PRODUCTION OF POLYSACCHARIDE FROM *GANODERMA NEOJAPONICUM* IMAZEKI

NIK HAFIZAH BINTI NIK UBAIDILLAH

THESIS SUBMITTED IN FULFILLMENT
OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCE
INSTITUTE OF BIOLOGICAL SCIENCES
UNIVERSITY OF MALAYA
KUALA LUMPUR
2015

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate : **Nik Hafizah Binti Nik Ubaidillah**

Matric. No. : **SGR 090043**

Name of Degree : **Master of Science**

Title of Thesis : **Production of Polysaccharide from *Ganoderma neojaponicum* Imazeki.**

Field of Study :**Fermentation Technology**

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ABSTRACT

Ganoderma species is the most popular medicinal mushroom for curing various diseases worldwide. *Ganoderma neojaponicum* (Imazeki), locally known as *cendawan senduk* was isolated from Bahau, Negeri Sembilan, Malaysia. This species has been used in natural healing and alternative medicine for external treatment of stomach ache in Malaysia. The most important therapeutic substance in *Ganoderma* spp. is polysaccharide. The aim of this study is to formulate low cost medium and optimize physical parameters for mycelial growth, β-glucan and total carbohydrate production from *G. neojaponicum* in shake flask and a 2-L stirred tank reactor (STR). Response surface methodology (RSM) was employed to obtain the best combinations of alternative carbon and nitrogen sources consisting of spent yeast (brewery industry) and brown sugar (sugar cane industry). Optimal growth medium requirements were 37.25 g/L spent yeast, 91.3 g/L brown sugar, 0.046 g/L Tween 80, with constant amount 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4 and 0.5 g/L $MgSO_4.7H_20$. The optimum physical parameters were temperature 26.72 ^oC, aeration 1.33 vvm, constant pH 6 and agitation 160 rpm. The optimal growth for *G. neojaponicum* was determined at 4 days of cultivation time with 25.32 g/L mycelial dry weight. The dried broth of *G. neojaponicum* showed higher total β-glucan 23.56±0.01% (w/v) and total carbohydrate 115.89±2.78 g/L compared to the dried mycelium. The mycelium and broth of *G. neojaponicum* were then partially purified for intracellular polysaccharide (IPS) and extracellular polysaccharides (EPS), respectively. The EPS of *G. neojaponicum* contained higher amount of total β-glucan and total carbohydrate compared to IPS, 9.41% (w/v) dan 80.81 g/L, respectively. The dried mycelium and broth as well as IPS and EPS were assessed for immunomodulatory

property. The concentration 1000 µg/mL of IPS showed the most significant immunostimulating effect of human immune response *in-vitro.* The concentration enhanced up to 225% of proliferation activity of macrophage cell (RAW264.7), increased 460% phagocytosis activity and decreased 49.91% cytotoxicity effect of human colon cancer cell (HT29) compared to untreated cells ($P<0.05$). The *G. neojaponicum* mycelium and spent mycelium had significant ($P<0.05$) effect of Nf-kB inhibition of HT29. The dried mycelium of *G. neojapocum* was evaluated for its safety by acute oral toxicity test. The dried mycelium of *G. neojapocum* at a dose of up to 2000 mg/kg body weight/day did cause neither mortality nor toxic effects on the rats in 14 days.

ABSTRAK

Ganoderma species adalah cendawan ubatan yang paling terkenal digunakan dalam menyembuhkan pelbagai penyakit di serata dunia. Dalam kajian ini, *Ganoderma neojaponicum* (Imazeki) yang dikenali sebagai cendawan senduk telah dipencilkan dari Bahau, Negeri Sembilan, Malaysia. Spesis ini telah digunakan untuk penyembuhan penyakit secara semulajadi dan perubatan tradisional untuk rawatan luaran bagi sakit perut. Bahan terapeutik terpenting dalam *Ganoderma* spp. adalah polisakarida. Tujuan penyelidikan ini ialah untuk memformulasi dan mengoptimumkan medium daripada sumber berkos rendah untuk pertumbuhan miselia, β-glucan dan polisakarida oleh *G. neojaponicum* dalam kelalang goncang dan Reaktor Tangki Teraduk- 2 L (2 L-STR). Kaedah Tindakbalas Permukaan (RSM) telah digunakan untuk mencari kombinasi paling baik bagi sumber alternatif karbon dan nitrogen dari yis tergunapakai (industri bir barli) dan gula merah (industri gula tebu). Keperluan media pertumbuhan yang optimum yang dinilai melalui RSM ialah 37.25 g/L yis tergunapakai, 91.3 g/L, gula merah, 0.046 g/L Tween 80, dengan kadar tetap 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4 dan 0.5 g/L $MgSO_4.7H_20$. Parameter fizikal didapati optimum pada suhu 26.72 °C, pengudaraan 1.33 vvm, pH 6 dan goncangan 160 rpm. Pertumbuhan *G. neojaponicum* didapati optimum pada hari ke-4 jangkamasa pemeraman sebanyak 25.32 g/L berat kering miselia. Larutan media *G. neojaponicum* didapati lebih tinggi dengan jumlah β-glucan 23.52±0.01% (w/v) dan karbohidrat, dan 115.89±2.78% (w/v) berbanding miselia keringnya. Larutan media dan miselia *G. neojaponicum* telah diekstrak masing-masing separa tulen untuk intrasellular polisakarida (IPS) dan ekstrasellular polisakarida (EPS). Jumlah β-glucan dan jumlah polisakarida lebih tinggi pada EPS berbanding IPS di dalam *G. neojaponicum*, 9.41% (w/v) dan 80.81 g/L, masing-masing. Miselia dan sisa larutan

media yang dikeringkan serta IPS dan EPS telah dijalankan pencirian immunomodulatori. Pada kepekatan 1000 µg/mL IPS telah menunjukkan kesan ransangan imun yang signifikan ke atas sel imun badan manusia secara *in-vitro* dengan meningkatkan proliferasi sel makrofaj (RAW264.7) sebanyak 225%, meningkatkan aktiviti fagositosis sebanyak 460% dan menurunkan kadar proliferasi sel kanser kolon manusia (HT29) sebanyak 49.91% berbanding sel tanpa rawatan (P<0.05). Ekstrak *G. neojaponicum* menunjukkan kesan positif ke atas perencatan Nf-ƙB pada HT29 (P<0.05). Miselia kering *G. neojaponicum* telah dijalankan ujian toksisiti akut oral. Miselia kering *G. neojaponicum* pada kandungan dos sehingga 2000 mg/kg berat badan/sehari tidak memberi kesan kematian dan toksik terhadap ujian tikus selama 14 hari.

ACKNOWLEDGEMENTS

In the name of Allah, Most Gracious and Most Merciful. Alhamdulillah, boundless praises be to Allah for granting me the ability and blessing to complete the research work, and finally the thesis. Highest appreciation goes to my supervisory committee members, Prof. Dr. Noorlidah Abdullah (main) and Prof. Dr. Vikineswary Sabaratnam for their supervision and continuous assistance. Their great help through constructive comments and recommendations during the experimental and thesis work has contributed significantly to making this research a success. To Assoc. Prof. Dr. Muhammad Suffian Muhammad Annuar and Assoc. Prof. Dr. Nurhayati Zainal Abidin, their advice and guidance on technical research input as well as material have deepened the knowledge exploration of the subject matter. I would like to acknowledge Prof. Yao Yi-Jian, Director of Fungarium (HMAS), Institute of Microbiology, Chinese Academy of Sciences, Beijing, China for his valuable findings in verification of *G. neojaponicum* taxonomy by morphology and molecular analysis.

I would like to say special thanks to my sponsors: Ministry of Agriculture (MOA Science Fund), University of Malaya (UM Postgraduate Grant), and Malaysian Agricultural Research and Development Institute (MARDI scholarship). I am also very grateful to all the staff and postgraduate students of Mycology Laboratory and Mushroom Research Centre for their assistance and moral support during my candidature. Last but not least, my heartfelt gratitude goes to my beloved parents: Hj. Nik Ubaidillah Nik Su and Hjh. Tengku Lijah Tengku Salim, and also to all my family members for their endless love, well wishes and unwavering encouragement. This work is inspired by and dedicated to my beloved son, Nik Muhammad Aaqil Hariz. Again, from the bottom of my heart, a million thanks to all of you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

AKP	Alkaline Phosphatase
Alb	Albumin
ALP	Alkaline phosphatise
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
β-1,6/ β-1,3	Beta-1,6/-1,3- glucans
B	Dried Broth
BALB/c	Albino
BMM	Bone Marrow-derived Macrophage
BS	Brown Sugar
C/N	Carbon/Nitrogen
Ca	Calcium
$CaCl_2•2H_2O$	Calcium Chloride Dihydrate
CCD	Central Composite Design
CD5+ / 4+	T cell
CD8+	Cytotoxic T cell
Cholt	Cholesterol
Cl	Chloride
ConA	Concanavalin A
CR3	Macrophage-1 Antigen
Crea	Creatinine
CSL	Corn Steep Liquor
DAG	Directed Acyclic Graph
DC	Denderic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNS	Dinitrosalicyclic Acid

ELISA	Enzyme-linked Immunosorbent Assay
EPS	Extracellular Polysaccharides
FBS	Fasting Blood Sugar
$FeSO_4 \bullet 7H_2O$	Ferrous Sulfate Heptahydrate
FFD	Full Factorial Design
FITC	Fluorecein Isothiocyanate
GAN	Ganoderan
Ge	Organic Germanium
GGT	Gamma Glutamyl Transpeptidase
GLB7	Protein-polysaccharide Fraction
GLC	Glucose
Hb	Haemoglobin
HCK	Hemopoietic Cell Kinase
HCl	Hydrogen Chloride
HPLC	High Performance Liquid Chromatography
HT29	Human Colon Cancer Cell Line
IFN	Interferon
IFN-γ	Inteferon-gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IL-2	Interleukin-2
IP3	Inisitol Triphosphate
IPS	Intracellular Polysaccharides
K	Potassium
K_2HPO_4	Dipotassium Hydrogen Phosphate
KH_2PO_4	Potassium Dihydrogen Phosphate
LPS	Lipopolysaccharides
M	Dried Mycelium
MAPK	Mitogen-activated Protein Kinase

MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MEA	Malt Extract Agar
mg	Miligram
MgSO4.7H20	Magnesium Sulfate Heptahydrate
ml	Milliliter
MLC	Multi-level Cell
MLS	Molasses
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide
Na	Sodium
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
Nf-κB	Nuclear Factor Kappa-light Chain-enhancer of Activated B cell
$(NH_4)_2SO_4$	Ammonium Sulphate
NK	Natural Killer Cells
NO	Nitric Oxide
OD	Optical Density
OPF	Oil Palm Frond
PBS	Phosphate Buffer Saline
PCV	Hematocrit (packed cell volume)
PDA	Potato Dextrose Agar
Phos	Phosphorus
PKC	Paroxysmal Kinesogenic Choreothetosis
PMA	Propylene-glycol Monomethyl-ether Acetate
POME	Palm Oil Mill Effluent
PSK	Polysaccharide- K
PSP	Polysaccharides-Peptide
PSPC	Polysaccharide-protein complex
RA	Rheumatoid Arthritis
RAW264.7	Mouse Leukaemic Monocyte Macrophage cell line

RBC	Red Blood Count
RPE	Reishi Powdered Extract
rpm	Rotary Per Minute
RPMI	Roswell Park Memorial Institute medium
RSM	Response Surface Modelling
SSG	*SclerotiniaSclerotiorum* Glucan
STR	Stirred Tank Reactor
SM	Dried Broth
SY	Spent Yeast
tBOOH	t-butyl Hydroperoxide
TCM	Traditional Chinese Medicine
T. Bil	Total Bilirubin
TLR-4	Toll-like Receptor 4
TNF-α	Tumour Necrosis Factor-alpha
T. Prot	Total Protein
Thromb	Thrombocytes-platelets
TLR	Toll-like Receptor
Trig	Triglyceride
U.A	Urine analysis
U937	Human Myelomonocytic Leukemia Cell line
uPA	Urokinase-type Plasminogen Activator
WBC	White Blood Count
YE	Yeast Extract

CHAPTER 1.0 INTRODUCTION

Ganoderma is a fungi that belongs to class basidiomycetes, order Polyporales and family Ganodermataceae. Currently, 250 *Ganoderma* spp. have been described worldwide. *Ganoderma* is also known as Ling zhi, Reishi or Youngzhi, is the most widely consumed medicinal mushroom based on its renowned medicinal properties. Numerous studies on *Ganoderma* spp. mainly in China, Korea, Japan and United States have shown its effectiveness in the treatment of a wide range of diseases. *Ganoderma neojaponicum* (Imazeki) is a wild tropical strain known locally among the indigenious people of Malaysia as 'Cendawan Senduk'. It has been used for external treatment of stomach ache in natural healing and alternative medicine in Malaysia (Lee *et al*., 2008). Also, *G. neojaponicum* has been used for medicinal purposes in China and as a traditional food ingredient in Taiwan (Pegler, 2002 and Chau & Wu, 2006).

Throughout the last three decades, *Ganoderma* spp. have been investigated pharmacologically and clinically. The nutraceutical products of *Ganoderma* spp. are widely used as remedies to treat more than 20 different illnesses including hypertension, hepatitis, cardiovascular problems and different types of cancer including leukaemia (Alice & Philip, 1996). *Ganoderma* spp. have been found useful in controlling immune functions and slowing down tumour growth (Wasser & Weis, 1999). *G. neojaponicum* is also regarded as having nutraceutical properties based on traditional used by indigenous people in Malaysia.

Generally, polysaccharides are the potent compounds for antitumour and immunomodulatory activities in *Ganoderma* spp. (Mizuno, 1999). The major polysaccharides responsible for the acclaimed medicinal properties of *G. lucidum* are β-1,3 and β-1,6-D glucans (Russell, 2008). *G. neojaponicum* has also been reported to contain β-

1,3-linked and β-1-6-linked D-glucose polysaccharides that have been identified as potential anti-tumour agents and may function to enhance the immune system (Takei, 2006). Polysaccharide presence in *G. neojaponicum* underscore the significance of study on this species for its medicinal purposes.

The commercial values of polysaccharides have been indicated by several indicators. In Japan, polysaccharide products have been researched and manufactured as immunotherapeutical drugs (biological response modifiers, BRM). The market prices of these polysaccharide product were sold at US$ 120 per bottle (20 mL) of sonifilan (*Schizophylum commune*) which is used for treating cervical cancer. US$ 120 per bottle (20 mL) of lentinan (*Lentinula edodes*) for stomach cancer and US$ 12 per pack (1 g) of krestin (*Trametes versicolor*) for cancers related to digestive organs, lungs and breast (Mizuno, 1999). Evidently polysaccharides have high commercial value globally given its medicinal purposes.

Although polysaccharide production of these mushroooms has been established and marketed throughout the world, most of the polysaccharide is being extracted from the fruiting body whilst the extract from submerged fermentation is not widely produced commercially. Hence, the aim of this study was to employ submerged fermentation for the production of polysaccharides from *G. neojaponicum* while exploring the potential of a Malaysian strain as an alternative for *G. lucidum* for commercial production in Malaysia.

To date, the extraction of polysaccharides from *G. neojaponicum* using submerged fermentation is yet to be fully researched. The polysaccharides produced by this species can be alternatively extracted using submerged fermentation technique. The polysaccharides in submerged fermentation can be produced in large quantities by upscaling production, an

approach which offers convenient control and easy downstream processing. Submerged culture can greatly decrease the cultivation period, enhance polysaccharide production compared with extracting polysaccharides from basidiocarps. The bioreactor can easily manipulate environmental conditions like temperature, agitation, dissolved oxygen and pH to obtain maximum yield of polysaccharide. This study also focused on the replacement of new low-cost cultivation medium and requires optimum physical parameters in shake flask and bioreactor.

Polysaccharide produced can be partially purified through ethanol precipitation. The polysaccharide is significant for evaluation of immunomodulatory properties of *G. neojaponicum*. Although the polysaccharide fraction of *G. lucidum* has been widely discussed for its immunomodulatory, anti-inflammatory, and anti-cancer properties, as far as we know, there are limited reports on the immunomodulatory properties of the IPS and EPS of *G. neojaponicum.*

Therefore suitable fermentation technology is needed to produce polysaccharides using *G. neojaponicum*. The following are the specific objectives of this study :

OBJECTIVES OF THE STUDY

a. To screen and formulate low-cost medium for optimum mycelium growth, β-glucan and total carbohydrate production by *G. neojaponicum*.

b. To optimize the physical parameters for intracellular and extracellular polysaccharides production in shake flask fermentation and a 2-L stirred tank reactor (STR) using optimised medium in (a).

c. To determine the immunomodulatory and antitumour effect of dried mycelium, dried broth and its polysaccharide extracts via macrophage and human cancer cell lines against cell proliferation, cytotoxicity, phagocytosis and NFκB activity.

d. To evaluate acute oral toxicity (*in-vivo*) of the dried mycelium of *G. neojaponicum* .

CHAPTER 2.0 LITERATURE REVIEW

2.1 *Ganoderma neojaponicum* (Imazeki)

Over 250 *Ganoderma* species have been described worldwide, and most of them are from the tropic (Moncalvo *et al.,* 1995). However, in therapeutic practices and literature citations, the *Ganoderma* species usually refer to the species of *Ganoderma lucidum*. Several *Ganoderma* species are widely used for medicinal purposes, e.g., *G. lucidum* (P. Kurtis), *G. luteum* (Steyaert), *G. tsugae* (Murrill), *G. applanatum* (Pers.: Wallr.) Pat., *G. australe* (Fr.) Pat., *G. capense* (Lloyd), *G. tropicum* (Jungh.) (Wasser, 2005). Traditionally, the genus *Ganoderma* have been classified by its morphological characteristics of basidiocarps such as size, colour and stipe attachment patterns (Steyaert, 1972 and Corner, 1983). *Ganoderma* spp. are classified based on species circumscription, phylogenetic relationships, host range and distribution of taxa (Moncalvo *et al.*, 1995).

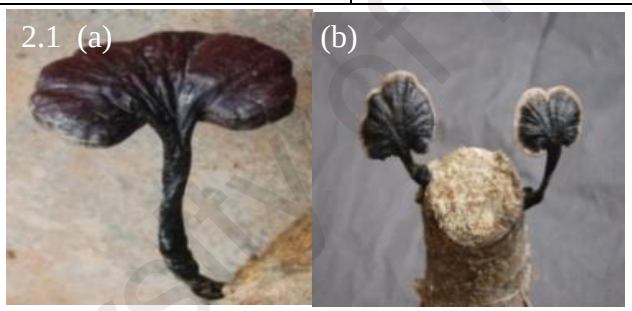
The morphological variations can be affected by environmental conditions (Pegler & Yao, 1996). The *Ganoderma* species may differ in colour based on its growing temperature, humidity, carbon dioxide content, and the existing nutrients (Moncalvo *et al.*, 1995). Furthermore, the maturation of basidiocarps takes a long period, in which environmental factors can affect the morphogenesis. Besides that, the geographical origins of *Ganoderma* spp. may also differ in traits including growth characteristics of basidiocarps and histo-anatomical characteristics (Kim *et al*., 2001). The morphology pattern of *Ganoderma* spp. can be confirmed based on analysis of DNA sequence (Moncalvo *et al.*, 2005).

Ganoderma neojaponicum (Imazeki) is one of the medicinal *Ganoderma* species that belongs to the Kingdom Fungi, Phylum Basidiomycota, Class Basidiomycetes, Order Aphyllophorales, Family Ganodermataceace and Genus *Ganoderma* (Moncalvo, 1995). The basidiocarp of *G. neojaponicum* is black or violet brownish in colour, like snakehead in shape and has long twisted stem with diameter up to 14 cm. *Ganoderma neojaponicum* grows naturally on decaying bamboos. The upper surface of the cap is varnished woody–textured but not glossy. Meanwhile, the lower surface has shade of numerous pores in bruising brown colour depending on its aging stage.

In this present study, a wild strain of *G. neojaponicum* was isolated from Bahau, Negeri Sembilan, Malaysia. The taxonomy of *G. neojaponicum* was confirmed based on morphology and molecular analysis by Prof. Yao Yi-Jian (Director from Institute of Microbiology, Chinese Academy of Sciences, Beijing, China). The morphology of *G. neojaponicum* is summarised in Table 2.1. The basidiocarp and mycelia of *G. neojaponicum* are shown in Figures 2.1 and 2.2.

Table 2.1: Morphological characteristics of *G. neojaponicum* Imazeki *

Morphology		***G. neojaponicum* Imazeki**
Pileus	Size	Up to 14 cm
	Position to stalk	centrally attached when in sessile form
	Growing edge colour	yellowish brown
	Colour	black or violet brown
	Surface	varnished but not glossy
	Shape	like snakehead or raised cobra
Pore Surface	Colour	bruising brown
	Size	tiny circular pores, nearly invisible to the naked eye

	Tubes size	Not determined
Stipe	Length	Up to 20 cm
	Thickness	2 cm
	Structure	long and thin stem; twisted
	Colour	varnished black or violet-brown and coloured like the cap
Flesh	Colour	dark brown flesh
	Texture	hard and woody texture
Basidiospore	Size	Not determined
	Shape	Ellipsoid, truncated at the apex, double-wall
	Hyphae	Dimitic, pale yellow to brown in colour
Ecology	Growing condition	Low land tropical forest
	Host plant	Grows saprotrophically on decaying bamboos
Basidiocarp	 Figure 2.1: Basidiocarp of *G. neojaponicum*	
Colony characteristic	Mycelia network	Thread-like filament
	Mycelia colour	White
	 Figure 2.2: *G. neojaponicum* mycelium	
Distribution	China, Japan, Malaysia	
Local name	Cendawan senduk (Malaysia)	

*(Nik Hafizah, 2009)

2.2 Medicinal property of *Ganoderma* spp.

A wide variety of pharmacologically active chemicals have been extracted from *Ganoderma* spp. These include terpenoids, sesquiterpenoids, triterpenoids, polysaccharides, amino acids, nucleotides, alkaloids, steroids, lactones, ergosterol, fatty acids, enzymes, polysaccharide-peptide complex, protein, heteropolysaccharides, triterpenoids, glycoprotein, nucleosides and organic germanium. All these compounds have different medicinal values.

The polysaccharide compounds of *Ganoderma* spp. that have been investigated are *G. tsugae, G. boninense, G. tropicum* and *G. applanatum* (Chang & Philip, 2004). The polysaccharides of *G. lucidum, G. tsugae* and *G. applanatum* were successfully tested using *in-vitro* study on antitumour and hypotensive activity (Grace *et al*., 2006). The crude polysaccharide might contain various components of bioactive compound such as β-glucan and other component such as glucuronoglucan, xyloglucan, unannoglucan, xylomannoglucan along with other active heteroglucans and protein complexes (Wasser & Weis, 1999). *G. neojaponicum* has also been reported to contain β-1, 3-linked and β-1-6-linked D-glucose polysaccharides that have been identified as potential anti-tumour agents and may function to enhance the immune system (Takei, 2006).

