Ganoderma lucidum*. *Journal of Applied Biology & Biotechnology*, 6(04), 41-45.

- Sievers, F., & Higgins, D. G. (2018). Clustal Omega for making accurate alignments of many protein sequences. *Protein Science*, 27(1), 135-145.
- Smith, B. J., & Sivasithamparam, K. (2000a). Internal transcribed spacer ribosomal DNA sequence of five species of *Ganoderma* from Australia. *Mycological Research*, 104(8), 943-951.
- Smith, B. J., & Sivasithamparam, K. (2000b). Isozymes of *Ganoderma* species from Australia. *Mycol. Res.*, 104(8), 952-961.
- Songulashvili, G. G., Elisashvili, V., Wasser, S. P., Hadar, Y., & Nevo, E. (2008). Effect of the carbon source and inoculum preparation method on laccase and manganese peroxidase production in submerged cultivation by the medicinal mushroom *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Aphyllphoromycetideae). *International Journal of Medicinal Mushrooms*, 10(1), 79-86.
- Steinkraus, K. H. (1984). Solid-state (solid-substrate) food/beverage fermentations involving fungi. *Acta Biotechnologica*, 4(2), 83-88.
- Stojkovic, D. S., Barros, L., Calhella, R. C., Glamoclija, J., Ciric, A., van Griensven, L., . . . Ferreira, I. (2014). A detailed comparative study between chemical and bioactive properties of *Ganoderma lucidum* from different origins. *International Journal of Food Sciences and Nutrition*, 65(1), 42-47.
- Subramaniyam, R., & Vimala, R. (2012). Solid state and submerged fermentation for the production of bioactive substances: a comparative study. *International Journal of Science and Nature*, 3(3), 480-486.
- Supramani, S., Ahmad, R., Ilham, Z., Suffian Mohamad Annuar, M., Klaus, A., & Abd Al Qadr Imad Wan-Mohtar, W. (2019a). 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.
- Supramani, S., Jailani, N., Ramarao, K., Zain, N. A. M., Klaus, A., Ahmad, R., & Wan, W. A. A. Q. I. (2019b). Pellet diameter and morphology of European *Ganoderma pfeifferi* in a repeated-batch fermentation for exopolysaccharide production. *Biocatalysis and Agricultural Biotechnology*, 19, 101118.
- Szedlay, G., Jakucs, E., Bóka, K., & Boldizsár, I. (1996). *Macro-and micromorphological characteristics of Ganoderma lucidum Karsten strains isolated in Hungary*. Paper presented at the Annales Historico Naturales-Musei Nationalis Hungarici.
- Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, 101(30), 11030-11035.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30(12), 2725-2729.