2.3 Submerged fermentation for production of polysaccharide and mycelium in *Ganoderma* spp.

Ganoderma spp. can be cultivated using submerged fermentation for mycelium and polysaccharide production. In submerged fermentation, the studies are usually undertaken in Erlenmeyer flasks and bioreactors. Submerged culture can greatly decrease

the cultivation period, enhance polysaccharide production compared with extracting polysaccharides from basidiocarps. The production of basidiocarps takes a minimum of 3-5 months to obtain high biomass yield. Meanwhile, mycelial biomass can be derived in a period of 2-3 weeks through submerged fermentation (Tang & Zhong, 2002a). According to Bae *et al*. (2000), submerged fermentation requires minimal space and has less risk of contamination. Hence, the polysaccharides of *G. neojaponicum* in submerged fermentation can be produced in large quantities by upscaling production, an approach which offers convenient control and easy downstream processing.

2.4 Medium component for submerged fermentation of *Ganoderma* spp.

The medium components such as sources of carbon and nitrogen, mineral salts and trace elements are essential in the formulation of media for submerged culture.for *Ganoderma spp.* growth and its metabolites production (Moreira *et al*., 1998 and Duta *et al*., 2004). The media parameters can influence the constitution, viscosity and structure of microbial polysaccharides and mycelial growth of *Ganoderma* spp.

2.4.1 Effect of carbon sources

The source of carbon in the growth medium was observed to be a crucial factor for the production of polysaccharides (Kim *et al*., 2002). The energetic source is represented by carbon, which will aid the growth of the microorganism. It may be a simple source, pure monosaccharide compound like glucose; alternatively, it may be a complex source, polymeric molecules like cellulose or starch. The subsequent growth trials in liquid media found glucose as the best carbohydrate source that supported the greatest mycelia growth of *Ganoderma* spp. (Yang & Liau, 1998a). This can be attributed to the direct linkage of these

components to metabolite biosynthesis of β-glucan and cell proliferation (Fang *et al*., 2002; Tang & Zhong, 2002b and Kim *et al*., 2002). Tang & Zhong (2002a) noted that sucrose is required for extracellular polysaccharides but not for cell growth. Meanwhile, lactose was suitable for cell growth and intracellular production of polysaccharides. Potato dextrose broth was used by Gao *et al.*, (2002a) as a source of carbon in the growth medium of *G. lucidum* to produce 16.3 g/L of mycelial dry weight and 6.5 g/L of EPS. Hence, carbon source is vital for the growth of *G. neojaponicum* mycelium in submerged fermentation.

2.4.2 Effect of nitrogen sources

Nitrogen source is the main stimulatory factor that encourages stimulation on microbial growth. The ideal nitrogen source for production of polysaccharides is malt extract, while yeast extract is the ideal source for β-glucans production. According to Wasser *et al*., (2003), the cell growth and β-glucan production in mushroom was greatly influenced by the type and concentration of the nitrogen source. Tables 2.2 and 2.3 shows the usage of nitrogen sources for the *Ganoderma* spp. cultivation in submerged fermentation. The ammonium nitrogen sources showed good growth of fungus but had low yields of β-glucan (Kanokarn, 2010). Most fungi grew well on a mixture of nitrogen sources compared to only having one nitrogen source. The inorganic sources may be supplied by ammonia gas, ammonium salts and nitrates. Meanwhile, amino acid, protein, peptone, and yeast extract are some forms of nitrogen sources (Slaughter, 1988). Therefore, *G. neojaponicum* requires nitrogen source either in their inorganic or organic form for the production of polysaccharides and mycelial dry weight.

Table 2.2: The carbon, nitrogen and mineral sources for *Ganoderma sp* in submerged fermentation

No.	Carbon Sources (g/L)	Nitrogen Sources (g/L)	Mineral Salt/Trace Element (g/L)	Other Sources (g/L)	Fermentation Condition	References
1.	Glucose (20)	Peptone (2); Yeast Extract (2)	KH_2PO_4,(0.46); K_2HPO_4 (1); $MgSO_4 7H_2O$ (0.5)	-	3 L in 5-L bioreactor, pH 5.0; 150 rpm; 25 ^oC; 2 vvm	Kim *et al*., (2002b)
2.	Glucose (60)	Yeast Extract (6)	KH_2PO_4 (0.5); $(NH_4)_2HPO_4$ (1)	Sigma antifoam 289; (5)	2 L in 3-L bioreactor, pH 6.0; 150 rpm; 25 ^oC; 2.5 vvm	Lee *et al*., (1999)
3.	Lactose (35)	Yeast Extract (2.5); Peptone (5)	$KH_2PO4.H_2O$ (1); $MgSO_4.7H_2O$ (0.5); Vitamin B_1, (0.05)	-	3.5 L in bioreactor, 150 rpm; 30 ^oC	Tang & Zhong, (2003b)
4.	Glucose (35)	Yeast Extract (5); Peptone (5)	$KH_2PO_4.H_2O$ (1); $MgSO_4.7H_2O$ (0.5); Vitamin B_1 (0.05)	-	50 mL in 250 mL flask, initial pH 5.5; 120 rpm; 30 ^oC	Fang & Zhong, (2002a)
5.	Glucose (35)	Peptone (2) ; Yeast Extract (10)	$KH_2PO_4.H_2O$ (1); $MgSO_4.7H_2O$ (0.5); Vitamin B_1 (0.05)	-	50 mL in 250 mL flask, initial pH 5.5; 120 rpm; 30 ^oC	Fang & Zhong, (2002b)
6.	Lactose (35)	Yeast Extract (2.5); Peptone 2	$KH_2PO_4.H_2O$ (1); $MgSO_4.7H_2O$ (0.5); Vitamin B_1 (0.05)	-	50 mL in 250 mL flask, initial pH 5.5; 120 rpm; 30 ^oC	Tang & Zhong, (2002a)
7.	Brown sugar (71.4)	Yeast Extract (2.28) ; Malt Extract, skim milk	KH_2PO_4, (0.5); K_2HPO_4 (0.5);$MgSO_4.7H_2O$ (0.5);	Olive Oil	100 mL in 500 mL flask, pH 5.5; 160 rpm; 34 ^oC	Chang *et al.*, (2006)
8.	Glucose (50)	Yeast Extract (5) ; Peptone (5)	$KH_2PO_4.H_2O(1)$; $MgSO_4.7H_2O$ (0.5); Vitamin B_1 (0.05)	-	45 mL in 250 mL flask; 120 rpm; 30 ^oC	Fang & Zhong, (2002a)
9.	Soluble Starch (40)	Peptone (4)	$KH_2PO_4.H_2O$ (1.5); $MgSO_4.7H_2O$ (1)	-	100 mL , pH 5; 150 rpm; 30 ^oC	Pang *et al*., (2007)
10.	Molasses (35); Glucose (50)	Yeast Extract (1) ; NH_4Cl (4)	KH_2PO_4 (0.5); K_2HPO_44 (0.5);$MgSO_4.7H_2O$ (0.5)	-	100 mL , pH 5; 150 rpm; 30 ^oC	Cheinyan, (2004)

2.4.3 Effect of mineral salt/trace element

The growth medium was supplemented with other trace elements and mineral sources such as potassium (KH_2PO_4), magnesium ($MgSO_4 \cdot 7H_2O$), various vitamins and trace metals which helped to produce high polysaccharides content from *Ganoderma* spp. Tables 2.2 and 2.3 also shows the usage of mineral salt and other trace elements for the production of polysaccharides from *Ganoderma* spp. The culture media with buffered inorganic salts (Lin *et al*., 1973) and low concentrations of ammonium phosphate (Lee *et al*., 1999) have been reported to produce good mycelial growth. Hence, mineral salt/trace element sources are significant for *G. neojaponicum* mycelia growth and polysaccharides production.

Table 2.3: The physical parameters and medium compositions for *Ganoderma* spp. in submerged fermentation

Physical Parameter	**Medium Composition**	**Mycelial dry weight (g/L)**	**EPS (g/L)**	**IPS (g/L)**
T = 30 °C, shaking 120 rpm, pH= 4.5	$KH_2PO_4 \cdot H_2O$ 1 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; vitamin B1 0.05 g/L; lactose 35 g/L; Yeast Extract 5 gm/L and Peptone 5 g/L .	14.02±0.21	1.22±0.23	2.55±0.01
T = 30 °C, shaking 120 rpm, pH= 4.5	Glucose 50 g/L; K_2HPO_4 0.5 g/L; KH_2PO_4 0.5 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; Yeast Extract 1 g/L ; NH_4Cl 4 g/L .	15.15±0.21	1.76±0.11	2.52±0.22
T = 30 °C, shaking 120 rpm, pH= 4.5	3 kg of peeled potatoes autoclaved, added with water to make up to 10-L; Glucose 20 g/L; olive oil 2%.	19.03±0.17	1.21±0.26	2.33±0.19

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2.4.4 Use of waste materials as low-cost growth media formulations

These waste materials can be formulated as alternative ingredients for low cost growth medium for the cultivation of mushrooms. For submerged fermentation, low renewable and abundantly available agricultural or industrial processing waste materials are necessary. These are potentially convertible into value-added product for *Ganoderma* spp. growth. Agricultural waste of palm oil mill effluent (POME) and oil palm frond (OPF) were successfully used as growth medium for *Saccharomyces cereviceae* and *Candida albicans* to produce intracellular protein using submerged fermentation (Loo *et al.*, 2002). Meanwhile, pineapple waste was also used as medium to grow *Aspergillus niger* in submerged fermentation (Dacera *et al*., 2009). The utilization of orange peel hydrolysate from citrus processing waste as medium for *Mucur indicus* for production of mycelial dry weight has also been demonstrated (Patrik, 2012). Furthermore, sugarcane baggasse was also used as medium for production of cellulose from *Aspergillus terreus* using submerged fermentation (El-Nawwi & El-Kader, 1996). As summarized in Table 2.4, spent yeast, brown sugar, molasses and corn steep liquor are rich in complex organic and inorganic nutrients, minerals and vitamins that are importantly needed for fungal growth (Dhampure Specialty Sugars Ltd, 2003; Hubert, 2006; Zabriskie *et al*., 1982; Steckley *et al*., 1979). Besides that, rice straw hydrolysate was used as a medium for *Candida arborea* growth for the production of fermentable sugars used as animal feed (Zheng *et al*., 2005). Spent yeast, a brewery by-product and molasses, a by-product of sugar industries can be used as cost-effective cultivation medium for

fungal growth (Amran , 2006 and Kim *et al*., 2006). Hence, these may potentially be convertible as carbon, nitrogen and minerals trace element sources for *G. neojaponicum* growth.

Table 2.4: The biochemical compositions of different low-cost medium sources (g/L)

Constituents	Brown Sugar[1]	Cane Molasses[2]	Corn Steep Liquor[3]	Spent Yeast[4]
Water	-	20	50	14.4
Total sugar	97.3	58.9	2. 5 (Dextose)	5.8
Protein	-	-	24	53.0
Fat	-	-	1.0	0.7
Calcium	85	1.5	-	0.29
Iron	1.30	0.2	-	-
Phosphorous	3.90	-	-	2.06
Potassium	100	0.2	-	-
Magnesium	23	0.1	-	0.31
Calcium	-	3.5	-	2.41
Vitamin C	0.50	-	-	-
Thiamine, B1	0.008	0.09	-	-
Riboflavin, B2	0.007	-	-	-
Niacin	0.082	-	-	-
Biotin	-	-	-	-
Pyridoxine	-	-	0.2	-
Zinc	0.18	-	-	316
Manganese	-	-	-	16
Crude Fibre	-	-	1.0	4.8
Ash	-	3.5	8.8	9.8
Nitrogen	-	0.5	7.5	31.7

0.09

Dhampure Specialty Sugars Ltd, 2003 [1] ; Hubert,2006 [2] ; Zabriskie et al.,1982 [3] and Steckley et al.,1979 [4].

2.5 Physical parameters affecting cultivation of *Ganoderma* spp.

There are many physical factors (pH, temperature, oxygen concentration and agitation) that influence the microbial polysaccharide and mycelial growth of *Ganoderma* spp in submerged fermentation. The mycelium can be dispersed within the substrate more uniformly; the media parameters can be easily manipulated and environmental conditions can be easily controlled in submerged fermentation conditions. Hence, the environmental conditions such as temperature, agitation, dissolved oxygen and pH can easily manipulate in the submerged fermentation to obtain maximum yield of polysaccharide of *Ganoderma* spp.

2.5.1 Effect of pH

pH is an important physical parameter to obtain optimum microbial growth. Studies by Chen & Yu (1993) revealed that culture pH clearly affects fungal growth. The initial pH in fermentation broth may influence fungal cell membrane, cell morphology and structure as well as the utilisation of different nutritional requirements. However, Bilgrami & Verma (1992) reported that mushroom mycelia are more tolerant to acidic media than basic media. Meanwhile, the decrease of mycelial growth at lower pH could be due to the toxicity of hyphae in very acidic pH conditions of the cultivation medium. Maximum mycelial growth and production of polysaccharides for *Ganoderma* spp. were obtained at initial pH of 7.0-6.0 (Kim, 2006). Table 2.3 shows the studies had been conducted at pH range 4.5-5.5 for mycelial weight and production of *Ganoderma* spp. polysaccharides. The EPS production from *G. lucidum* was the highest ever recorded in the literature, a constant pH of 6.0

favoured exopolysaccharide productions with yields of 20.04 g/L on day 6 (Fang & Zhong, 2002c). The pH range may also be achievable for polysaccharides and mycelial growth of *G. neojaponicum*.

2.5.2 Effect of temperature

Temperature is one of the most crucial environmental factors in mycelial growth which requires careful control (Brancato & Golding, 1953). This characteristic explains why *Ganoderma* spp. are often found in hot climates and tropical regions. Table 2.3 shows the cultivation temperature for *Ganoderma* spp. varied from 25-34 ^{o}C. *Ganoderma lucidum* isolated from different regions generally grow at optimal temperature of 30 ^{o}C (Lin *et. al*, 1973, Yang & Liau, 1998a). The highest EPS production from *G. lucidum* was 22 g/L reported in the literature at temperature of 25 ^{o}C by bistage control in 3-L air lift bioreactor (Jong & Birmingham, 1992). The temperature range attained could also be displayed by *G. neojaponicum.*

2.5.3 Effect of agitation and aeration

Microorganisms normally vary in terms of their oxygen requirements. The largest utilisation of oxygen during growth of aerobic microorganisms is for respiration. Oxygen serves as a terminal electron acceptor for oxidative reactions to supply energy for cellular activities (Forage *et al.*, 1985). Productivity of several fermentations is limited by the availability of oxygen. Therefore, it is important to consider the factors which affect the efficiency in supplying microbial cells with oxygen (Standbury & Whitaker, 1984). The agitation provided in the fermentation process affects the formation of mycelium. High

agitation speed was necessary during fermentation to avoid cell sedimentation. Meanwhile, extremely high agitation speed has been shown to increase the shear stress on the mycelium, which in turn, decreases biomass production (Yang & Liau, 1998a). The production of polysaccharides from *G. lucidum* was favoured at low oxygen tension (Fang & Zhong, 2002a). Tables 2.2 and 2.3 show the submerged cultivation of *Ganoderma* spp. was optimized at agitation speed of 120-160 rpm and aeration of 2-2.5 vvm.

It has been reported that agitation speed for *G. lucidum* is approximately at 100 rpm (Lin *et.al.,* 1973). Tang & Zhong *et al*. (2002c) observed that high initial oxygen supply increased mycelial biomass production. Physical parameters such as pH, temperature, agitation, (aeration) oxygen supply and cell morphology are important in producing high yields of polysaccharides/bioactive metabolites during submerged fermentation in shake flask and bioreactor (Duta *et al*., 2004). Tables 2.2 and 2.3 shows the common physical parameters that have been studied in *Ganoderma* spp. Similar to *G. lucidum*, *G. neojaponicum* could also attain the same range of agitation and aeration for optimum yields of polysaccharides/bioactive metabolites in shake flask and bioreactor for submerged fermentation.

2.5.4 Factors affecting pellet size

It has been discovered that the additions of surfactants, vegetable oil and fatty acids can enhanced mycelial dispersion, biomass and production of polysaccharides (Cassiano *et al*., 2007). Most fungi especially basidiomycetes tend to produce morphologically and physiologically heterogeneous pellets of

variable sizes, and are often in large sizes. Growth of this polypore in submerged culture can be in the form of pellets (when shaken) or filamentous mat (under static condition). This will make representative sampling difficult (Gibbs *et al*., 2000). In submerged cultures, the morphology of filamentous microorganisms usually differ between pelleted form and the dispersed forms; this is determined by the respective culture conditions. In its dispersed form, mycelium comprises branched and unbranched hyphae (freely dispersed) as well as aggregates (clumps) (Cox *et al*., 1998).

The dispersion of mycelial growth is desirable because of the increased homogeneity and ease of sampling. The anti-foam agent used in the medium to stimulate growth was olive oil; it was discovered to have positive effects on the biomass production for *G. lucidum*. Its presumed partial incorporation of lipids in the cell membrane results in the stimulation which in turn helps nutrients to be absorbed from the medium. According to Park *et al.,* (2002), vegetable oil tested at certain levels did stimulate mycelial growth and polysaccharides formation in *G. lucidum* while, higher concentrations might cause inhibitory effect. It has been suggested that surfactants affect cell membrane permeability. Several qualities of the cell surface, such as surface antigens, hormone binding, cell recognition, adhesiveness and adsorption can be ascribed to polysaccharides or are somehow affected by them (De Souza, 1989). Acid polysaccharides contain negatively charged residues. Because of their negative charge, they are highly hydrated molecules, which avidly bind cations and may form gels of varying pore sizes and

charge density, acting in cell osmoregulation (De Souza, 1989). Thus, acid polysaccharides act as sieves to regulate the traffic of ions going in and out of the cell and consequently play a role in cell morphogenesis, cell differentiation and cellular growth (De Souza, 1989). Similar to *G. lucidum*, this factor is also vital for *G. neojaponicum* to attain optimum yields of polysaccharides in shake flask and bioreactor for submerged fermentation.

2.6 Polysaccharides

The polysaccharides were related structurally to a distinct class of macromolecules. Three main groups of polymers exist in polysaccharides structure; these are polymers of mannose, peptides polymers of glucose and polymers of N-acetylglucosamine. The polysaccharide has differing unique structure for the different species available. Polysaccharides derived from *Sclerotium* spp. is called scleroglucan, pullulan from *Pullularia pullulan*, curdlan from *Alcaligenes faecalis*, dextran from *Leuconostoc mesenteroides*, xanthans from *Xanthomonas compestris*, and algenates from *Azotobacter vinelandii*. *Ganoderma* spp. have been reported to produce more than over 100 types of biologically active polysaccharides extracted from its mycelium and basidiocarps

2.6.1 β-glucan

In the fungal cell wall, the carbohydrate layer is composed mainly of β-(1,3)-glucan (55% of cell wall carbohydrate), α-(1-3)-glucan (28%), β-(1,6)-glucan (6%) and chitin (0.5%) (Hochstenbach *et al*.,1998). The most potent bioactive compound in fungal polysaccharides used for medicinal purposes is β-D-glucan. β-D-glucan can be classified into two subtypes based on the mode of

glucose linkages: long chains of 1500, β-1,3-D-glucose units and short chain of 150, β -1,6-D-glucose units (Klis *et al*., 2002). Figure 2.3 shows the primary structure of β-D glucans of polysaccharide. β-D glucan consists of a linear backbone of β-(1,3)-linked D-glucopyranosyl groups, which have different degrees of branching from the C6 position (Wasser, 2005).

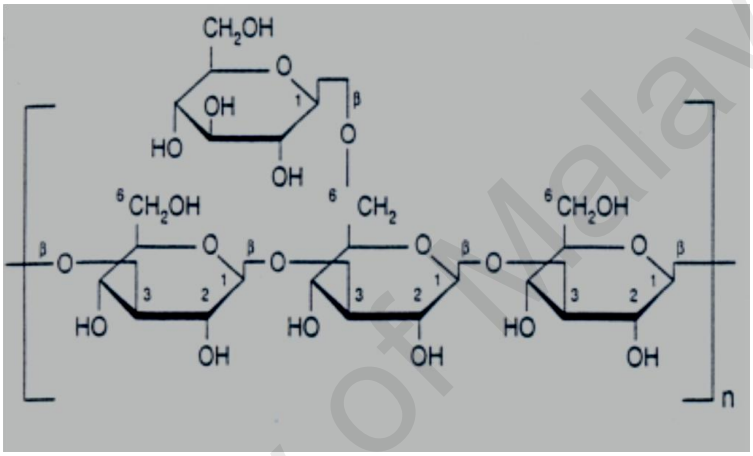


Figure 2.3: Primary structure of β-D-glucan (Kidd, 2000)

The designations of α and β-glucans are determined by the position on the sugar molecule at which the bonding occurs. Although many studies have been conducted with regard to β-glucan, there is not much information available about α-glucan. The function of α-glucan is unclear; it is yet to be known if α-glucan plays a role in maintaining cell shape. In contrast, it is widely accepted that β-glucan is the main support of cell shape and is medically important. *G. neojaponicum* has also been reported contain β-1,3-linked and β-1-6-linked D-glucose polysaccharides that have been identified as potential anti-tumour agents and may function to enhance the immune system (Takei, 2006).

2.6.2 Extraction, purification and validation of homogeneity of polysaccharides

There has been improvement in approaches and techniques employed in the extraction and purification of polysaccharides in clinical research over the years. One of the established procedures for extraction, fractionation and purification of polysaccharides from fruiting bodies or culture mycelia was developed by Mizuno (1999). In submerged fermentation, the polysaccharide can be further purified for intracellular polysaccharides (IPS) and extracellular polysaccharides (EPS). The IPS is extracted from mycelium while EPS is secreted in the growth medium (broth).

The dried mycelium and broth can be extracted for crude polysaccharides by using hot water process. The hot water extract method is a boiling procedure used to concentrate the bioactive compound to the desired amounts or suitable level for therapeutic usage (Mizuno, 1999). In addition, Takashi (2009) also reported that the crude polysaccharides could be extracted using hot water, hot alkali solution or cold alkali solution. The amount of polysaccharides of *G. lucidum* had increased up to 5 mg/mL by cold alkali method compared to hot water extraction with only 1 mg /mL (Takashi ,2009). The use of salts, alkali and DMSO can also be applied for extraction of polysaccharides (Mizuno *et al*., 1984). A study by Tang & Zhong (2002) reported that the crude polysaccharide fraction obtained from *G. lucidum* by hot water extraction had generated immunostimulatory activity in mice. To date, the extraction and functional characterization of polysaccharides isolated from *G. neojaponicum* is far from

complete. Therefore, the same procedure of other *Ganoderma* spp. may also applied to extract polysaccharide from *G. neojaponicum*.

The process of eliminating low molecular weight substances is needed to produce partially purified polysaccharides (Mizuno, 1996). The partial purification of β-glucan can be achieved by removing all impurities of the component of crude polysaccharides using ethanol precipitation. The partial purification of EPS (filtered broth) from medicinal mushrooms is done by adding 96% ethanol (volume ratio 1:1) to precipitate EPS and then accumulated by centrifugation and dissolving in distilled water. Finally, the extracts are dialysed against distilled water for duration of two days. Some studies reported that precipitation could also be used to recover more EPS (filtered broth) with the application of three to four volumes of 95–96% ethanol. There have also been cases where two volumes of acetone were used (Lee *et al*., 1999). According to Ji *et al*. (2004), the partially purified polysaccharides could be conducted by ethanol precipitation at the ratio of 1:4 as described in Figure 2.4. There are also other ways of using 1% ammonium oxalate, where 5% NaOH can be used after hot water technique for extraction to purify IPS (mycelium) (Habijanic *et al*., 2001). Solution of 1 M NaOH at temperature of 60 °C for duration of 1 hour also used to extract IPS.

In general, purification of high molecular weight polysaccharides from mushrooms can be done using gas chromatography (GC) and high performance chromatography (HPLC). These methods have largely replaced the previous

methods of paper chromatography and thin-layer chromatography (TLC), which were initially used in determining mono and disaccharides. This change could be attributed to their advantages in terms of separation efficiencies, quantification and speed of analysis of polysaccharide extracts.

Recent studies have established a more efficient procedure to purify and validate of the purity of polysaccharide extract by a combination of methods such as ethanol concentration, fractional precipitation, acidic precipitation with acetic acid, ion-exchange chromatography, gel filtration and affinity chromatography. The fractionation procedures such as Sephadex G-200 can then be utilised to analyse the homogeneity of the exopolysaccharides through gel filtration (Babitskatya *et al*., 2000). The purity level of β-D-glucan was obtained at 87.5% from *Lentinus edodes* through ethanol precipitation, freeze-drying in liquid nitrogen and purified using a carbohydrate analysis column (Babitskatya *et al*., 2000)

For the isolation of pure polysaccharides extract from *Agaricus blazei* and *Sparassis crispa*, a combination of extractions with hot water and cold alkali (NaOH) was used, followed by fractionation using ion-exchange chromatography on DEAE-Sephadex A-25 (Ohno *et al*., 2001). Besides that, Mizuno (1999) also used similar protocols involving hot water extraction, gel filtration and ion-exchange chromatography for isolating purified polysaccharides from *Agaricus blazei*.

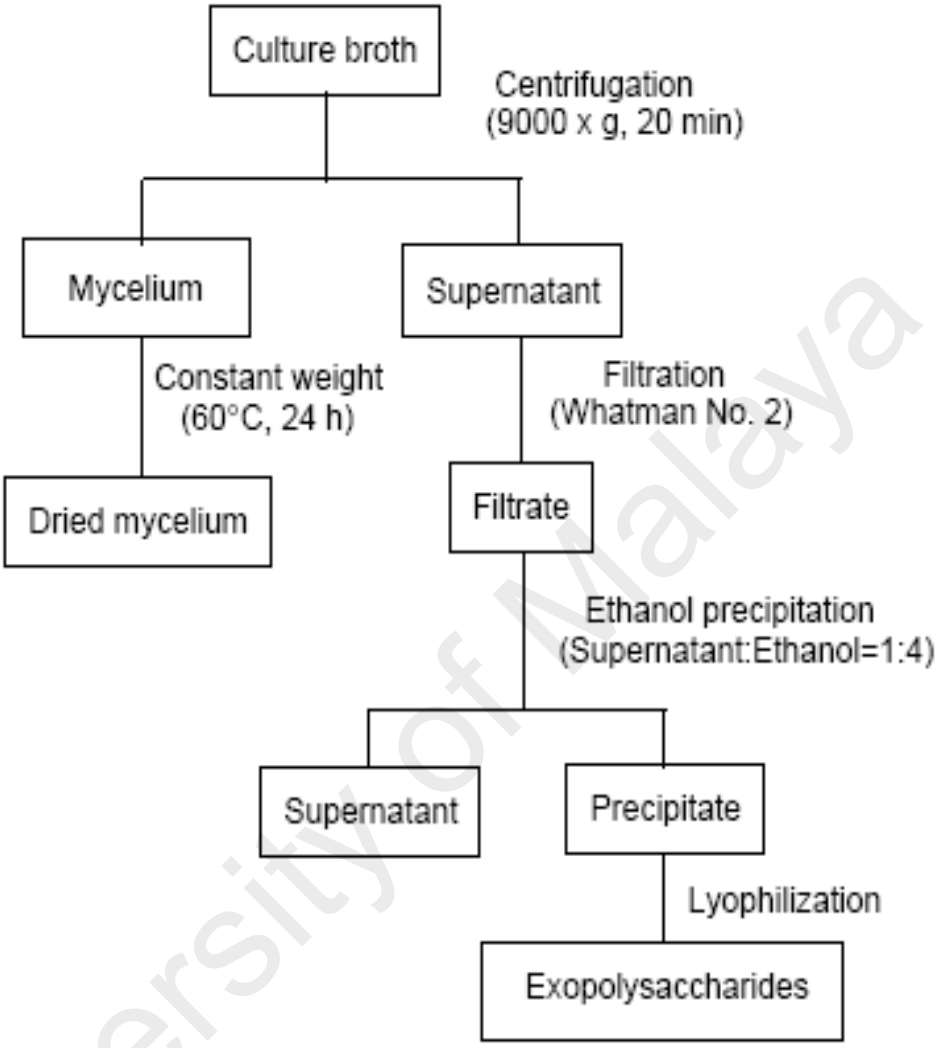


Figure 2.4: The process of partially purified polysaccharide extraction in submerged cultivation (Ji *et al.*, 2004)

2.6.3 The molecular weight of polysaccharide fractions

The different types of polysaccharide of basidiomycetes have different medicinal properties. The polysaccharides differ greatly due to their chemical constitution composition and configuration. In addition, their physical properties also contribute to this variation. Solubility in water, size of molecules, branching rate and form can be linked to variation in activities.

The polysaccharide fraction of each mushroom species has unique molecular structures of β-D-glucan which influence different biological activities (Ohno *et al*., 2000 and Adachi *et al*., 1990). The molecular structure of (1,3)- β-glucans in the helical of the glucan backbone (triple-stranded) contain residues with the presence of hydrophylic groups located on the outside surface of the helix. The diffraction of the β-D-glucan structures can be analysed using NMR and X-ray for the degree of triple-stranded and winding helix structure (Bluhm & Sarco, 1977). The different degree of β-D-glucopyranosyl branch units in β-(1-6)-bonding system affects its structure, size of molecules and solubility in water (Mizuno, 1995).

The molecular weight of most polysaccharides ranged from 4×10^5 to 1×10^6 in the primary structure (Su *et al*., 1997). Meanwhile, according to Mizuno *et al*., (1996), the medicinal properties of β-D-glucan had molecular weight range $1x10^4$ to $1x10^6$ kDa. In terms of effectiveness, high molecular weight glucans seem to perform better compared with those of low molecular weight (Mizuno *et al*., 1996).

It has been discovered that polysaccharides with higher molecular weight of $1x10^5$ kDa and above exhibited more powerful antitumour effects and more active in terms of immunomodulatory activities. A report by Maeda *et al*., (1988) also agreed that the high molecular weight glucans was more effective on immunomodulatory activity compared to low molecular weight of glucans. The molecular weight and structure of polysaccharides with immunomodulating activities from medicinal mushroom species is summarized in Table 2.6. β-glucans extract of *G. lucidum* had influenced the biological activity as immunomodulating agent (Mizuno *et al*., 1999). Besides that, according to Bohn & BeMillar (1995), β-glucans extract in *G. lucidum* that are soluble in alkali have been demonstrated to show antitumour activity. Meanwhile, Takei (2006) reported that *G. neojaponicum* also contained polysaccharides that have been identified as potential anti-tumour agents. Yet, the molecular weight of *G. neojaponicum* need to be further studied.

Table 2.5: The molecular weight and structure of polysaccharides with immunomodulating activities from medicinal mushroom species (Mohamad-Fata, 2007)

Compound	Mushroom Species	Molecular Structure	Molecular Weight (KDa)
Sclerotinia Sclerotiorum Glucan (SSG)	*Sclerotinia Sclerotiorum*	1,6-Monoglucosylbranched 1,3-β-Dglucan	≥2000
D-fraction	*Grifola Frondosa*	1,6-Monoglucosylbranched 1,3-β-Dglucan	1000
Schizophyllan	*Schizophyllum Commune*	1,6-Monoglucosylbranched 1,3-β-Dglucan	350
Grifolan	*Grifola Frondosa*	1,6-Monoglucosylbranched 1,3-β-Dglucan	500
Lentinan	*Lentinus Edodes*	1,6- Monoglucosylbranched 1,3-β-Dglucan	500
Polysaccharide-K (PSK)	*Coriolus versicolor*	1,3 and 1,6-Monoglucosylbranched 1,4-β-Dglucan with binding to aspartic, glutamic and other acidic amino acids	100
Polysaccharide-protein complex (PSPC)	*Tricholoma lobayense*	Consists of galactose, glucose, mannose, fucose, arabinose and rhamnose with binding to aspartic, glutamic and other acidic amino acids	150
Prolysaccharides-Peptide (PSP)	*Coriolus Versicolor*	Consists of glutamic and aspartic acids	100

2.7 The effects of polysaccharides of *Ganoderma* spp. on immune cells

Polysaccharides of *Ganoderma* spp. have demonstrated considerable pharmacological effects particularly its role in modulating immune responses. The mechanism involved in the immunomodulatory effect on the immune cells is summarized in Table 2.6. The polysaccharide from *Ganoderma* spp. affects the recognition of receptors, differentiation of immune cells and cytokines (including interleukins) as well as the proliferation of cell-mediated immune responses. Besides this, polysaccharides from *Ganoderma* spp. have been linked to mitogenicity and the activation of immune effector cells such as macrophages as well as T and NK cells.

Macrophages play an essential role in cellular immunity, a forefront in the immune system. According to Gao *et al.*, (2002), immune effector cells such as cytokines, interleukins and interferons as well as tumour necrosis factor-γ (TNF-α) are stimulated in immune response systems. As a result of stimulation of these immune effector cells, cytokines such as interferon (INF), interleukins (IL) and tumour necrosis factor (TNF)-α were affected as reported in Table 2.5. According to Bohn and BeMiller (1995), the mode of immunopotentiation by (1-3)-β-D-glucan includes activation of cytotoxic macrophages, helper T cells and NK cells as well as the promotion of T cell differentiation. Therefore, the β-glucan present in *G. neojaponicum* may also trigger similar immunomodulatory function in curing tumour or cancer cells.

Table 2.6: The effects of polysaccharides of *Ganoderma* spp. on immune cells

Immune cells	Effects	References
Macrophages	1. Improve phagocytosis 2. Promote IL-1, TNFα production and TNFα mRNA expression 3. Stop oxidant tBOOH-induced oxidative injury 4. Protect mitochondrial membrane and alleviate membrane injury by free radicals 5. Increase [Ca2+]i Induce IP3 and DAG formation 6. Raise PKC activity 7. Activate macrophages via TLR4 and TLR4-modulated protein kinase signalling pathways	Lin *et al.*,(2005) Li *et al*., (2000) Tang & Zhong (2002a)
Neutrophils	1. Increase in PKC, MAPK, HCK and tyrosine kinase Lyn activities 16 2. Restrict spontaneous and Fas-induced apoptosis by activation of Akt-regulated signalling pathways	Hsu *et al*., (2004)
Dendritic cells	1. Encourage maturation and immune response initiation induced by DC 2. Promote cytotoxicity of specific CTL induced by DC 3. Raise IFNγ and granzyme B production and mRNA expression 4. Induce activation and maturation of human DC by the NF-κB and p38 5. MAPK pathways	Cao & Lin (2002) Lin *et al.*, (2005)
Natural killer cells	1. Improve activity of NK cells 2. Increase NK-cell-mediated cytotoxicity	Lin *et al.*, (2005) Chien (2004)
T lymphocytes	1. Increase lymphocyte proliferation induced by ConA and MLC 2. Promote IL-2, IFN-γ production 3. Increase DNA synthesis and improve activity of DNA polymerase α 4. Raise the percentage of CD5+, CD4+ and CD8+ T-lymphocytes 5. Raise production of IP3 and DAG 6. Increase activities of PKA and PKC	Lei (1993)
B lymphocytes	1. Increase lymphocyte proliferation induced by LPS 2. Increase in secretion of immunoglobulin 3. Stimulate expression of PKC 4. Decrease mucosal specific IgA response of young adult mice orally 5. Immunised with cholera toxin	Cao & Lin (2003) Bao (2002)

2.7.1 Role of macrophages in the immune system

Macrophages eliminate pathogenic microorganisms, decompose parts of cells, foreign particles and also granulocytes, which produce mediators utilised by cells in communicating with one another within the immune system. Phagocytosis is an initial and critical part in host defence against pathogen particularly in its role in triggering the adaptive immune response. Many studies suggested that phagocytotic activities are stimulated in the macrophage cell and its mediators (Lin *et al*., 2007 and Medici *et al*., 2005). According to Laskin (2009), macrophages serve as antigen-presenting cells in triggering adaptive immune response. A number of research have indicated the ability of polysaccharides to enhance phagocytic activity, or stimulate the production of hydrogen peroxide, NO or cytokines in macrophage cells (Hatcher & Lambrecht, 1993 and Matsuguchi *et al*., 2003).

The polysaccharide fraction of *G. lucidum* is a strong stimulator to the macrophages (Oh *et al*., 1998). Polysaccharide extract (100 µg/mL) was demonstrated to increase phagocytic activity of BALB/c mouse peritoneal macrophages and chicken macrophage BM2CL cells against FITC-labelled *Candida albicans* by 55.2% and 21.2%, respectively. Macrophages are also one of the components that are vital for tumour rejection. According to Wasser (2002), cancer cells are not attacked directly by the polysaccharides of mushrooms. Instead, host mediated immunomodulatory response is enhanced by the polysaccharides through their role in activating the immune system to destroy cancer cells (Wasser & Weis, 1999).

Furthermore, the polysaccharides produce antitumour effects by triggering various immune responses in the host. These activities result in the induction of various types of antitumour effector cells such as cytotoxic T cells, NK cells and macrophages. According to Bohn & BeMiller (1995), macrophages have a highly selective cytotoxicity towards cancer cells *in vitro*; with evidence show they may also destroy malignant cells *in vivo*. T cell competence seems to be needed for selection of macrophage resistance; this suggests that in response to a tumour challenge, these two cell types interact in the intact host.

The water extract polysaccharides from *Ganoderma* spp. are demonstrated to increase the production of cytokines, including IL-1, IL-6, IFN-γ and TNF-α by human macrophages (Wang *et al*., 1997). These showed anti-proliferative, differentiation and apoptosis inductive activities against HL-60 and the U937 leukemic cells (Wang *et al*., 1997 and Lee *et al*., 1995). Macrophages released IFN-γ and TNF-α which acted in synergy to restrict growth of leukemic cells. Besides this, the reduction in amount of oxygen free radicals produced by *G. lucidum* polysaccharide antagonised the respiratory burst induced by PMA in murine peritoneal macrophages (Li *et al*., 2000).

The β-D-glucans, also polysaccharides of D-glucose monomers linked by β-glycosidic bonds have been reported to activate the innate host defences by binding to specific macrophage receptors (Mueller *et al*., 2000). Numerous research have reported the responsiveness of macrophages towards β-glucan treatment (Suzuki, 1990). The β-glucans are also categorised as biological response modifiers which affected macrophages activities (Ishibashi, 2001). The activation of macrophages

by β-D-glucans releases cytokines, nitric oxide (NO) and other mediators in the immune system. Meanwhile, CR3 receptors on macrophages are bound by β-D-glucans to internalise for priming a series of molecular action in the immune system. Besides this, β-D-glucans are powerful stimulators for murine and human macrophages using *in-vitro* and *in-vivo* model systems (Wang *et al*., 1997 and Gao *et al*., 2004). It is observed that cell proliferation of RAW264.7 cell lines was restricted on ganoderic acid, improvement in production of NO in the RAW264.7 Macrophages was attributed to the ganoderic acid extract from *G. lucidum* (Han *et al*., 1998). RAW264.7 macrophages cell line may also show similar immunomodulatory response to the polysaccharide extract of *G. neojaponicum*.

The β-glucan-related polysaccharides of the higher fungus activate macrophage and release NO, which is a vital chemical messenger for the induction of several biological responses. The macrophages capture β-glucans through the Dectin-1 receptor with or without TLR-2/6. Next, the large β-glucan molecules are internalised and fragmented into smaller sized β-glucan fragments within the macrophages. Next, they are transported to the marrow and endothelial reticular system and are then released. These small β-glucan fragments are ultimately taken up by the circulating granulocytes, monocytes or macrophages via the complement receptor (CR)-3. The monoclonal antibody tagged tumour cells cause the immune response to trigger phagocytotic action.

Therefore, the β-glucan molecules in *G. neojaponicum* may also trigger the similar posibble immune function in curing tumour or cancer cells.

2.7.2 Role of NfkB in the immune system

NF-κB is one of the transcription factors that plays a prominent function in the immune system as it relates to regression of tumours. There is high likelihood that many tumours will be solely resistant to restriction of NF-κB. However, the sensitisation of these cancer cells to apoptosis induced by other agents provides NF-κB restriction the opportunity to be the latest therapy to treat solid tumours (Lin *et al*., 1995). Figure 2.5 illustrates the schematic diagram of the β-glucans recognition by specific receptors on the cell surface and the transcription of several genes in both adaptive and innate immune reactions caused by activation of NF-κB. Usually, the ligand-receptor complex can be internalised into endocytic vesicles, thus activating the nuclear factor NF-kB. NF-κB shifts to the nucleus to bind to response elements of NF-κB target genes. One of the effects of NF-κB activation is the activation of several NF-kB target genes; this leads to the cytokines being produced, such as ROS and tumour necrosis factor α (TNFα). NF-κB-regulated enzyme NO synthase then produces nitric oxide (NO). In bone marrow macrophages experiments, the fungal β-glucans have been reported to control the production of NO (Lee *et al*., 2008).

The function of NF-κB in the regulation of genes is crucial for cell growth, anti-apoptosis, the cell cycle and cellular adhesion. Constitutive activation of NF-κB, though not altogether, has been found in many solid tumours. In fact, NF-κB is stimulated in response to several agents employed in cancer treatment. As a result, the activation of NF-κB in tumours contributes to tumour growth and cancer cell survival as well as in the resistance of cancer to present therapies. The NF-κB

molecules are dimmers which are made up of p65/RelA and p50 (Mercurio, 1997 and Ghosh *et al*., 1998). The constitution of the dimmers and the cell type in which it is functioning are deemed as contributing factors to the transcriptional specificity of NF-κB (Perkins *et al*., 1992, Kunsch *et al*., 1992 and Hansen *et al*., 1994).

Ganoderma spp. are reported to have anti-inflammatory and anti-cancer properties due to the restriction of NF-κB (Sliva *et al*., 2003). *Ganoderma lucidum* mycelium (1.6 mg/mL) was activated kappa B DNA binding activity in RAW264.7 cells (Kuo *et al*.,2006). *Ganoderma* spp. also suppresses cell adhesion and cell migration of highly invasive breast and prostate cancer cells, suggesting its potency to decrease tumour invasiveness. *Ganoderma* extract restricts active NF-κB, displaying strong restriction of cancer cell migration. The therapeutic effect in *Ganoderma* spp. for anti-cancer and anti-inflammatory actions was attributed to its immunomodulating activity. The evidence for the anti-cancer effects of *G. lucidum* has been reviewed by Yuen & Gohel (2005). Therefore, polysaccharides of *G. neojaponicum* as anti-tumour agents, indicating NF-κB as one of prominent elements in the immune system that relates to regression of tumours.

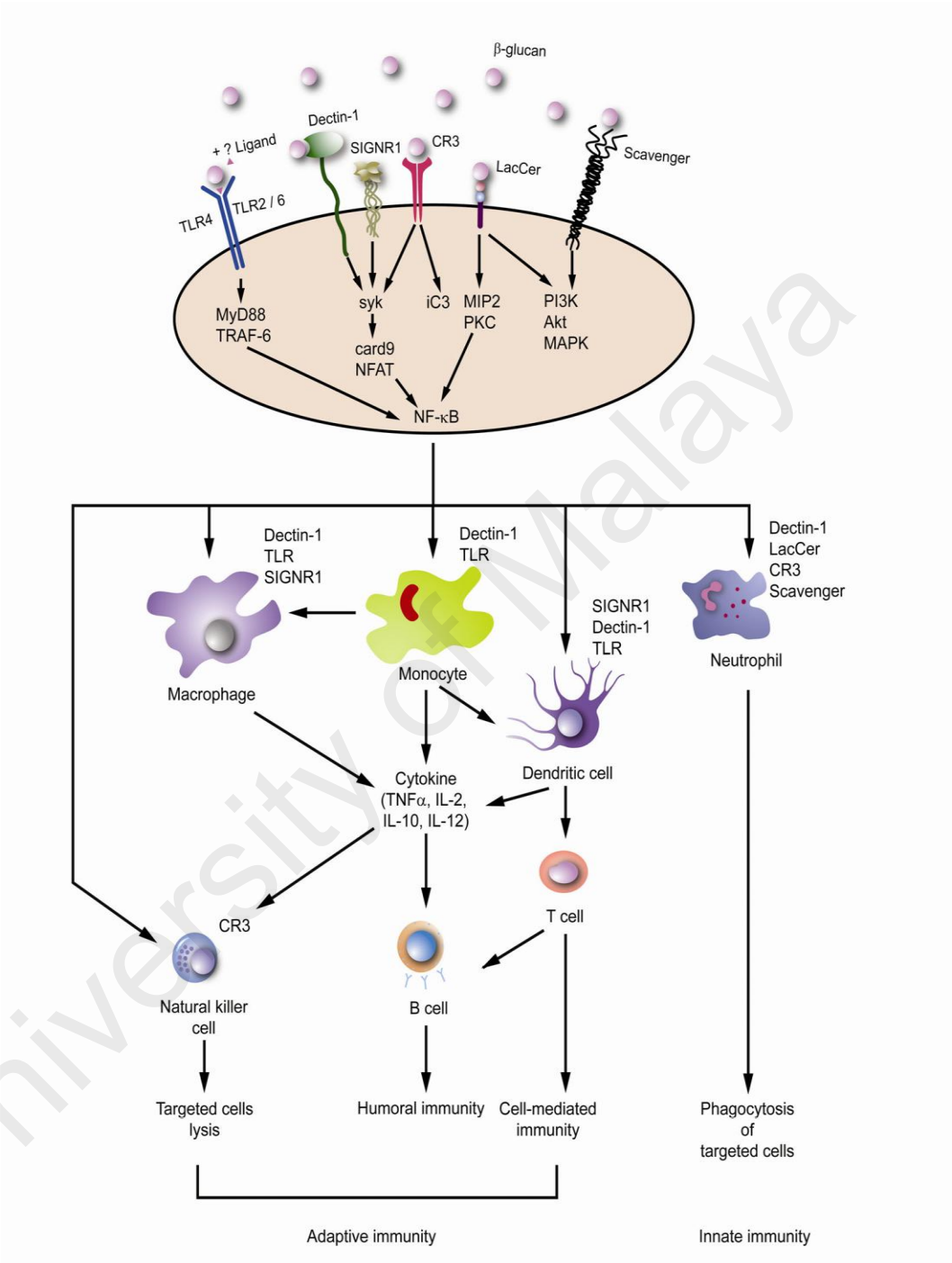


Figure 2.5: Schematic representation of the β-glucans recognition by specific receptors on the cell surface and activation of NF-κB leading to transcription of many genes in both adaptive and innate immune responses (Godfrey, 2009)

CHAPTER 3.0 MATERIALS AND METHODS

3.0 Experimental Plan

The flow chart of the experimental plan and the four objectives are shown in Figure 3.1.

3.1 *Ganoderma neojaponicum* strain

The strain of *G. neojaponicum* (KLUM61076) was collected from Bahau, Negeri Sembilan and deposited at the University of Malaya Culture Collection, Mushroom Research Centre (Kuala Lumpur, Malaysia). The *G. neojaponicum* was maintained as stock culture on malt extract agar (MEA, Oxoid) as in Appendix A1.