- Tang, Y.-J., Zhang, W., Liu, R.-S., Zhu, L.-W., & Zhong, J.-J. (2011). Scale-up study on the fed-batch fermentation of *Ganoderma lucidum* for the hyperproduction of ganoderic acid and *Ganoderma* polysaccharides. *Process Biochemistry*, 46(1), 404-408.
- Thyagarajan, A., Jedinak, A., Nguyen, H., Terry, C., Baldrige, L. A., Jiang, J., & Sliva, D. (2010). Triterpenes from *Ganoderma lucidum* induce autophagy in colon cancer through the inhibition of p38 mitogen-activated kinase (p38 MAPK). *Nutrition and cancer*, 62(5), 630-640.
- Tian, Y., Xu, Z., Zheng, B., & Martin Lo, Y. (2013). Optimization of ultrasonic-assisted extraction of pomegranate (*Punica granatum L.*) seed oil. *Ultrasonics - Sonochemistry*, 20(1), 202-208.
- Villares, A., Mateo-Vivaracho, L., & Guillamón, E. (2012). Structural features and healthy properties of polysaccharides occurring in mushrooms. *Agriculture*, 2(4), 452-471.
- Vu, D., Groenewald, M., de Vries, M., Gehrman, T., Stielow, B., Eberhardt, U., . . . Verkley, G. J. M. (2019). Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in Mycology*, 92(92), 135-154.
- Wagner, R., Mitchell, D. A., Lanzi Sasaki, G., Lopes de Almeida Amazonas, M. A., & Berovič, M. (2003). Current techniques for the cultivation of *Ganoderma lucidum* for the production of biomass, ganoderic acid and polysaccharides. *Food technology and biotechnology*, 41(4), 371-382.
- Wan-Mohtar, W. A. A. Q. I., Ab Kadir, S., & Saari, N. (2016a). The morphology of *Ganoderma lucidum* mycelium in a repeated-batch fermentation for exopolysaccharide production. *Biotechnology Reports*, 11(C), 2-11.
- Wan-Mohtar, W. A. A. Q. I., Ab. Latif, N., Harvey, L. M., & McNeil, B. (2016b). Production of exopolysaccharide by *Ganoderma lucidum* in a repeated-batch fermentation. *Biocatalysis and Agricultural Biotechnology*, 6, 91-101.
- Wan-Mohtar, W. A. A. Q. I., Abd Malek, R., Harvey, L. M., & McNeil, B. (2016c). Exopolysaccharide production by *Ganoderma lucidum* immobilised on polyurethane foam in a repeated-batch fermentation. *Biocatalysis and Agricultural Biotechnology*, 8, 24-31.
- Wan, W. A. A. Q. I., Mahmud, N., Supramani, S., Ahmad, R., Zain, N. A. M., Hassan, N. A., . . . Halim-Lim, S. A. (2018). 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*, 3(4), 481-492.
- Wu, G., Qian, Z., Guo, J., Hu, D., Bao, J., Xie, J., . . . Wang, Y. (2012). *Ganoderma lucidum* extract induces G1 cell cycle arrest, and apoptosis in human breast cancer cells. *The American journal of Chinese medicine*, 40(03), 631-642.

- Xing, J. H., Song, J., Decock, C., & Cui, B. K. (2016). Morphological characters and phylogenetic analysis reveal a new species within the *Ganoderma lucidum* complex from South Africa. *Phytotaxa*, 266(2), 115-124.
- Xu, P., Ding, Z.-Y., Qian, Z., Zhao, C.-X., & Zhang, K.-C. (2008). Improved production of mycelial biomass and ganoderic acid by submerged culture of *Ganoderma lucidum* SB97 using complex media. *Enzyme and Microbial Technology*, 42(4), 325-331.
- Yang, F.-C., & Liao, C.-B. (1998). The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochemistry*, 33(5), 547-553.
- Yang, F. C., Yang, M. J., & Cheng, S. H. (2009). A novel method to enhance the mycelia production of *Ganoderma lucidum* in submerged cultures by polymer additives and agitation strategies. *Journal of the Taiwan Institute of Chemical Engineers*, 40(2), 148-154.
- Yang, H.-l., Wu, T.-x., & Zhang, K.-c. (2004). Enhancement of mycelial growth and polysaccharide production in *Ganoderma lucidum* (the Chinese medicinal fungus, 'Lingzhi') by the addition of ethanol. *Biotechnology Letters*, 26(10), 841-844.
- Yue, Q.-X., Song, X.-Y., Ma, C., Feng, L.-X., Guan, S.-H., Wu, W.-Y., . . . Cui, Y.-J. (2010). Effects of triterpenes from *Ganoderma lucidum* on protein expression profile of HeLa cells. *Phytomedicine*, 17(8-9), 606-613.
- Zhou, X., Li, Q., Zhao, J., Tang, K., Lin, J., & Yin, Y. (2007). Comparison of rapid DNA extraction methods applied to PCR identification of medicinal mushroom *Ganoderma* spp. *Preparative Biochemistry and Biotechnology*, 37(4), 369-380.