3.2 Screening of low cost materials as carbon and nitrogen sources by radial growth measurement

The low cost materials (brown sugar, spent yeast, molasses and corn steep liquor) were investigated as new sources for cultivation medium of *G. neojaponicum* growth using plate agar technique.

3.2.1 Carbon and nitrogen sources; and determination of carbon and nitrogen content

The source of molasses was a by-product of sugar refinery by-product collected from Central Sugar Refinery Sdn Bhd, Shah Alam, Selangor. Meanwhile, the source of spent yeast a by-product of brewery industry was from Carlsberg Brewery (M) Berhad, Petaling Jaya, Selangor. Brown sugar, a product cane industry by-product was obtained from Malayan Sugar Manufacturing Co. Bhd. While corn steep liquor, a by-product of corn industry was purchased from Sigma-Aldrich (M) Sdn. Bhd.

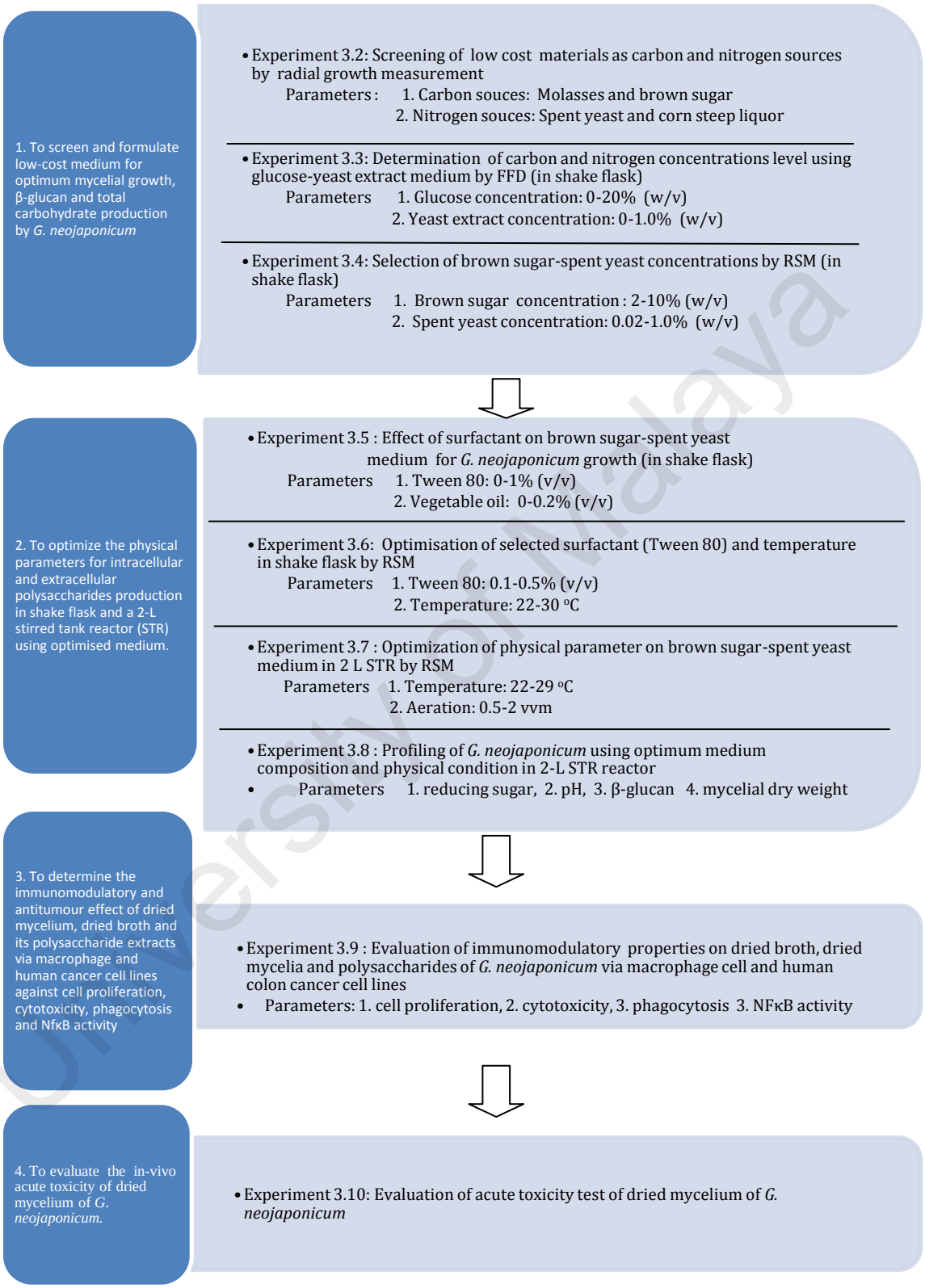


Figure 3.1: The flow chart of experimental plan

The raw materials consisted of brown sugar, spent yeast, corn steep liquor and molasses were analysed for total carbon and nitrogen. The total carbon was determined using furnace method while total nitrogen was analysed using Kjeldhal Method (AOAC, 1980).

3.2.2 Experimental Design and statistical analysis

The selection of suitable combination of carbon and nitrogen sources as low cost substrates was designed by Design Expert software (Minitab Version 16) via full factorial design (FFD). Range of carbon investigated was as determined in Section 3.2.1. Two levels of full factorial designs constructed using MINITAB® software (Minitab Inc. USA) were used after a series of preliminary experiments. Table 3.1 shows the levels of four factors with a total of 60 runs were carried out in the experiment. Each factor was examined at high level (+1) and low level (-1). The experiments were conducted in one block with three center points and three replicates. The response variable was the radial extension rate (mm/day). Details for the factorial design and response surface were as described by Montgomery (1997).

Table 3.1: Levels of factors used for selection of low cost materials in FFD

Factor	Name	Level	
		-1	+1
X1	% C	2	10
X2	% N	0.02	0.1
X3	Carbon source	MLS	BS
X4	Nitrogen source	CSL	SY

Abbreviations: MLS: Molasses BS: Brown sugar CSL: Corn Steep Liquor SY: Spent Yeast.

3.2.3 Low- cost medium formulation and experimental set up

The carbon and nitrogen content in low cost medium formulation were supplemented with a basal medium. The formulation of low cost media were calculated as Appendix A2-A5. The basal medium was consisted of 4% (w/v) bactoagar, 0.05% (w/v) KH_2PO4, 0.05% (w/v) K_2HPO4, 0.05% (w/v) $MgSO4.7H_2O$ (Appendix A7). The media were mixed and autoclaved at 121 ^oC for 20 minutes. The sterilized media were poured into petri dishes and allowed to solidify at room temperature.

G. neojaponicum inoculum was prepared as shown in Section 3.1 (page 36). One 5-mm disc containing mycelium was cut from the periphery of the colony and inoculated on the media in petri dish. The culture was incubated at 25±2 ^oC in static condition for seven days.

3.2.4 Measurement of radial growth rate

Radial extension rate was measured daily interval (every 24 hours) as a rate of change in the colony's radius (mm/day). The size was defined as the average of four diameter measurements along lines crossing at right angles. The readings of colony radius were measured starting from the centre of the disk to the periphery of the colony until one of the strains reached the edge of the Petri dish. Radial growth rate was determined by the slope and intercept of a linear graph. Obtained linear equations were used to model linear growth as described by Baumer *et al.*, (2008).

3.3 Determination of carbon and nitrogen concentrations requirement as for carbon and nitrogen sources using glucose-yeast extract medium in shake flask

The commercial medium containing yeast extract (Merck, Germany) and glucose (Fluka, Germany) were used as reference to determine the carbon and nitrogen concentration requirement for mycelial growth yield of *G. neojaponicum*. The carbon source (glucose) at range of 10-20% (w/v) and nitrogen source (yeast extract) was tested at 0–1% (w/v). The medium was prepared as described in Appendix A6.

G. neojaponicum inoculum was prepared as described Section 3.1 (page 36). The inoculum was punched at 5 mm of agar plate (culture plugs) from the periphery of the colony. Ten plugs were inoculated into the 100 mL medium supplemented with basal medium in 250 mL Erlenmeyer flasks. The basal fermentation medium was consisted of 0.05% (w/v) KH_2PO_4, 0.05% (w/v) K_2HPO_4 and 0.05% (w/v) $MgSO_4.7H_2O$ at pH 6.0. The medium was autoclaved at 121^oC for 20 minutes. The culture was incubated at 25±2 ^oC, 160 rpm for six days.

3.3.1 Determination of mycelial dry weight

The culture broth was centrifuged at 10,000 rpm, 4 ^oC for 10 minutes using refrigerated ultracentrifuge (Beckmann Coulter, Germany). The supernatant was discarded and the filtrate was dried at 50±2 ^oC to obtain constant weight. The mycelial dry weight was expressed as gram of dry weight per litre (g/L).

3.3.2 Statistical analysis

Data analysis for selection of carbon and nitrogen levels in commercial medium was analysed by Duncan multiple range test via SAS Ver 9.3 (SAS Institute, Cary, USA) and differences between treatment means were considered significant at $P< 0.05$.

3.4 Optimization of brown sugar and spent yeast concentrations in shake flask fermentation by RSM

Spent yeast and brown sugar were suitable as carbon and nitrogen sources for *G. neojaponicum* growth as determined from Experiment 3.3 . The amount of carbon and nitrogen range for *G. neojaponicum* were set based on Experiment 3.3. The parameters investigated in this experiment were spent yeast and brown sugar concentrations ranges 2-10% (w/v) carbon content for brown sugar and 0.02-0.1% (w/v) nitrogen content for spent yeast.

3.4.1 Experimental design and statistical analysis

Response surface analysis was conducted using Central Composite Design (CCD) for two factors consisting of 39 runs with five centre points and three replicates. Table 3.2 shows the two levels of factors of carbon and nitrogen concentration medium used in this experiment. Each factor was examined at high level (+1) and low level (-1) as stated in Table 3.2. The experiments were conducted in one block with three replicates. The response variable was taken as mycelial dry weight. The determination of mycelial dry weight can be refered to Section 3.3.1 on page 40.

Table 3.2: Levels of factors in CCD for spent yeast and brown sugar

Substrates	Factor	Name		Level		
				-1	0	+1
Brown Sugar	X_1	Carbon Content	(% w/v)	2	6	10
			g/L	1.76	5.23	8.8
Spent Yeast	X_2	Nitrogen Content	(% w/v)	0.02	0.06	0.1
			g/L	1	3	5

3.4.2 Preparation of seed culture as inoculum

Stock culture was prepared as described Section 3.1 (page 36). The seed culture was prepared by inoculating ten plugs of stock culture to the 100 mL medium. The medium contained brown sugar-spent yeast supplemented with basal medium (0.05% (w/v) KH_2PO_4, 0.05% (w/v) K_2HPO_4 and 0.05% (w/v) $MgSO_4.7H_2O$) in 250 mL Erlenmeyer flask (Appendix A5). The medium was adjusted at pH 6.0 and autoclaved at 121^oC for 20 minutes. The culture was incubated at 25±2 ^oC, 160rpm for six days. Ten percent of seed culture (10% (v/v)) was then used as inoculums for subsequent experiment.

3.4.3 Model verification

To verify the model prediction of the response, comparison between experimental value and predicted value were performed under optimal condition as predicted by the model. The five replicates of experimental value were conducted using 6% (w/v) carbon content in brown sugar and 0.075% (w/v) of nitrogen content in spent yeast. Meanwhile, five replicates of predicted value were conducted using 6% (w/v) carbon content in brown sugar and 0.06% (w/v) of nitrogen content in spent yeast.

3.5 Effect of surfactants on brown sugar-spent yeast medium for *G. neojaponicum* growth and yield of polysccharides in shake flask

The brown sugar (6% (w/v)) -spent yeast (0.075% (w/v)) medium formulation supplemented with basal medium (described Section 3.4.2 page 42) was further enhanced by addition of surfactants for increasing the *G. neojaponicum* growth and its polysaccharides. This experiment was carried out to select between two sources of surfactants namely vegetable palm oil (Seri Murni, cooking palm oil, Malaysia) at range (0-1% (v/v)) and Tween 80 (Sigma-Aldrich, USA) at range (0-1.2% (v/v)). The surfactant levels was selected as described by Dominguesa *et al*., (2000) and Cassiano *et al*., (2007).

The preparation of inoculum and seed culture *G. neojaponicum* can be refered to Section 3.4.2 (page 42). Ten percent of seed culture (10% (v/v)) was inoculated to the 100 mL brown sugar-spent yeast medium supplemented with surfactant in 250 mL Erlenmeyer flask (Appendix A5). The culture was incubated at 25±2 ^{o}C, 160 rpm for six days.

The determination of the mycelial dispersion of *G. neojaponicum* was observed based on size of the pellet formation in the cultivation flask. The mycelium and broth were separated by ultracentrifugation (Beckman Coulter, Germany) at 10,000 rpm for 10 minutes. The sediment was washed twice with the same volume of distilled water and freeze dried for two days. This sediment was classified as dried mycelium (M). While, the supernatant was the culture broth of *G. neojaponicum* then also freeze dried for 2 days and classified as dried broth (B).

3.5.1 Extraction of polysaccharides from mycelium and broth of *G. neojaponicum*

The mycelium and broth of *G. neojaponicum* were separated by ultra centrifugation (Beckman Coulter, Germany) at 10,000 rpm for 10 minutes. The broth was further partially purified using ethanol precipitation. The broth was added to double volumes of 95% (v/v) ethanol and left overnight at 4 ^{o}C to precipitate. Subsequently, were dried to remove the residual ethanol with a freeze dryer for 2 days. This extract was classified as ethanol precipitate of extracellular polysaccharides (EPS).

The mycelium was washed twice with the same volume of distilled water and freeze dried. 1 g of the freeze dried mycelium was ground into powder and dissolved with 10 mL of distilled water (1:10 w/v) pre-sterilized at 121 ^{o}C for 30 minutes. Double volumes of 95% (v/v) ethanol was added to aliquots of the extract and left overnight at 4 ^{o}C to precipitate the ethanolic extract of IPS. This extract was classified as ethanol precipitate of intracellular polysaccharides (IPS). This procedure was carried out as described by Hseih *et al*., (2005).

3.5.2 Determination of total carbohydrate in dried mycelial (M), dried broth (B) and polysaccharides extracts (IPS and EPS).

The content of total carbohydrate was determined by phenol-sulphuric acid assay according to Dubois *et al*. (1956). 0.1 g of M, B, IPS and EPS were diluted into 1 mL distilled water in test tube. Then, 1 mL of 5% (v/v) phenol solution and 5 mL of concentrated sulphuric acid were added to each tube. The mixtures were allowed to stand at room temperature for 10 minutes. The prepared aliquots were read using spectrophotometer (Cary WinUV, Agilent,

USA) at 490 nm absorbance. The percentage of total carbohydrate was determined using standard curve of glucose as in Appendix B1. The standard curve obtained was a linear graph of glucose concentration ranging from 0-1000 mg/ mL.

3.5.3 Determination of β-glucan content in in dried mycelial (M), dried broth (B) and polysaccharides extracts (IPS and EPS).

The Mushroom and Yeast β-glucan kit (K-YBGL, Megazyme, Ireland) was used in determining the β-glucan content. The contents of total glucan, *α*-glucan and β-glucan were calcullated as in Appendix D1. For total glucan, 0.1 mL of prepared extract were mixed with exo-1,3,β-glucanase and β-glucosidase in 40 ^{o}C of water bath for 60 minutes. Then, 3 mL of glucose oxidase was added to each tube and incubated for another 20 minutes. The aliquots were measured at an absorbance of 510 nm. The content of β-glucans was calculated as a difference between the total glucan and α-glucan. Meanwhile the solubilisation and partial hydrolysis of α-glucan were as described in Appendix C1. For α-glucan, 0.1 mL of prepared aliquots was added to 0.1 mL sodium acetate buffer followed by the addition of 3 mL of glucose oxidase to each tube and incubation at 40 ^{o}C for another 20 minutes. The absorbance of the α-glucan solution was read at 510 nm against a reagent blank. The β-glucan content was calculated by substraction of total glucan and α-glucan as detailed in Appendix D1.

3.5.4 Statistical analysis

The analysis of data for selection of types and concentration of surfactants were performed using Duncan multiple range test by SAS Version 9.3 (SAS

Institute, Cary, USA) and differences between treatment means were considered significant at $P<0.05$.

3.6 Optimisation of Tween 80 concentration and temperature in shake flask by RSM

The range of Tween 80 concentrations was prepared as determined in Section 3.5. The optimisation of Tween 80 concentration and temperature were conducted in this experiment by RSM in shake flask fermentation. The temperature range was set in this study was based on literature study as summarized in Table 2.2 on page 11. The parameters investigated in this experiment were temperature and Tween 80 concentration on spent yeast-brown sugar medium.

3.6.1 Statistical experimental design analysis

This experiment was designed by Central Composite Design (CCD) via Response Surface Methodology (RSM) (Minitab Ver. 16). The experiment was conducted with two factors in one block consisting of 39 runs with five center points and three replicates. Each factor was examined at high level (+1) and low level (-1) as referred to in Table 3.3. Table 3.3 shows the levels of factors used in the experiment. Response variables used in the response surface model were mycelial dry weight and β-glucan content.

Table 3.3: Levels of factors used for Tween 80 and temperature by CCD

Factor	Name		Level		
			-1	0	+1
X_1[a]	Tween 80 Concentration	% w/v	0.1	0.3	0.5
X_2[b]	Temperature	^oC	22	26	30

3.6.2 Experimental set up

The preparation of inoculum and seed culture can be refered to Section 3.4.2 (page 42). The determination of mycelial dry weight is as described to Section 3.3.1 page 40. The determination of β-glucan content is as illustrated in Section 3.5.3 on page 45.

3.6.3 Model verification

To verify the model prediction of the response, comparison between experimental value and predicted value were performed under optimal conditions as predicted by the model. The five replicates of experimental value were conducted using 0.046% (w/v) at a temperature of 26 ^{o}C. Meanwhile, five replicates of predicted value were conducted using 0.05% (w/v) Tween 80 and at a temperature of 26 ^{o}C.

3.7 Optimization of physical parameter of growth and polysaccharides yield of *G. neojaponicum* in brown sugar-spent yeast medium in a 2-L stirred tank reactor (STR) by RSM

Physical condition is an important parameter to be optimized in submerged fermentation. This study selected temperature and aeration rate as the most crucial physical factors for scale-up process for *G. neojaponicum* as referred to Felse & Panda, (2000). Meanwhile the selection of aeration rate range in this experiment was based on the summary reviewed by Wagner (2003b) while, the selection of temperature for *G. neojaponicum* as determined in Section 3.6 on page 46.

3.7.1 Experimental design

This experiment was based on the designed by Central composite Design (CCD) via Response surface methodology (RSM) (Minitab Ver .16). Response surface analysis was run on two factors consisting of 39 runs with five centre points and three replicates. Table 3.4 shows the levels of factors used in the experiment. The response variable was mycelial dry weight .

Table 3.4: Levels of factors used in the Central Composite Design for aeration and temperature

Factor	Name		Level		
			-1	0	+1
X_1	Aeration	vvm	0.5	1.25	2.0
X_2	Temperature	^oC	22	26	30

3.7.2 Fermentation condition of STR reactor

The *G. neojaponicum* was investigated for upsccaling production using 2-L STR reactor (Biostat ®A plus, B. Braun International, Germany) (Figure 3.2). The STR reactor (Biostat ®A plus, B. Braun International, Germany) is a stainless steel top plate fermenter that equipped with inlets for pH and oxygen electrodes, inoculation, sampling and temperature controlling sensor, air sparger, acid, alkali and antifoam inlets. Two six-bladed turbine impellers with a diameter of 52 mm mounted on the agitator shaft were used for agitation (Figure 3.3). During the fermentation process, the pH profile was measured using a pH probe. The probe was calibrated using buffers at pH 4.0 and 7.0 prior to the fermentation sterilisation. A dissolved oxygen probe was used to measure the Dissolved Oxygen Transfer (DOT).

Figure 3.2: A Biostat A plus 2-L stirred tank reactor

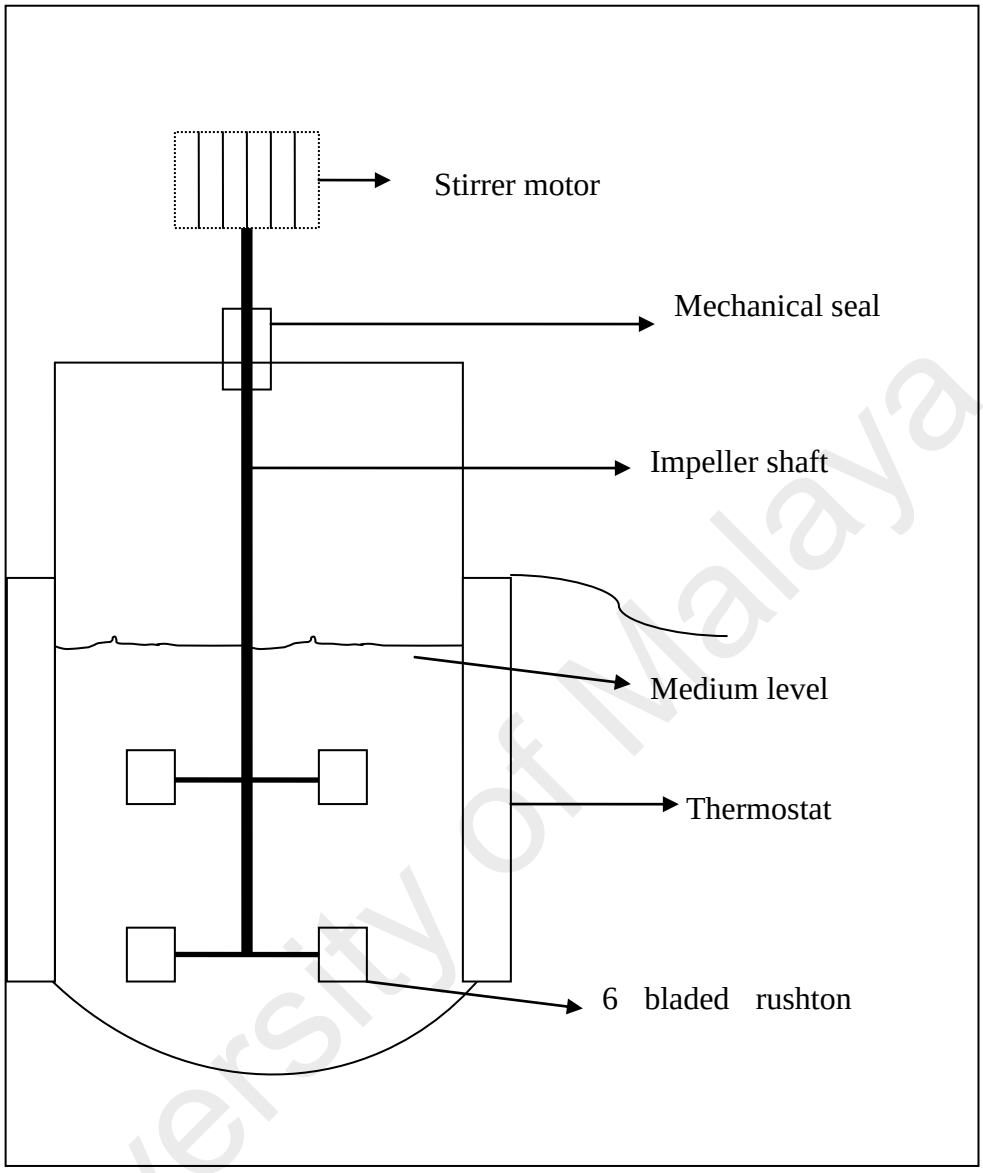


Figure 3.3: A schematic diagram of 2-L stirred tank reactor

3.8 Profiling of reducing sugar, growth and polysaccharides yield of *G. neojaponicum* using optimum medium composition and physical condition in 2-L bioreactor

The growth profile of *G. neojaponicum* was conducted in optimum spent yeast-brown sugar medium to obtain suitable cultivation time in 2-L STR reactor. The optimum cultivation medium and physical condition was prepared as obtained from previous Experiment 3.7. The optimum medium formulation was 5.74% (w/v) brown sugar, 0.06% (w/v) spent yeast, 0.46% (v/v) Tween 80 with constant basal medium. The optimum physical condition was at temperature 26.71 ^{o}C, aeration 1.33 vvm, pH 6 and 160 rpm. The cultivation medium and physical condition of bioreactor experiment was prepared as shown in Appendix A7. The preparation of inoculum and seed culture can be refered to Section 3.4.2 (page 42). Ten percent of seed culture (10% (v/v)) was inoculated to a 1-L working volume in 2-L stirred tank reactor (STR) . *G. neojaponicum* was profiled based on mycelial growth, pH and β-glucan and reducing sugar content. The profiles were conducted at every two-day time interval during six days of cultivation period.

3.8.1 Extraction of polysaccharides and determination of polysaccharide yield

The mycelium and broth of *G. neojaponicum* were separated by ultracentrifuge (Beckman Coulter, Germany) at 10,000 rpm for 10 minutes. The mycelium was washed twice with the same volume of distilled water and freeze dried. The culture broth of *G. neojaponicum* was freeze dried for 2 days. The freeze dried mycelium (M) and dried broth (B) were further extracted for EPS and IPS using ethanol precipitation as described in Section 3.5.1 on page 44.

The content of total carbohydrate was determined by phenol-sulphuric acid assay according to Dubois *et al*., (1956) as in Section 3.5.2 on page 44. The determination of β-glucan content was conducted as in Section 3.5.3 on page 45.

3.8.2 Determination of reducing sugar

The reducing sugar was assayed according to DNS method (Miller, 1959). The di-nitrosalicylic (DNS) acid solution was prepared by adding 1g of DNS (Fluka, Germany) to 20 mL of 1 N NaOH (Merck, UK) in the 100 mL of volumetric flask. This was followed by the addition of 50 mL of distilled water to the flask. Thereafter, 30 g of potassium tartrate (Fluka, Germany) was added for colour stabilization and the volume made up to 100 mL distilled water. To 1 mL of sample, 1 mL of DNS solution was added in a test tube followed by the addition of 2 drops of 1 N NaOH (Merck, Germany). The test tube was then heated in boiling water for 5 minutes and cooled under running water. 10 mL distilled water was added to the content of the test tube before reading in a spectrophotometer (Cary Win UV, Agilent, USA) at 540 nm absorbance. The percentage of reducing sugar was determined using a standard curve of glucose as in Appendix B3. The standard curve was a linear graph of glucose concentration that ranged from 0-1000 mg/mL.

3.8.3 Statistical analysis

The Statistical Analysis System (SAS) Version 9.3 (SAS Institute, Cary, USA) was employed in performing statistical analysis. Data were provided as Mean±SD; *t*-tests were employed in carrying out statistical analyses and statistical significance was considered only at *P*-values less than 0.05.

3.9 Production of *G. neojaponicum* polysaccharides at optimum condition and evaluation of its immunomodulatory properties

The production of *G. neojaponicum* polysaccharides were produced at optimum condition using 2-L STR reactor as described previously in Experiment 3.8 page 51 at day 4 of cultivation time. Four *G. neojaponicum* extracts consisted of dried broth (B), dried mycelium (M), IPS and EPS extracts were extracted as described in Section 3.5.1 page 44 and evaluated for immunomodulatory properties. The positive control used was yeast β-glucan extract (Megazyme, Germany) and the negative control used was cell culture medium without sample. The evaluations of immunomodulatory and antitumour properties of of *G. neojaponicum* were conducted using *in-vitro* test via macrophage (RAW264.7). Human colon cancer cell lines (HT29) against cell proliferation, cytotoxicity, phagocytosis and NFκB activity.

3.9.1 Cell culture

Two types of cell cultures were used macrophage cell (RAW264.7) and human colon cancer cell (HT29) for immunomodulatory assay. The RAW264.7 cells was obtained from ATCC (USA) and was maintained in DMEM medium (Invitrogen, Carlsbad, USA) containing 100 U/mL penicillin (PAA, Austria), 100 ug/mL streptomycin (PAA, Austria) and 10% v/v (PAA, Austria) fetal bovine serum. The cells were cultured to confluence in sterile tissue culture flasks; they were then carefully detached by scraping. Meanwhile, the human colon cancer cell of HT29 (ATCC, USA) was maintained in RPMI1640 (PAA, Austria) containing 100 U/mL penicillin (PAA, Austria), 100 µg/mL streptomycin (PAA, Austria) and 10% v/v (PAA, Austria) of fetal bovine serum. Both cells were grown in an incubator at 37 ^{o}C in a humidified atmosphere containing 5% CO_2

(Shel Lab, USA). The protocol for the maintenance and aseptic handling of cells is detailed in Appendix C1-C6.

3.9.2 Proliferation and cytotoxicity effects

The proliferation activity of *G. neojaponicum* extracts was investigated of RAW264.7 macrophages while, cytotoxicity effect of *G. neojaponicum* extracts were investigated of human colon cancer cells (HT29). 1 x 10^6 cells/well of HT29 or RAW264.7 cells were nurtured in 96-well microplates with complete medium. The cells were treated for 24 h with 100-1000 µg/mL of *G. neojaponicum* extracts. After incubation, 20 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, USA) at 5 mg/mL concentration was added into 170 µL of fresh medium in each well and incubated for 4 h at 37 ^oC and 5% CO_2 (Shel Lab, USA). Thereafter, the medium was removed from each well and DMSO (100 µl) (Merck, Germany) extraction buffer was added to each well to extract and solubilise the formazan crystal. The solution was vigorously mixed to dissolve the reacted dye and incubated for 20 minutes at 37 ^oC and 5% CO_2 . Finally, the plate was read on a ELISA Reader (Versamax with SoftMax Pro 5.3, USA) with 570 nm wavelength. The calculation was as below:

$$\text{Cytotoxicity / proliferation rate (\%)} = \frac{\text{(Absorbance of sample - Absorbance control)}}{\text{Absorbance of control}} \times 100\%$$

3.9.3 Phagocytotic ability

The ability of phagocytosis was assessed by neutral red uptake as previously reported (Weeks *et.al*., 1987). RAW264.7 cells were seeded at a

density of 1 x 10^6 cells per well in 96-well microplates. The calculation of cells was followed as Appendix A8. The cells were treated with 100-1000 µg/mL of *G. neojaponicum* extracts for 24 hours. Then, 0.075% (w/v) of neutral red was added. The plates were kept in an incubator for 30 minutes. Next, the cells were rinsed to remove leftover dye and were then blotted dry. After that, the cells were resuspended in ethanol (50% v/v) containing glacial acetic acid (1% v/v), and absorbance was measured at 540 nm in a microplate reader (Versamax with SoftMax Pro 5.3, USA)

The calculation of absorbance was translated into phagocytotic ability as calculation below:

$$\text{Phagocytotic ability} = \frac{\text{Absorbance Sample- Absorbance control}}{\text{Absorbance Control}} \times 100\%$$

3.9.4 Inhibition of NF-κB

The 1.5 x 10^6 cells/mL of HT 29 were stimulated with 500 µg/mL and 1000 µg/mL of the polysaccharides in 6-well multiwell cell culture plates and were subsequently incubated at 37 ^oC overnight. The quantification of NF-κB was carried out by NF-κB/p65 ActivELISA (Imgenex, San Diego, USA). The NF-κB ActivELISA system is a sandwich ELISA. It quantified the nuclear levels of p65 that may have positive relationship with the activation of NF-κB pathway. The free p65 was captured by anti-p65 antibody followed by alkaline phosphatase (AKP)-conjugated secondary antibody using calorimetric detection in ELISA micro plate reader (Versamax with SoftMax Pro 5.3, USA) at a wavelength of 405 nm. Details of the protocols of NF-κB ActivELISA is described in Appendix

D2. The inhibition and activation of the cells were determined by comparing them with the negative control (cells without polysaccharides). The protein in NF-κB / p65 was measured using BCA protein kit (Pierce, Thermo Fisher, USA) (Appendix D3).

3.9.5 Statistical analysis

The analysis of bioactivity data was performed using *t*-tests SAS Version 9.3 (SAS Institute, Cary, USA) (SAS, 1990). Statistical significance was considered at *P*-values less than 0.05.

3.10 Oral acute toxicity of dried mycelium of *G. neojaponicum*

The dried mycelium of *G. neojaponicum* was selected as a representative sample of *G. neojaponicum* extracts produced in this study to evaluate its safety analysis using *in-vivo* acute oral toxicity test. The dried mycelium of *G. neojaponicum* was prepared using optimum medium and physical conditions obtained from previous experiment using 2-L STR reactor (as described in Section 3.8 on page 51). 50 g of dried mycelium of *G. neojaponicum* was then sent to an independent specialist services laboratory at SIRIM, Selangor, Malaysia for *in-vivo* toxicology analysis. The blood samples of the rats were sent to the pathology laboratory in UPM, Selangor for haematology analysis. The data were analysed based on Compilation Report No. R220/11/B19/03 (Appendix E7).

The dried mycelium of *G. neojaponicum* at 2000 mg/kg concentration was given orally force-fed to Sprague Dawley rats. The rats were observed over 14 days for mortality and physical/ behavioural changes as described by OECD (1989). Two groups consisting of control and test group with ten Sprague Dawley rats were used for acute

oral toxicity testing. The test group (five rats) were administered a single dose of the dried mycelium of *G. neojaponicum* (2000 mg/kg) orally. The dried mycelium were solubilised in water and were administered orally to the test group while, the control group (five rats) were given a normal diet without sample. The volume administered was 10 mL/kg body weight. After the extract was administered, the animals were closely monitored for 14 days for any symptoms of toxicity or mortality.

CHAPTER 4.0 RESULTS

4.1 Screening of low cost materials as carbon and nitrogen sources by radial growth measurement

The low cost materials investigated for *G. neojaponicum* cultivation medium for carbon and nitrogen sources were molasses, brown sugar, corn steep liquor and spent yeast. Carbon and nitrogen content of the low cost materials were calculated as shown in Appendix A2-A6 and displayed in Table 4.1. The brown sugar and molasses contained 98.99% (w/v) and 85.77% (w/v) of carbon content were potential carbon sources for *G. neojaponicum* cultivation medium. The spent yeast and corn steep liquor contained 2.0% (w/v) and 2.9% (w/v) of nitrogen were potential sources of nitrogen in subsequent experiments for *G. neojaponicum* growth.

Table 4.1: Total carbon and nitrogen content in low cost materials

*Carbon and Nitrogen sources	Total Carbon % (w/v)	Total Nitrogen % (w/v)
Brown sugar (BS)	98.99	N.D (<0.2)
Spent Yeast (SY)	25.63	2.0
Molasses (MLS)	85.77	N.D (<0.2)
Corn Steep Liquor (CSL)	48.22	2.9

*** Analysis was done by Consolidated Laboratories Sdn Bhd.**

The low cost materials were investigated as cultivation medium for *G. neojaponicum* growth in plate agar studies. The four raw materials (molasses, brown sugar, corn steep liquor and spent yeast) used as carbon and nitrogen sources as determined from Section 3.2.1 were sources for cultivation medium formulations of *G. neojaponicum*. The combination of carbon and nitrogen sources and its concentrations were designed by two level full factorial statistical designs (FFD) for *G. neojaponicum*

growth as in Table 4.2. The range of nitrogen concentration was 0.02-0.1% (w/v) while, carbon concentration was 2 - 10% (w/v).

Table 4.2: Experimental design and responses for mycelial growth rate of *G. neojaponicum* using FFD

Run	Factor				Response
	Carbon sources	Nitrogen sources	Nitrogen Concentration	Carbon concentration	Mycelial growth rate
	X_1	X_2	X_4 % (w/v)	X_3 % (w/v)	(mm/day)
1	MLS	CSL	0.02 (-1)	2 (-1)	8.76±0.08
2	MLS	CSL	0.02 (-1)	10 (+1)	6.62±0.10
3	MLS	CSL	0.06 (0)	6 (0)	12.17±0.03
4	MLS	CSL	0.1 (+1)	2 (+1)	15.33±0.03
5	MLS	CSL	0.1 (+1)	10 (+1)	7.79±0.01
6	BS	CSL	0.02 (-1)	2 (-1)	18.3±0.02
7	BS	CSL	0.02 (-1)	10 (+1)	13.60±0.03
8	BS	CSL	0.06 (0)	6 (0)	15.93±0.05
9	BS	CSL	0.1 (+1)	2 (+1)	14.72±0.04
10	BS	CSL	0.1 (+1)	10 (+1)	17.83±0.03
11	BS	SY	0.02 (-1)	2 (-1)	17.33±0.08
12	BS	SY	0.02 (-1)	10 (+1)	13.96±0.02
13	BS	SY	0.06 (0)	6 (0)	20.86±0.02
14	BS	SY	0.1 (+1)	2 (+1)	16.90±0.06
15	BS	SY	0.1 (+1)	10 (+1)	18.03±0.03
16	MLS	SY	0.02 (-1)	2 (-1)	18.20±0.03
17	MLS	SY	0.02 (-1)	10 (+1)	11.27±0.02
18	MLS	SY	0.06 (0)	6 (0)	17.33±0.03
19	MLS	SY	0.1 (+1)	2 (+1)	17.63±0.02
20	MLS	SY	0.1 (+1)	10 (+1)	13.40±0.03

Abbreviation: MLS-Molasses, SY-Spent Yeast, BS-Brown Sugar, CSL-Corn Steep Liquor. Samples were run in triplicates with three samples at center points. Standard deviation was calculated from three independent samples.

From this results it showed that, the combination of brown sugar and spent yeast at 0.06% (w/v) and 6% (w/v), respectively, promoted the most effective mycelial growth of 20.86±0.02 mm/day. Figure 4.1 shows the growth of *G. neojaponicum* on different medium formulations in petri plates. Regression analysis of the growth rate was also conducted with generated R-squared having high predictability levels (92.98%) of variability for mycelial growth rate production as in Table 4.3.

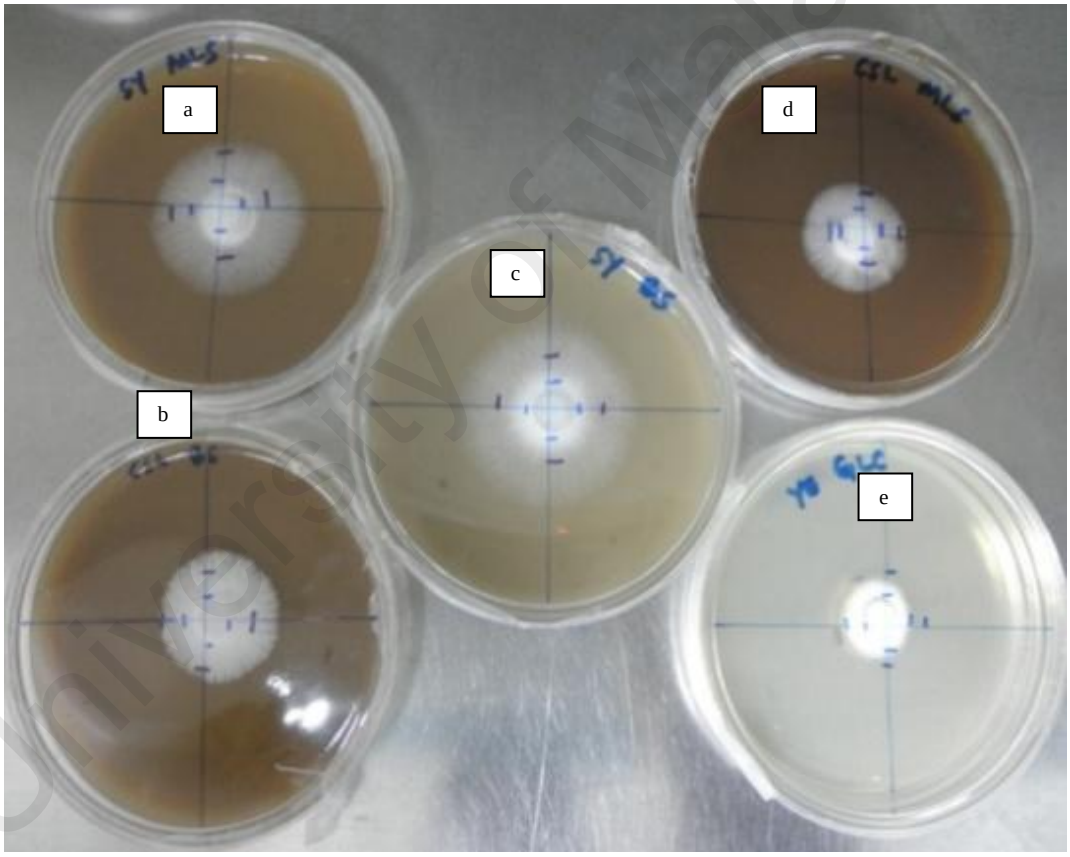


Figure 4.1: The radial growth of *G. neojaponicum* on different medium formulations. (a) Molasses and spent yeast (b) Brown sugar and corn steep liquor (c) Brown sugar and spent yeast (d) Molasses and corn steep liquor (e) Glucose and yeast extract.

Table 4.3: Analysis of variance (ANOVA) for mycelial growth rate of *G. neojaponicum* of Full Factorial Design (FFD)

Analysis of Variance (ANOVA) for GROWTH RATE (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	4	1.81783	1.76057	0.440142	62.73	0.000
2-Way Interactions	6	1.50497	1.50475	0.250791	35.74	0.000
Curvature	1	0.32450	0.32450	0.324496	46.24	0.000
Residual Error	42	0.29471	0.29471	0.007017		
Lack of Fit	3	0.26863	0.26863	0.089544	133.92	0.000
PureError	39	0.02608	0.02608	0.000669		
Total	58	4.20037				

S = 0.0837669 R-Sq = 92.98% R-Sq(adj) = 90.31%

Subsequently, analysis of variance of the growth rate showed F-value of 62.73 implying that the model is significant. Of all the carbon and nitrogen sources examined the combination of brown sugar and spent yeast, the most effectively promoted the mycelial growth with a 20.86±0.02 mm/day. Thus, the combination of brown sugar and spent yeast was selected for further optimization in cultivation medium of *G. neojaponicum* using response surface methodology (RSM).

4.2 Determination of suitable level of carbon and nitrogen concentrations for growth of *G. neojaponicum* using glucose -yeast extract medium in shake flask.

The suitable range of carbon and nitrogen concentration level for *G. neojaponicum* growth was determined using glucose as carbon and east extract as nitrogen in a shake flask fermentation. The experiment was conducted at a range of 0-1% (w/v) nitrogen and 0–20% (w/v) carbon using glucose and yeast extract, respectively as cultivation medium. The commercial glucose medium (Fluka, Germany) contains 99.98% (w/v) of total carbon while a total nitrogen of 11% (w/v) was found in yeast extract (Merck, Germany).

Table 4.4 shows *G. neojaponicum* mycelial dry weight (g/L) in the cultivation medium consisting of glucose and yeast extract as carbon and nitrogen sources, respectively. Table 4.4 also shows that 0.1% (w/v) was the most suitable nitrogen concentration needed for the growth of *G. neojaponicum.* Carbon at 10-20% (w/v) did not significantly increase the mycelial dry weight formation of *G. neojaponicum*. The highest amount of mycelial dry weight was 30.1±0.02 g/L at 0.1% (w/v) nitrogen and 10% (w/v) carbon of medium composition.

Table 4.4: Mycelial dry weight of glucose-yeast extract medium in shake flask incubated at 26 ^{o}C, 160 rpm for 6 days.

YE (%)	GLC (%)	N	Mycelial dry weight (g/L) (Mean ± SD)
0	10	3	23.8 ± 0.19^{ab}
0	15	3	27.8 ± 0.14^{ab}
0	20	3	28.6 ± 0.14^{ab}
0.1	0	3	23.0 ± 0.06^{ab}
0.1	10	3	30.1 ± 0.02^{a}
0.1	15	3	29.4 ± 0.11^{a}
0.1	20	3	28.9 ± 0.12^{a}
0.5	0	3	24.1 ± 0.05^{ab}
0.5	10	3	29.2 ± 0.06^{ab}
0.5	15	3	28.2 ± 0.01^{ab}
0.5	20	3	26.0 ± 0.06^{ab}
1	0	3	26.3 ± 0.02^{ab}
1	10	3	28.2 ± 0.04^{ab}
1	15	3	27.1 ± 0.09^{ab}
1	20	3	27.7 ± 0.10^{ab}

Abbreviation: GLC: Glucose and YE: Yeast extract. For each concentration of YE and GLC, same letter within column indicate significant differences between observed mycelial dry weight

This result shows that carbon and nitrogen up to 0.1% (w/v) and 10% (w/v) respectvely were suitable for the growth of *G. neojaponicum* in shake flask fermentation. Therefore, the ranges of 2–10% (w/v) of carbon and 0.02-0.1% (w/v) nitrogen were subsequently used in the formulation of brown sugar-spent yeast medium.

4.3 Selection of spent yeast and brown sugar concentrations using RSM

The brown sugar and spent yeast were the C/N sources used for optimal condition in developing medium formulation of *G. neojaponicum* production. The ranges of carbon and nitrogen contents from spent yeast and brown sugar were adjusted respectively at 2-10% (w/v) and 0.02-0.1% (w/v) based on the preliminary results in Section 4.2 (page 61). A total of 39 trial runs were conducted using a Central Composite Design (CCD) to investigate the two factors (brown sugar and spent yeast concentrations) with five centre points and three replicates. The variables of each constituent at level -1, 0 and +1 are presented in Table 4.5.

Table 4.5: Experimental design and response of spent yeast and brown sugar on *G. neojaponicum* mycelial dry weight and C/N ratio using CCD incubated at 26°C, 160 rpm for 6 days in shake flask culture.

Run Order	Pt Type	Factors		Response	
		Brown Sugar (% w/v)	Spent Yeast (% w/v)	C/N Ratio	Mycelial dry weight (g/L)
1	-1	10	0.06	3.11	21.23
2	-1	6	0.02	5.80	9.15
3	-1	10	0.06	3.11	21.82
4	0	6	0.06	1.74	19.97
5	0	6	0.06	1.74	18.57
6	0	6	0.06	1.74	18.42
7	1	10	0.1	1.76	18.47
8	1	2	0.02	1.76	9.28
9	0	6	0.06	1.74	19.72
10	0	6	0.06	1.74	19.57
11	1	2	0.1	0.15	0.98
12	1	10	0.1	1.76	1.88
13	0	6	0.06	1.74	1.92
14	1	2	0.1	0.15	0.97
15	1	2	0.02	1.76	0.75
16	0	6	0.06	1.74	1.98
17	0	6	0.06	1.74	1.83
18	-1	2	0.06	0.41	1.87
19	1	2	0.1	0.15	0.97
20	0	6	0.06	1.74	1.64
21	1	10	0.02	9.84	1.26
22	0	6	0.06	1.74	1.84
23	-1	2	0.06	0.41	1.74
24	0	6	0.06	1.74	1.93
25	0	6	0.06	1.74	1.81
26	-1	10	0.06	3.11	2.08
27	-1	6	0.02	5.80	1.94
28	0	6	0.06	1.74	1.84
29	0	6	0.06	1.74	1.98
30	0	6	0.06	1.74	1.88
31	1	10	0.02	9.84	1.36
32	1	10	0.1	1.76	1.87
33	1	10	0.02	9.84	1.37
34	1	2	0.02	1.76	0.73
35	-1	6	0.1	0.95	1.60
36	-1	6	0.02	5.80	0.91
37	-1	2	0.06	0.41	0.99
38	-1	6	0.1	0.95	1.69
39	-1	6	0.1	0.95	1.69

The response surface model was studied for mycelial dry weight (g/L) and C/N ratio. Analysis of variance (ANOVA) for mycelial dry weight is presented in Table 4.6 (a). The model was indicated as fit and can sufficiently explain the variation seen in mycelial dry weight with the design levels of brown sugar and spent yeast as explained in Table 4.6 (a) and (b).

Table 4.6 (a) and (b): Analysis of variance for mycelial dry weight and C/N ratio obtained from CCD

4.7 (a) Analysis of Variance for Mycelial dry weight (g/L)

Source	DF	Seq SS	Adj SS	AdjMS	F	P
Regression	5	5.881825.88182	1.17636	27.93	0.000	
Linear	2	2.650922.65092	1.32546	31.47	0.000	
Square	2	3.131893.13189	1.5659	537.18	0.000	
Interaction	1	0.099010.09901	0.0990	12.35	0.135	
Residual Error	35	1.630121.63012	0.04657			
Lack-of-Fit	5	0.309280.30928	0.06186	1.40	0.251	
Pure Error	30	1.320841.32084	0.04403			
Total	38	7.27153				

S = 0.2052 R-Sq = 80.9% R-Sq(adj) = 78.0%

4.7 (b) Analysis of Variance for C/N

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	3	208.242	208.242	69.414	67.52	0.000
Linear	2	182.474	182.474	91.237	88.75	0.000
Brown Sugar (% w/v)	1	76.768	76.768	76.768	74.67	0.000
Spent Yeast (% w/v)	1	105.706	105.706	105.706	102.82	0.000
Square	1	25.768	25.768	25.768	25.07	0.000
Spent Yeast (% w/v)* Spent Yeast (% w/v)		125.768	25.768	25.768	25.07	0.000
Residual Error	35	35.981	35.981	1.028		
Lack-of-Fit	5	35.981	35.981	7.196	0.000	0.000
Pure Error	30	0.000	0.000	0.000		
Total	38	244.224				

S = 1.01392 R-Sq = 85.27% R-Sq(adj) = 84.00%

DF: Degree of freedom; Seq SS: sequential sum of squares; Adj SS: adjusted sum of squares; Adj MS: adjusted mean square

The R-square indicated 80.89% of variability for mycelial dry weight. F-test for regression was significant at level of 5% (P<0.05). The lack-of-fit (0.251) is not significant at level of 5% (P>0.05); this indicated that the experimental data obtained

fitted well with the model. The analysis of variance (ANOVA) for carbon to nitrogen (C/N) ratio is presented in Table 4.7 (b). The obtained R-square value indicated that C/N ratio variability of 85.27% was observed in this experiment. The lack-of-fit (0.000) was not significant at level of 5% (P>0.05); this indicated that the data from the experiment suited the model. F-test for regression was significant at level of 5% (P<0.05) indicating that the model is suitable and can reasonably explain the variation noted in C/N ratio with the design levels of brown sugar and spent yeast. The calculation formula of the amount of carbon and nitrogen as well as carbon and nitrogen ratio are shown in Appendix A2. The results of C/N ratio and mycelial dry weight is shown in Table 4.7.

Table 4.7 (a) and (b): Estimated regression coefficients for mycelial dry weight and C/N ratio using CCD

4.8 (a)Estimated Regression Coefficients for Mycelial dry weight (g/L)

Term	Coef	SECoef	T	P
Constant	-0.1024	0.04920	38.770	0.000
Brown Sugar	0.1478	0.04837	6.941	0.000
Spent Yeast	39.1697	0.04837	3.844	0.001
Brown Sugar*Brown Sugar	-0.0081	0.07129	-1.832	0.076
Spent Yeast*Spent Yeast	-316.063	0.07129	-7.093	0.000
Brown Sugar*Spent Yeast	0.5677	0.05924	1.533	0.135

S = 0.2052 R-Sq = 80.9% R-Sq(adj) = 78.0%

4.8 (b)Estimated Regression Coefficients for C/N

Term	Coef	SE Coef	T	P
Constant	5.9520	70.2213	7.892	0.000
Brown Sugar (% w/v)	0.5162	922.065	0.2390	8.641	0.000
Spent Yeast (% w/v)	-182.873	-2.423	0.2390	-10.140	0.000
Spent Yeast (% w/v)* Spent Yeast (% w/v)	1019.0	81.631	0.3257	5.007	0.000

S = 1.01392 R-Sq = 85.27% R-Sq(adj) = 84.00%

Coef: Coefficient regression; SE Coef: Standard Error Coefficient; T: T-Test value; P: Probability value

The regression analysis in Table 4.7 (a) shows that spent yeast and brown sugar had positive linear effects on dry weight at 5% level (P<0.05). Among the two factors tested, spent yeast had high impact on the mycelial dry weight, given the high linear coefficient of 39.17 as shown in Table 4.7 (a). The probability of C/N ratio can be observed in Table 4.7 (b). The linear and square interaction were significant at 5% level (P<0.05). Regression analysis was conducted to fit the response function (mycelial dry weight). Models were generated using linear square type as mentioned in equations [1] and [2]. Reduced models were generated for the significant variables. The polynomial equation showed positive quadratic effect on mycelial dry weight, which indicate that mycelial dry weight increased as the level of spent yeast and brown sugar was increased. This phenomenon might be due to the fact that the cultivation medium was closely associated with mycelial dry weight. The negative second order polynomial equation was found to provide explanation about C/N ratio of *G. neojaponicum*. The negative quadratic effect indicated that the C/N ratio decreased as the amount of combination of spent yeast and brown sugar was increased.

Hence, the term was omitted from the polynomial equation [1] utilised for the model which represents mycelial dry weight g/L (Y) as a function of brown sugar (X1) and spent yeast (X2) concentrations. The C/N ratio in equation [2] shows opposite effect on the mycelial dry weight in equation [1]. The model of C/N ratio is expressed in equation [2] which represents C/N ratio (R) as function of brown sugar (X1) and spent yeast (X2) concentrations.

$$\mathbf{Y= -0.1024+0.147(X1) + 39.1697(X2) - 316.063(X2)^2} \quad [1]$$

$$\mathbf{R= 5.95207+0.5162(X1) -182.873(X2) +1019(X1)^2} \quad [2]$$

The surface and contour plots in Figure 4.2 show that the maximum mycelial dry weight at 21.5 g/L can be achieved with 10% (w/v) carbon in brown sugar and 0.075% (w/v) nitrogen in spent yeast; the C/N ratio is at 2.9 using spent yeast and brown sugar as medium for *G. neojaponicum.*

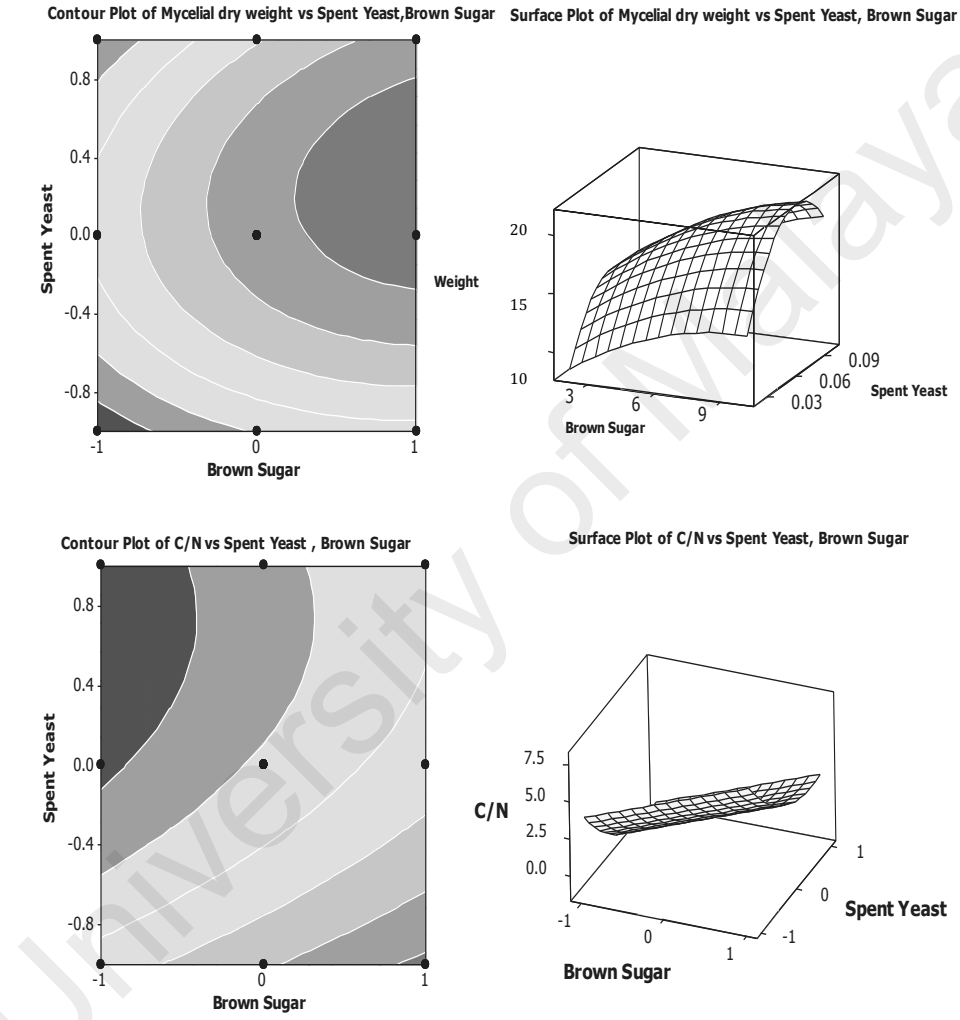


Figure 4.2: Three-dimensional plots and corresponding contour plots of the combination of spent yeast and brown sugar in shake flask by *G. neojaponicum*.

4.3.1 Model verification

To verify the model prediction of the response, all experiments were performed in five replicates under the optimal growth condition as predicted by the model in shake flask culture. Table 4.8 shows the comparison of predicted and experimental values for the response variables. The model showed good prediction for both variables (mycelial dry weight and C/N ratio). The good correlation between predicted and observed values confirmed that the response model was suitable for the intended optimization.

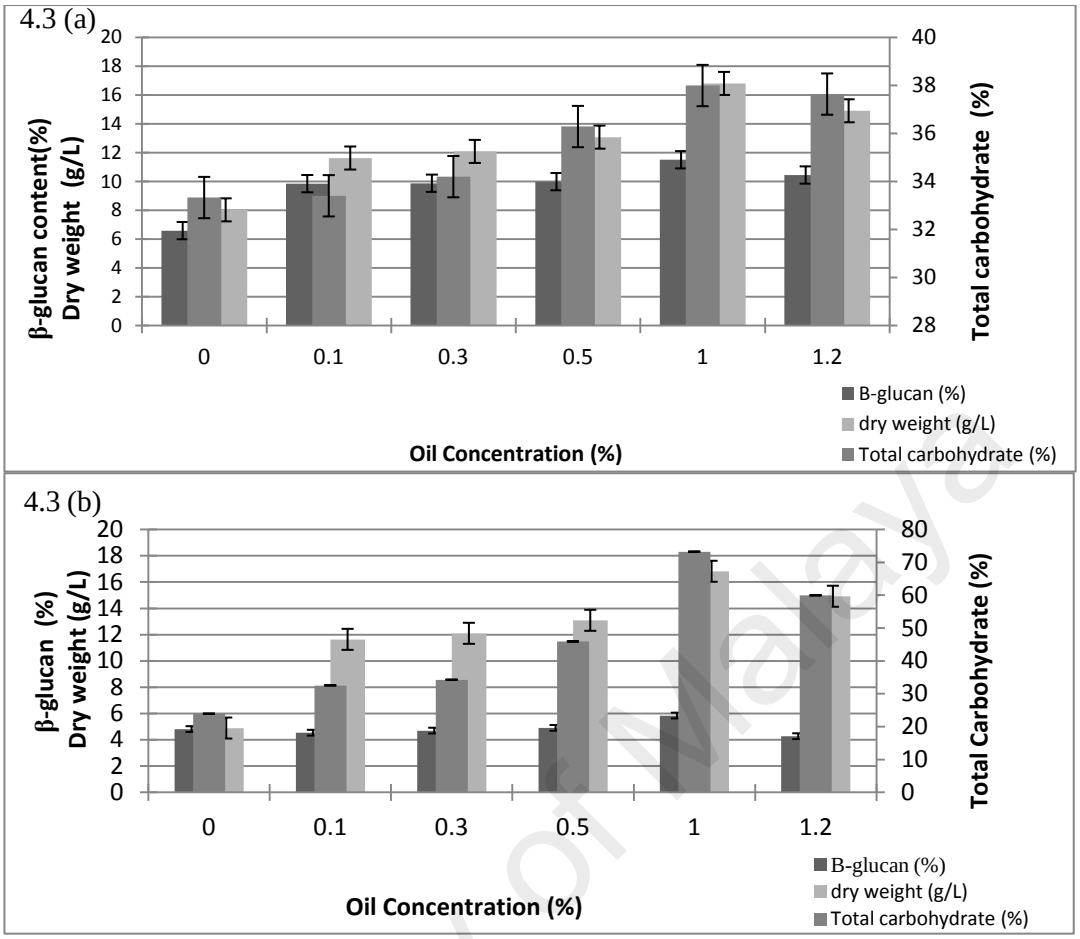
Table 4.8: Comparison of predicted and experimental values for the response variables.

Factors/ Response variables	Predicted value	Observed value
Spent yeast	0.06% (w/v)	0.08% (w/v)
Brown sugar	10% (w/v)	10% (w/v)
Mycelial dry weight	20.23±0.02 g/L	21.18±0.04 g/L.
C/N ratio	1.74±0.00	2.9±0.01

4.4 Effect of brown sugar-spent yeast medium supplemented with surfactants for production of β-glucan and carbohydrate by *G. neojaponicum*

The effect of brown sugar-spent yeast medium supplemented with surfactant was conducted to increase the total β-glucan and carbohydrate production by *G. neojaponicum* in shake flask culture. Polyoxyethelene sorbitan mono-oleate or Tween 80 (Sigma, USA) or vegetable oil (Seri Murni, Malaysia) was added into the culture medium in this study. The experiment was conducted using vegetable oil (0 – 1.2% (w/v)) and Tween 80 (0 -1% (w/v)) in the cultivation medium. The medium was

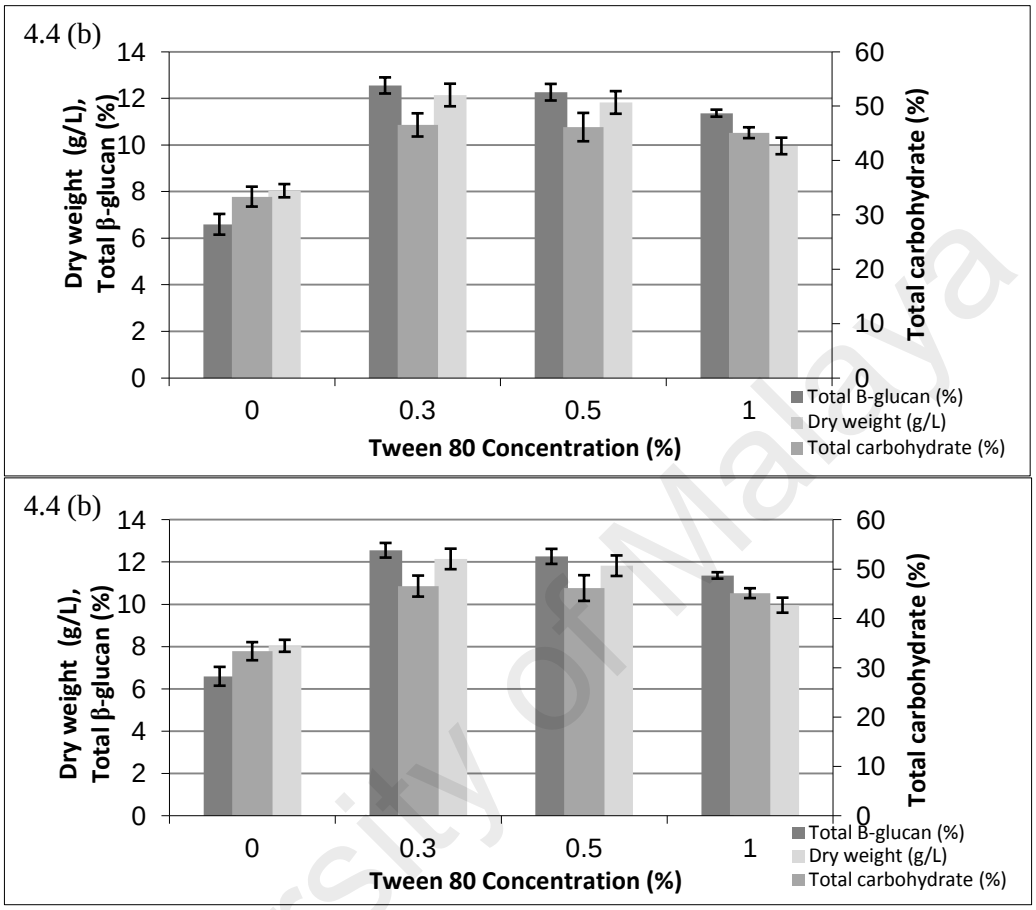
optimised from the previous experiment (Section 4.1 page 58 and section 4.3 page 63). The medium consisted of 37.25 g/L of spent yeast, 91.3 g/L brown sugar, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4 and 0.5 g/L $MgSO_4.7H_20$. The physical parameters were fixed (at 26 ^oC, 160 rpm, pH 6, 6 days of duration time) in this experiment. The effectiveness of surfactant sources in the cultivation medium is to promote mycelial dry weight, β-glucan and total carbohydrate production. The highest dry weight was obtained in dried broth with 9.543 g/L and dried mycelium with 16.81 g/L extracted from 1.0% (v/v) oil concentration. The dried mycelium recorded the highest amount of β-glucan and total carbohydrate production at 11.51% (v/v) and 3.71% (v/v) respectively, at the same concentration of oil [Figure 4.3 (a)]. The highest yields of β-glucan and total carbohydrate production on dried broth, 4.33% (v/v) and 70.68% (v/v) respectively, were detected in the medium containing 1.0% (v/v) vegetable oil [Figure 4.3 (b)].



Figures 4.3 (a) and (b): Effect of vegetable oil concentration (0-1.2% (v/v)) on β-glucan, dry weight and total carbohydrate yield in *G. neojaponicum* dried mycelium (a) and dried broth (b).

Therefore, 1% (v/v) of oil was considered as an optimum condition for mycelium growth and carbohydrate production from *G. neojaponicum.* As higher concentrations of oil were added to the medium, it produced higher viscosity and caused formation of emulsion in the medium. Figures 4.4 (a) and (b) shows the effects of Tween 80 on dried mycelium and dried broth of *G. neojaponicum.* Figure 4.4 (a) shows that the highest mycelial dry weight and β-glucan yield of dried broth was when 0.3% (v/v) Tween 80 was added to the culture medium. The highest mycelial dry weight, β-

glucan and total carbohydrate were also obtained at 0.3% (v/v) Tween 80 in dried mycelium with 12.14 g/L, 12.55% and 46.52%, respectively [Table 4.4 (b)].



Figures 4.4 (a) and (b): Effect of Tween 80 concentration (0-1.0% (v/v)) on β-glucan, dry weight and total carbohydrate yield of *G. neojaponicum* dried mycelium (a) and dried broth (b).

From this finding, the use of Tween 80 in culture medium of *G. neojaponicum* showed enhancement of mycelial dry weight and β-glucan production as well as total carbohydrate in shake flask fermentation. Therefore, Tween 80 was added for further optimization of spent yeast-brown sugar cultivation medium using a statistical model via RSM in shake flask, and in a STR reactor by *G. neojaponicum.*

4.4.1 Observation of pellet size formation

The mycelial dispersion of *G. neojaponicum* was observed based on pellet size formation in shake flask fermentation. The homogenous pellet size was important for sampling out the mycelial dry weight accurately from a bioreactor. The use of Tween 80 in culture medium showed better dispersion of mycelial pellets formed and enhanced mycelial dry weight in shake flasks. The pellets size of *G. neojaponicum* was reduced and dispersed in the fermentation medium with the addition of Tween 80. Figures 4.5 (a) to (e) shows the changes in pellet morphology at different Tween 80 concentrations ranging from 0 to 1.0% (v/v). The pellets formed were the smallest size and evenly distributed at 0.5% (v/v) of Tween 80. The addition of optimal concentration of Tween 80 had positive effect on the enhancement of mycelial growth due to better dispersion and homogeneity of mycelial dry weight. The mycelium size changes in the medium containing oil as shown in Figures 4.5 (f) to (j). As higher concentrations of oil were added to the medium, it produced higher viscosity and caused formation of emulsion in the medium. The amount of polysaccharide extract was reduced due to difficulties of separation of mycelium in this emulsion medium. Therefore, Tween 80 was selected as the best source of surfactant to produce well dispersed small mycelial pellet in shake flask culture.

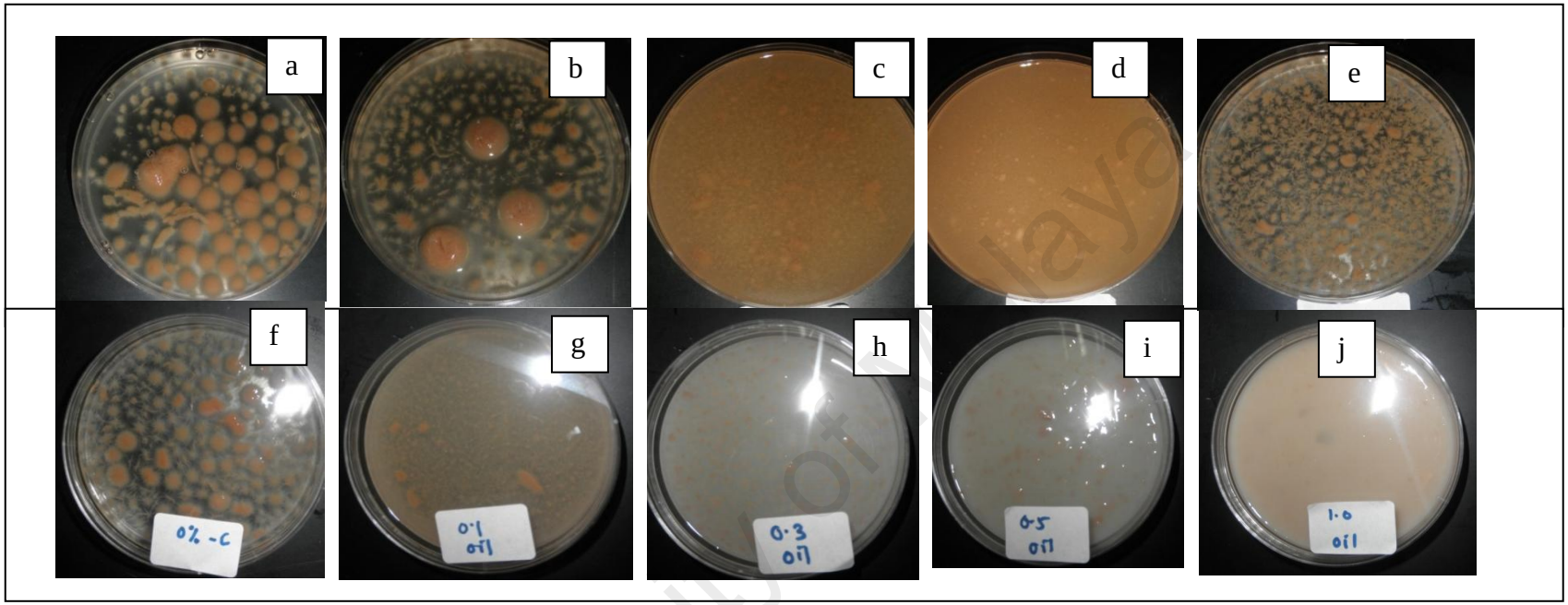


Figure 4.5: The changes in pellet morphology at different concentrations of Tween 80 (a) 0% (b) 0.1% (c) 0.3% (d) 0.5% (e) 1.0% and different concentrations of oil (f) 0% (g) 0.1% (h) 0.3% (i) 0.5% (j) 1.0% in medium containing 37.3 g/L of spent yeast, 91.3 g/L brown sugar, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4 and 0.5 g/L $MgSO_4.7H_20$ at pH 6. The liquid culture was incubated at 26 ^oC and 160 rpm for six days. The photographs of liquid cultures were captured in petri dishes for better clarity of mycelial dispersion.

4.5 Optimisation of Tween 80 concentration and temperature in shake flask by RSM

The selection of the optimum concentrations of Tween 80 and temperature on brown sugar-spent yeast medium was carried out based on mycelial dry weight and β-glucan content of *G. neojaponicum* production. The response surface statistical design was employed to optimise the concentrations of Tween 80 and temperature on brown sugar-spent yeast medium simultaneously in shake flask fermentation. The levels of factors used in this central composite design were 0.1% to 0.5% (w/v) (Table 4.9) Tween 80 at temperature of 22 ^{o}C to 30 ^{o}C.

Table 4.9: Levels of factors used for Tween 80 and temperature in the CCD

Factor			Level		
			-1	**0**	**+1**
X_1 [a]	Tween 80 concentration	% (w/v)	0.1	0.3	0.5
X_2 [b]	Temperature	^{o}C	22	26	30

The models were generated using the linear square type and were mentioned in the model equations [3] and [4]. Reduced models were generated for the significant variables. Regression analysis was conducted to fit the response function (mycelial dry weight) to data from the experiment. Based on the results obtained, the model was expressed by equation [3], which symbolised mycelial dry weight yield (*Y*) as a function of Tween 80 concentration (X_1) and temperature (X_2):

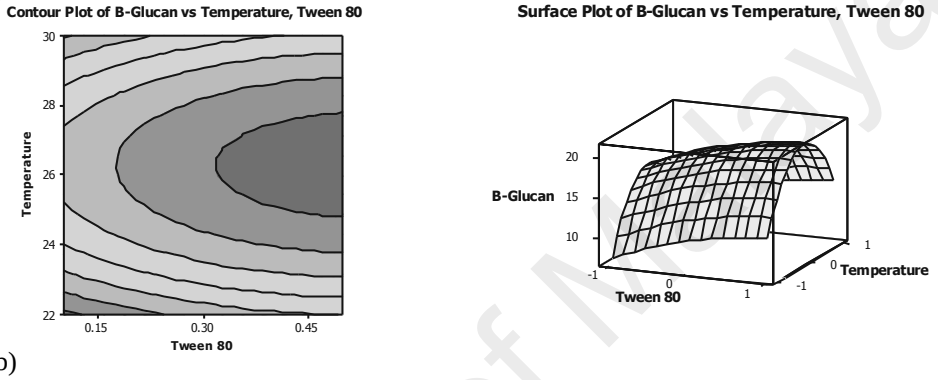
$$\boldsymbol{Y = -69.7901 + 12.8459(X_1) + 6.76137(X_2) - 0.129811(X_1X_2)} \qquad \textbf{[3]}$$

At 95% confidence level, the regression was statistically significant ($P<0.05$) with high regression coefficient (R^2=0.766) as in Tables 4.10 (a) and (b). It shows that

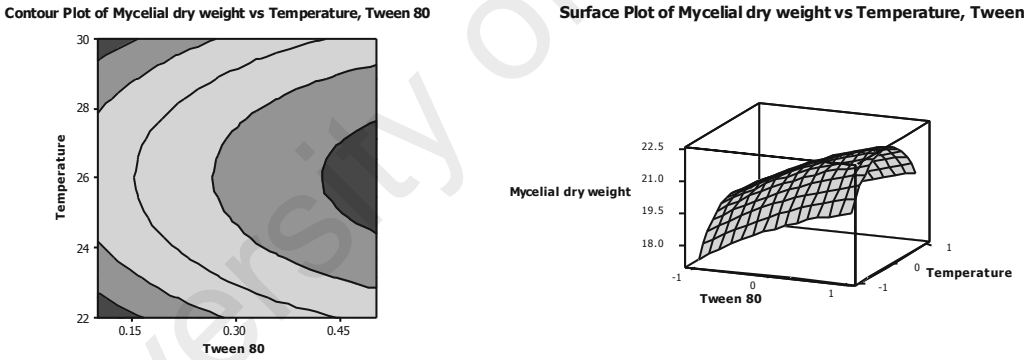
the maximum mycelial dry weight at 22.21 g/L and β-glucan content 20.56% (w/v) could be achieved at 0.4692% (w/v) Tween 80 and temperature of 26 ^{o}C. Figures 4.6 (a) and (b) show the response surface and contour plots derived from equation [3] and [4].

$$\boldsymbol{P=-314.623 +27.580X1+24.981X2-28.517(X1)^{1}-0.475(X2)^{2}} \quad \textbf{[4]}$$

4.6 (a)



4.6 (b)



Figures 4.6: Three-dimensional plots and corresponding contour plots of the effect of (a) Tween 80 and (b) temperature brown sugar-spent yeast medium of *G. neojaponicum* in shake flask culture

Tables 4.10: Estimated coefficients for (a) mycelial dry weight and (b) β-glucan using CCD.

4.10 (a) Estimated Regression Coefficients for Mycelial dry weight (g/L)

Term	Coef	SECoef	T	P
Constant	-69.7901	12.6922	-5.499	0.000
Tween 80	12.8459	4.7158	2.724	0.010
Temperature	6.7614	0.9979	6.775	0.000
Tween 80*Tween 80	-9.3791	7.6660	-1.223	0.230
Temperature*Temperature	-0.1298	0.0192	-6.773	0.000

S = 0.8827 R-Sq = 76.6% R-Sq(adj) = 73.8%

4.10 (b) Estimated Regression Coefficients for β-glucan (% w/v)

Term	Coef	SECoef	T	P
Constant	-314.623	48.8758	-6.437	0.000
Tween 80	27.580	18.1597	1.519	0.138
Temperature	24.981	3.8429	6.501	0.000
Tween 80*Tween 80	-28.517	29.5206	-0.966	0.341
Temperature*Temperature	-0.475	0.0738	-6.435	0.000

S = 3.399 R-Sq = 65.3% R-Sq(adj) = 61.2%

4.5.1 Model verification

To verify the model prediction of the response, five independent experiments were performed under the optimal growth condition as predicted by the model in shake flask fermentation. At observed values of 0.46% (v/v) Tween 80 at temperature of 26 ^{o}C, the mycelial dry weight and β-glucan content were at 23.67 g/L and 21.12% (w/v), respectively. These values were very close to the predicted values of dry weight at 22.21 g/L and β-glucan content at 20.56% (w/v). Hence, from these findings, the optimum concentration of surfactant of Tween 80 was 0.46% (v/v) at temperature of 26 ^{o}C; it added to the optimal brown sugar-spent yeast medium for subsequent experiments of *G. neojaponicum* cultivation in a STR reactor.

4.6 Optimization of physical parameters for *G. neojaponicum* growth, β-glucan and total carbohydrate production grown in brown sugar-spent yeast medium for in 2-L STR using RSM

The determination of physical parameters was conducted on brown sugar-spent yeast medium for *G. neojaponicum* cultivation in a 2-L STR reactor. The fermentation medium used was determined in the previous section 4.5 (page 75) which comprised of 37.25 g/L brewery spent yeast, 91.3 g/L brown sugar, 4.6 mL/L Tween 80, 0.5 g/L $MgSO_4 \cdot 7H_20$, 0.5 g/L KH_2PO_4 and 0.5 g/L K_2HPO_4. A total of 9 experiments were conducted using central composite design to obtain the optimal physical conditions of aeration and temperature for cultivation of *G. neojaponicum* in 2-L STR reactor. The variables of each constituent at levels -1, 0 and +1 are given in Table 4.11.

Table 4.11: Levels of factors used in the CCD for aeration and temperature

Factor			Level		
			-1	**0**	**+1**
X_1	Aeration	vvm	0.1	0.15	0.2
X_2	Temperature	^oC	22	26	30

In this study, temperature and aeration rate were selected as the most crucial physical factors for scale-up process for *G. neojaponicum* as suggested by Felse & Panda (2000). The other physical parameters were kept constant with pH 6 at agitation speed 160 rpm. The cultivation of *G. neojaponicum* in 2-L STR reactor was carried out for 6 days of cultivation. The variables were carried out at values ranging from 0.1 to 0.2 vvm of aeration and 22-30 ^oC of temperature. In this reactor, two factors of temperature and aeration rates consisted of 9 runs with 1 centre point. Each run was

carried out in duplicates. Table 4.12 shows the experimental design and responses of physical parameters for *G. neojaponicum*.

Table 4.12: Experimental design and responses for physical parameters of *G. neojaponicum* in 2-L STR reactor using RSM

Run Order	Pt Type	Temp (^oC)	Aeration (vvm)	Mycelial dry weight (g/L)	β-glucan (%)	Total carbohydrate (%)
1	0	25.5	1.25	27.21±0.16	21.34±0.01	76.90±1.81
2	-1	29	1.25	25.58±0.15	19.23±0.39	73.98±0.85
3	-1	22	1.25	16.43±0.01	18.67±0.09	64.89±0.86
4	1	22	0.5	13.21±0.02	15.92 ±0.41	58.97±0.02
5	1	29	0.5	11.11±0.11	11.26±0.11	60.26±0.09
6	1	29	2	16.78±0.11	16.11±0.09	60.26±0.03
7	-1	25.5	2	18.32±0.17	19.79±0.24	70.26±0.12
8	-1	25.5	0.5	11.67±0.13	12.56±0.04	50.25±0.15
9	1	22	2	11.82±0.05	8.90±0.39	32.9±0.45

A three-dimensional response surface plot was drawn with the vertical axis representing Regression analysis. It was conducted to fit the response function (mycelial dry weight) to the experimental data. Based on the results derived, the model was expressed using equation [5], which symbolised mycelial dry weight (Y_{MB}) as function of aeration (X1) and agitation (X2): The models were generated using the linear square type and were mentioned in the model equation [5]. Figure 4.7 shows the response surface and contour plots obtained from equation [5]. It shows that the maximum mycelial dry weight at 25.32 g/L could be achieved at a temperature 26.72 ^oC and aeration of 1.33 vvm. Figure 4.7 (a) shows the response surface and contour plot of mycelial dry weight of *G. neojaponicum* in a 2-L stirred tank reactor.

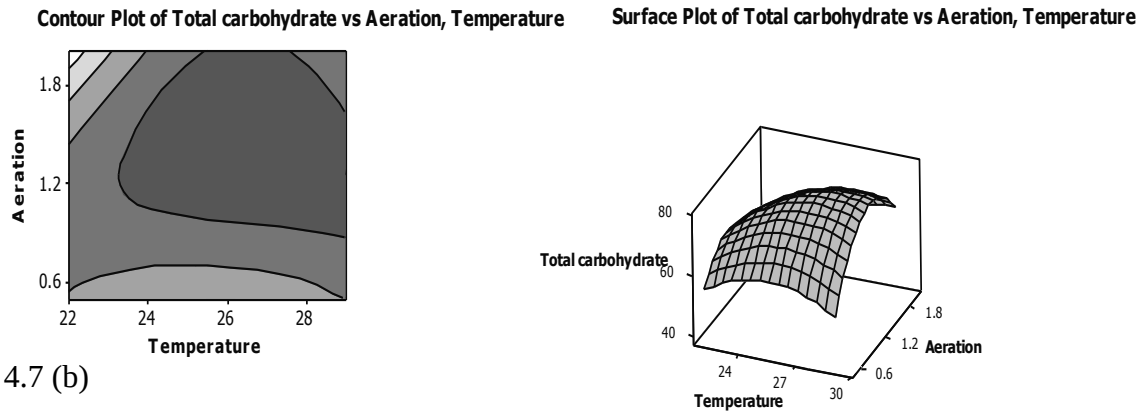
The maximum level of mycelial dry weight was obtained at 25.31 g/L, at temperature of 26.72 ^{o}C and aeration 1.33 vvm. The polynomial equation for the mycelial dry weight of *G. neojaponicum* in a 2-L stirred tank reactor was regressed as in equation [5] where, Y_{MB} is the mycelial dry weight and X1 is the code for temperature. X2 is the code for aeration.

$$\boldsymbol{Y_{MB}=-181.603+14.104X1+28.801X2-0.280(X1)^2-16.680(X2)^2} \quad [5]$$

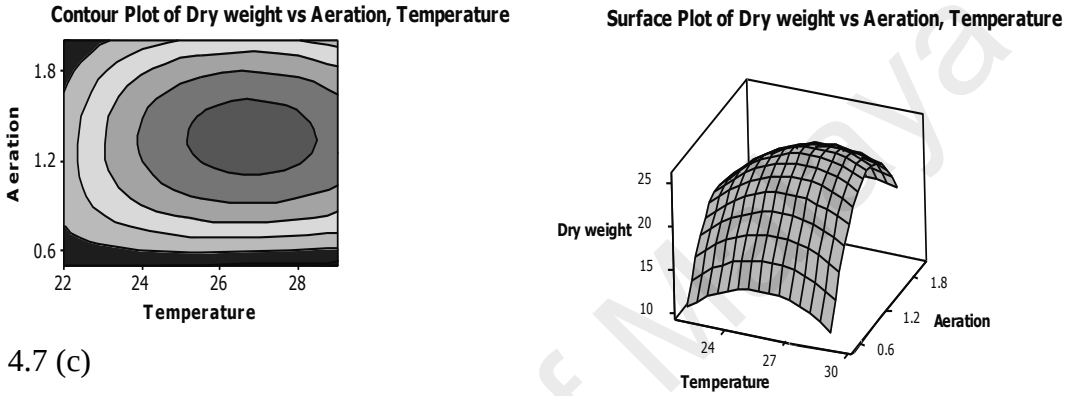
The polynomial model with linear-square term of mycelial dry weight had significant effect on aeration and temperature as shown in Table 4.13 (a). At 95% confidence level, the regression was statistically significant (P<0.05) with high regression coefficient (R^2=0.8824), reflecting a good fit between the observed value and predicted value. In the second surface plot shown in Figure 4.7 (b), regression analysis was conducted to suit the response function (β-glucan) to the experimental data. From the results obtained, the model was expressed using equation [6], which symbolised production of β-glucan (P_{MB}) as a function of aeration (X_1) and temperature (X_2). The third-dimensional plot in Figure 4.7 (c) of the polynomial model for total carbohydrate was regressed by equation [7] with reduction in terms under consideration and was expressed by, P_{PS} is the production of carbohydrate; X1 is the code for temperature.At 95% confidence level, the regression was statistically significant (P<0.05) with high regression coefficient (R^2=0.865). It showed that the maximum mycelial dry weight at 25.31 g/L could be achieved at temperature 26.72 ^{o}C and aeration 1.33 vvm.

The models were enhanced to generate significant variables. The contour plot in Figure 4.7 (c) shows the highest value of total carbohydrate at 73.56 g/L which could be obtained at a temperature of 26.7 ^{o}C and aeration of 1.33 vvm.

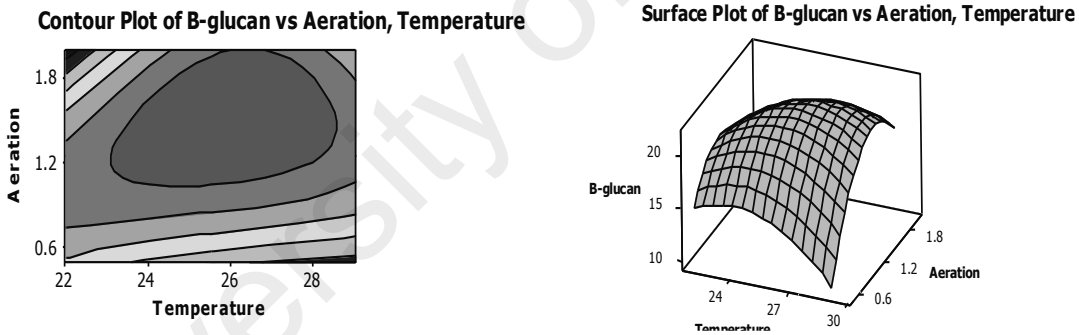
4.7 (a)



4.7 (b)



4.7 (c)



Figures 4.7: Three-dimensional plots and corresponding contour plots for the effect of aeration and temperature on *G. neojaponicum* growth in 2-L stirred tank reactor. (a) mycelial dry weight (b) β-glucan content and (c) total carbohydrate

The models were generated using linear square type and were mentioned in the model equation [7]. The value of X1 is positive linear coefficient, which means an increase of temperature implies an increase of carbohydrate production.

$$P_{BG} = -122.339 + 11.198X_1 - 0.244(X_1)2 \quad [6]$$

$$P_{PS=} -122.339 + 11.198X1 - 0.244(X1)^2 \quad [7]$$

The aeration time was insignificant (0.863) as shown in the regression model values in Table 4.13. The analysis of variance of the regression model was significant as shown by the probability value (P<0.05). The R^2 value of the model was 0.7284, reflecting the observed and predicted values being suitable for each other.

Table 4.13: Estimated coefficient interactions of (a) mycelial dry weight, (b) β-glucan content and (c) total carbohydrate of *G. neojaponicum* from CCD

4.13(a)Estimated Regression Coefficients for mycelial dry weight (g/L)

Term	Coef	SECoef	T	P
Constant	-181.603	66.6022	-2.727	0.020
Temperature	14.104	5.2202	2.702	0.021
Aeration	28.801	10.2623	2.806	0.017
Temperature*Temperature	-0.280	0.1019	-2.743	0.019
Aeration*Aeration	-16.680	2.2201	-7.513	0.000
Temperature*Aeration	0.604	0.3364	1.796	0.100

R-Sq = 88.24

4.13(b) Estimated Regression Coefficients for β-glucan (%)

Term	Coef	SECoef	T	P
Constant	-122.339	54.4873	-2.245	0.046
Temperature	11.198	4.2706	2.622	0.024
Aeration	-2.409	8.3956	-0.287	0.779
Temperature*Temperature	-0.244	0.0834	-2.928	0.014
Aeration*Aeration	-10.125	1.8163	-5.575	0.000
Temperature*Aeration	1.130	0.2752	4.107	0.002

R-Sq = 84.38%

4.13 (c) Estimated Regression Coefficients for Total carbohydrate (g/L)

Term	Coef	SECoef	T	P
Constant	-308.294	217.767	-1.416	0.185
Temperature	28.099	17.068	1.646	0.128
Aeration	5.907	33.554	0.176	0.863
Temperature*Temperature	-0.575	0.333	-1.725	0.112
Aeration*Aeration	-27.554	7.259	-3.796	0.003
Temperature*Aeration	2.422	1.100	2.202	0.050

R-Sq = 72.84%

4.6.1 Model verification

To verify the model prediction of the response, three independent experiments were performed under the optimal growth condition as predicted by the model in STR reactor. Model verification was conducted under optimised condition with temperature 26.71 ^{o}C and aeration 1.33 vvm. The observed experimental values at mycelial dry weight (25.31 g/L), total carbohydrate (73.56 g/L) and β-glucan content (21.34 % w/v) were very close to the predicted values with mycelial dry weight (26.57 g/L), total carbohydrate (75.56 g/L) and β-glucan content (21.57% w/v). Hence, from these findings, the physical parameters of temperature 27 ^{o}C and 1.33 vvm aeration were used for subsequent experiments on brown sugar-spent yeast medium of *G. neojaponicum* cultivation in a STR reactor.

4.7 Growth profiles of *G. neojaponicum* using optimum medium formulations and physical parameters in 2-L STR

This profiling of *G. neojaponicum* was conducted using optimum medium formulation and physical conditions in STR reactor as determined from previous sections 4.6 page 79. Fermentation medium and environmental conditions comprised 37.25 g/L brewery spent yeast, 91.3 g/L brown sugar, 4.6 ml/L Tween 80, 0.5 g/L $MgSO_4 \cdot 7H_20$, 0.5 g/L KH_2PO_4 and 0.5 g/L K_2HPO_4 at agitation speed of 160 rpm, temperature of 27 ^{o}C and aeration of 1.33 vvm. The medium was set at initial pH 6 and 10 % (v/v) inoculum size. The cultivation of *G. neojaponicum* in 2-L stirred tank

reactor was carried out till day 6 of cultivation. The time profiles of dry weight, reducing sugar and pH are shown in Figure 4.8.

The maximum mycelial dry weight was obtained at 25.32 g/L on day 4 of cultivation. At this stage, the values of pH and yields in the culture broth slowly decreased from pH 5.95 to 4.6 and 7.30 to 5.21 g/L, respectively. pH indicating measure of acidity concentration of hydrogen ions H+ in solution (Tim, 2002). Based on the growth curve, it can be seen that the logarithmic phase requires plenty of nutrition and sufficient oxygen supply during day 2 to day 4 of the fermentation process. The dissolved oxygen inside STR was reduced on day 4. The oxygen deficiency could have been primarily responsible for the observed effect. These results showed that almost all the sugar was consumed by *G. neojaponicum*. The reducing sugar decreased from 7.33 to 5.21 g/L which showed glucose consumption by *G. neojaponicum* as the fermentation progressed. The residual glucose in reducing sugar was reduced to 5.2 g/L on day 6 of the cultivation period with pH 4.6. The glucose consumption was directly related to the cell growth. Later, the mycelial dry weight decreased to 20.56 g/L on day 6 of fermentation period.

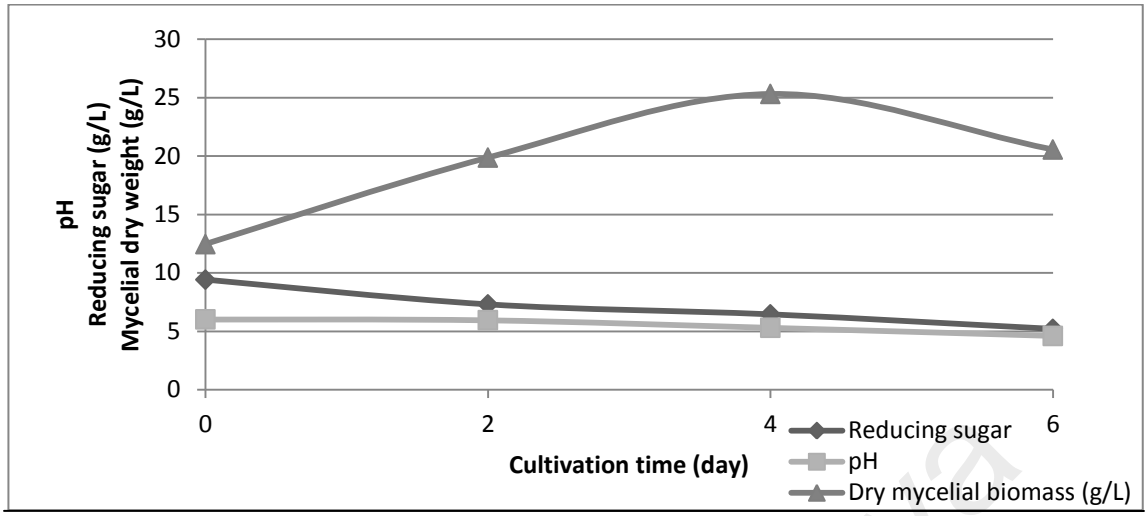
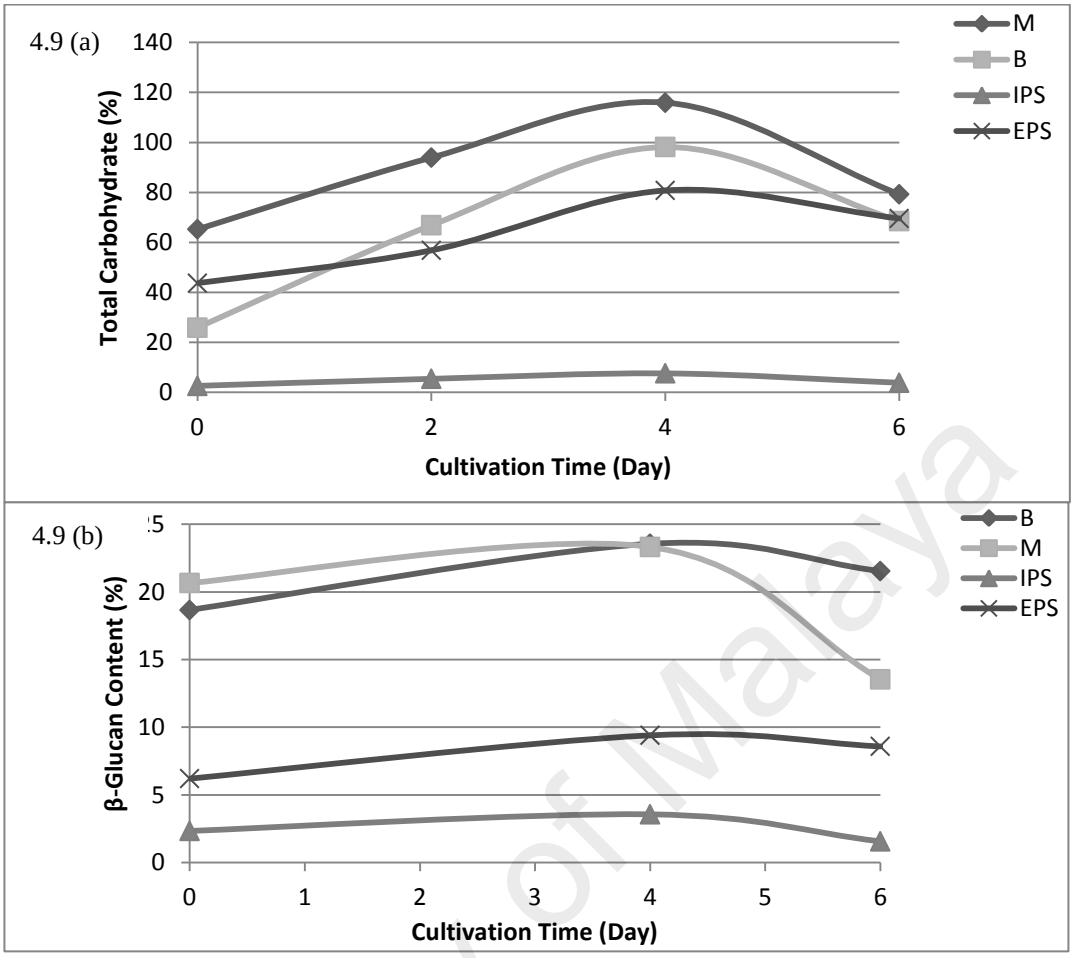


Figure 4.8: Time profiles of mycelial dry weight, pH and reducing sugar on brown sugar-spent yeast medium of *G. neojaponicum* in 2-L STR reactor at 27 ^{o}C , 160 rpm at 1.33 vvm.

The time profiles of β-glucan and total carbohydrate on dried mycelium (M), dried broth (SM), and extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS) are shown in Figures 4.9 (a) and (b). The contents of β-glucan and total carbohydrate production for EPS and IPS [Figures 4.9 (a) and (b)] attained the highest values on day 4, which are parallel with mycelial dry weight. The production of mycelial dry weight reached the maximum value of 25.32% (w/v) on day 4.

The IPS extract had lower amount of β-glucan and total carbohydrate as compared with EPS extract at 7.65% (w/v) and 3.67% (w/v), respectively, While, the broth extract had higher amount of β-glucan and total carbohydrate as compared with mycelium extract at 115.89% (w/v) and 23.56% (w/v), respectively. The β-glucan content was observed to be parallel with total carbohydrate in EPS and IPS of *G. neojaponicum*. Hence, these findings proved that the highest β-glucan content and total carbohydrate were obtained on day 4 of fermentation time in *G. neojaponicum* mycelium



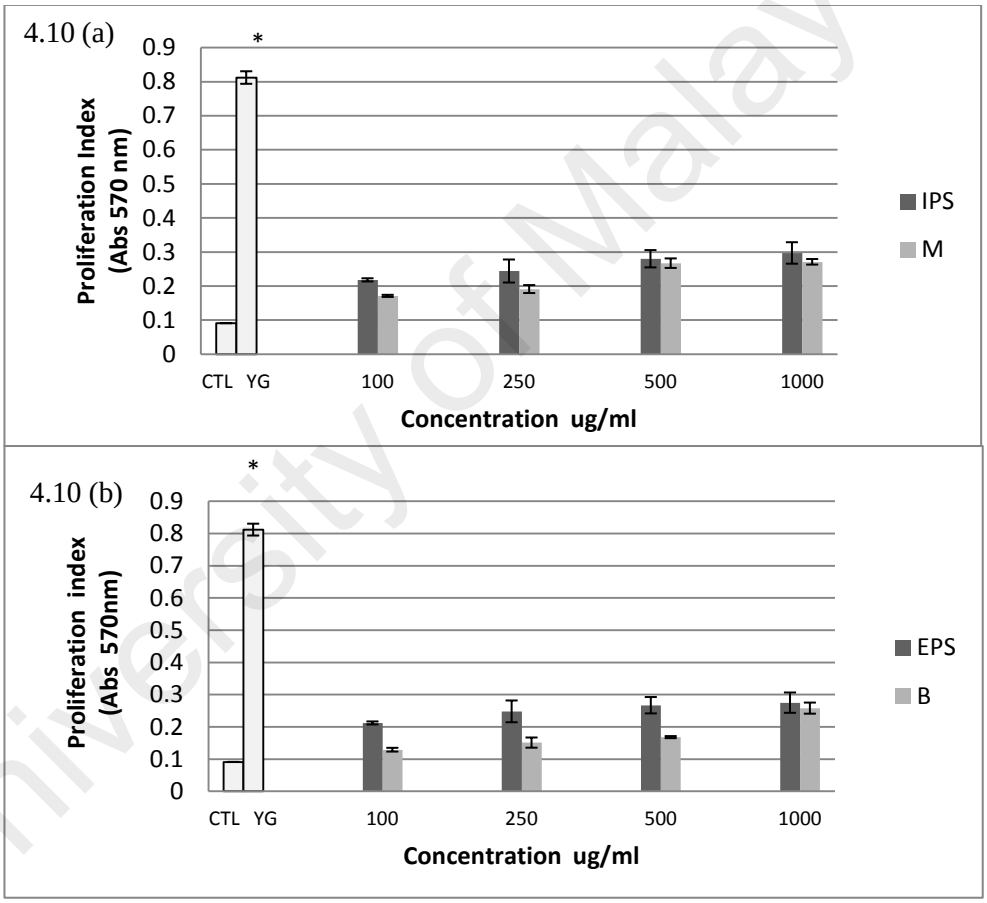
Figures 4.9 (a) and (b): Time profiles of (a) total carbohydrates and (b) β-glucan content of dried mycelium (M), dried broth (B), extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS) of *G. neojaponicum* in 2-L STR reactor.

4.8 Evaluation of immunomodulatory properties of dried mycelium, broth and its polysaccharides from *G. neojaponicum*

The evaluation of immunomodulatory and antitumour effect of dried mycelium, dried broth and its polysaccharide extracts were determined via macrophage and human cancer cell lines against cell proliferation, cytotoxicity, phagocytosis and NfκB activity.

4.8.1 Effect of *G. neojaponicum* extracts on proliferation of RAW264.7 macrophages

The dried mycelium and broth as well as polysaccharide extracts (IPS and EPS) of *G. neojaponicum* were evaluated for proliferation activities using RAW264.7 macrophages. Figures 4.10 (a) and (b) show that the highest proliferation activity was at concentration of 1000 ug/mL for all *G. neojaponicum* extracts tested.



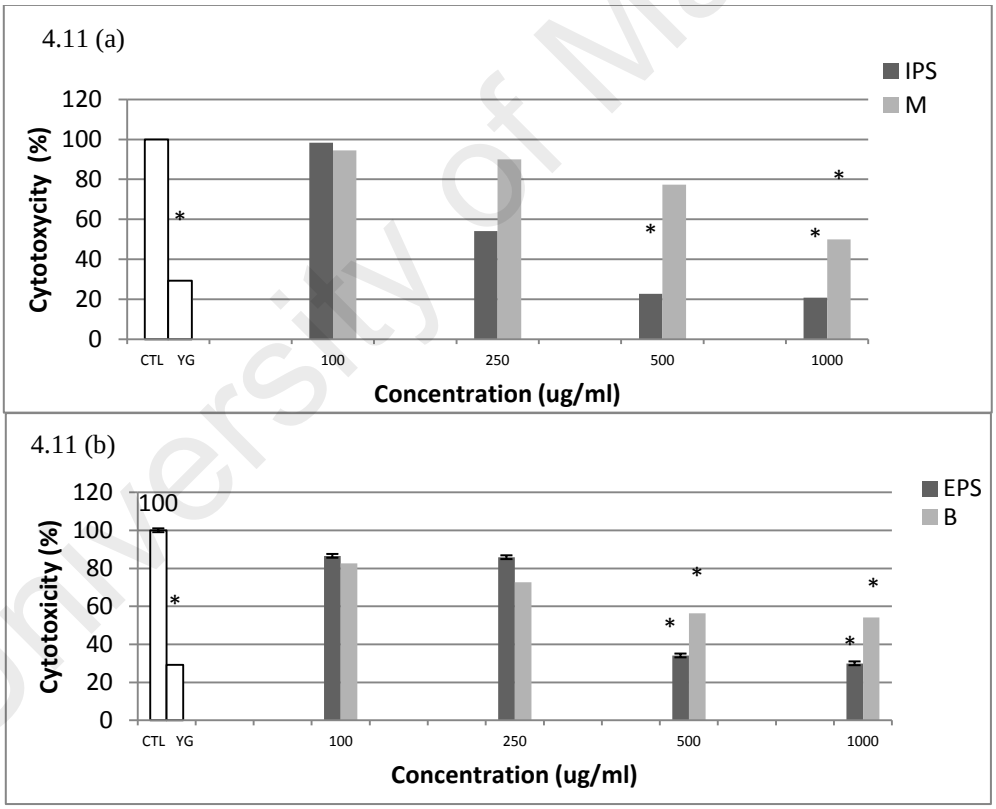
Figures 4.10 (a) and (b): Effect of proliferation by *G. neojaponicum* extracts of RAW264.7 macrophages. (a) Dried mycelium (M) and intracellular polysaccharide (IPS) (b) Dried broth (B) and extracellular polysaccharide (EPS)

Note: The results were calculated by dividing experimental data by control values. Experiments were performed in triplicate, and error bars represent the standard deviations of the mean. *Statistically significant difference in comparison with the control, P<0.05. YG-cell treated with yeast glucan as positive control; CTL- cell treated with DMEM medium as negative control.

The positive control was β-glucan from yeast glucan extract. The yeast glucan showed significant proliferation index of 0.81 as compared to control of 0.09. The IPS and EPS extract shows higher proliferation index compared to mycelium or broth of *G. neojaponicum*. The proliferation activities of the polysaccharides extracts of *G. neojaponicum* of the macrophages were found to be dose-dependent.

4.8.2 Cytotoxicity effect of *G. neojaponicum* extracts of human cancer cell line (HT29).

The *G. neojaponicum* extracts of M, B, IPS and EPS were investigated for cytotoxicity effects of human colon cancer cell line (HT29). Figures 4.11 (a) and (b) show the cytotoxic effect of *G. neojaponicum* of the HT29.



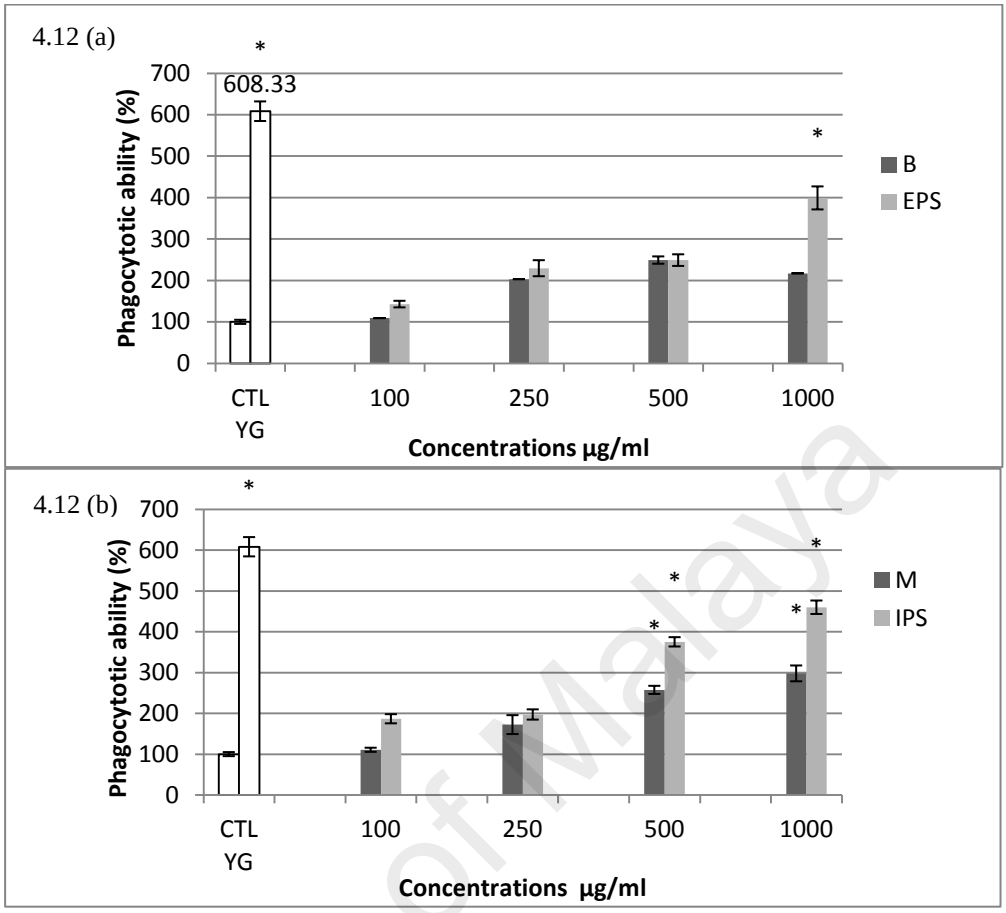
Figures 4.11 (a) and (b): Cytotoxicity effects of *G. neojaponicum* of human colon cancer cell line (HT29). (a) Dried mycelium (M) and intracellular polysaccharide (IPS) (b) Dried broth (B) and extracellular polysaccharide (EPS).

Note: YG- cell treated with yeast glucan as positive control; CTL- cell treated with DMEM medium as negative control. *Significant compared with negative control (P<0.05) (Mean ± S.E. (n=5).

The *G. neojaponicum* extracts had cytotoxic effects of human colon cancer cell as compared to control. The *G. neojaponicum* extracts at concentration of 1000 ug/mL had cytotoxicity effect at 20.83% (M), 54.22% (B), 49.91% (IPS) and 29.98% (EPS), respectively. Amongst all, the lowest cytotoxic effect of *G. neojaponicum* dried mycelium was observed at concentration 1000 ug/mL with 20.83%.

4.8.3 Phagocytotic ability of *G. neojaponicum* extracts of RAW264.7 macrophages.

The dried mycelium, dried broth, IPS and EPS of *G. neojaponicum* were evaluated for phagocytotic ability by neutral red uptake assay of RAW264.7 macrophages. Figures 4.12 (a) and (b) show the phagocytotic ability of *G. neojaponicum* extracts after 24 hours treated with 100-1000 µg/mL concentrations The RAW264.7 macrophages demonstrated increment of phagocytotic ability as compared to negative control after treating with 100-1000 µg/mL of *G. neojaponicum* extracts. The highest phagocytotic ability was IPS of *G. neojaponicum* at concentration of 1000 µg/mL (460%). Hence, IPS of *G. neojaponicum* had the most potent phagocytotic ability of macrophages.

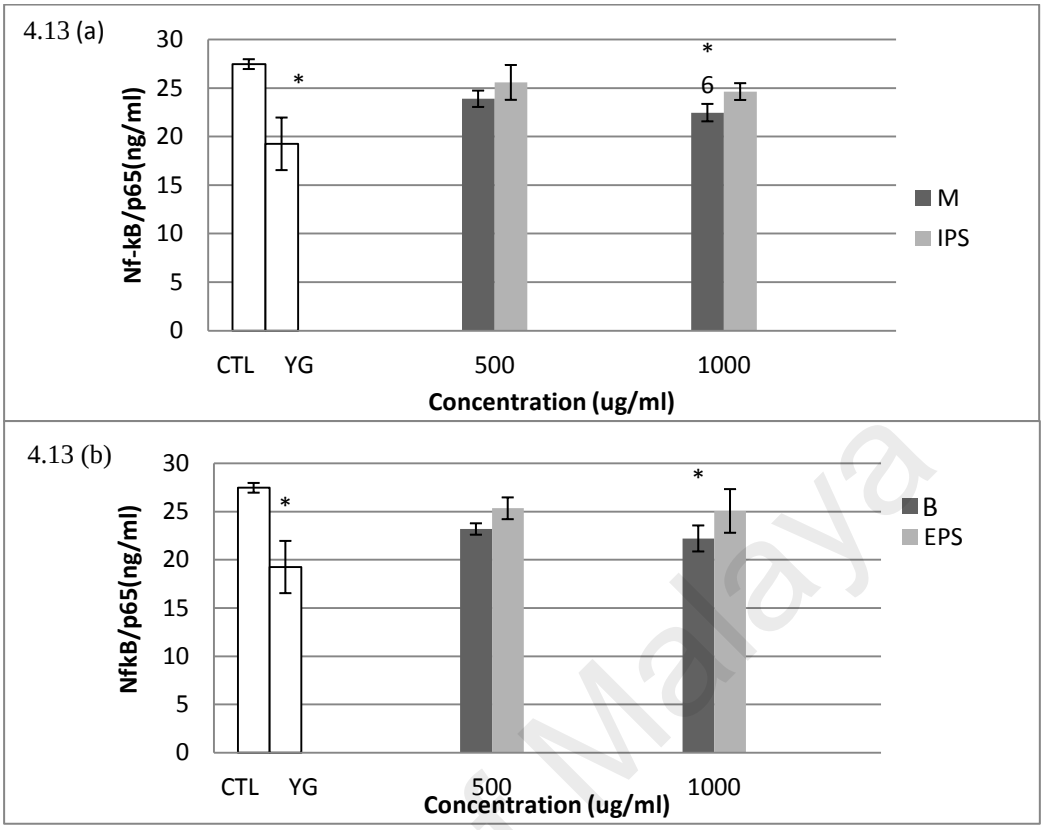


Figures 4.12 (a) and (b): Phagocytotic ability of (a) dried mycelium (M) and intracellular polysaccharide (IPS) (b) dried broth (B) and extracellular polysaccharide (EPS) of *G. neojaponicum*.

Note: YG- cell treated with yeast glucan as positive control; CTL- cell treated with DMEM medium as negative control. *Significant compared with negative control (P<0.05) [Mean ± S.E. (n=5)].

4.8.4 NF-κB inhibition of *G. neojaponicum* extracts of human colon cancer cell HT29.

The quantifications of NF-κB activity of *G. neojaponicum* extracts were conducted as preliminary analysis to evaluate the inflammation and immunomodulation of the immune system using *in- vitro* colon cancer cell lines. Figures 4.13 (a) and (b) show the Nf-kB inhibition of dried mycelium, broth and polysaccharide of *G. neojaponicum* of human colon cancer cell (HT29).



Figures 4.13 (a) and (b): Nf-kB inhibition of *G. neojaponicum* of human colon cancer cells (HT29). (a) Dried mycelium (M) and intracellular polysaccharide (IPS) of *G. neojaponicum* (b) Dried broth (B) and extracellular polysaccharide (EPS)

Note: YG- cell treated with yeast glucan as positive control; CTL- cell treated with DMEM medium as negative control. *Significant compared with negative control (P<0.05) [Mean ± S.E. (n=5)]

The reduction patterns were observed of Nf-κB value of human colon cancer cell (HT29) after 24 hours treatment of *G. neojaponicum* extracts. The *G. neojaponicum* mycelium and broth had significant (P<0.05) effect of Nf-kB inhibition while, no significant (P<0.05) effect for IPS and EPS. The Nf-κB inhibition of yeast glucan (positive control) also shows significant (P<0.05) different as compared to untreated cell.

4.9 Oral acute toxicity of dried mycelium of *G. neojaponicum*

The dried mycelium of *G. neojaponicum* was evaluated for its safety to Sprague Dawley rats. The appearance and general behavioural pattern of the rats were observed during 14 days after the administration of the dried mycelium. The result shows that the animals in both control group and treated group were normal. Individual body and organ weights of the control group and treated group are summarised in Table 4.14. No significant values (P<0.05) were obtained between the organ and body weights of treated group.

Table 4.14: Summary of body and organ weights of treated Sprague-Dawley rat treated with dried mycelium of *G. neojaponicum*

Organ/Body	Treated Group (g)	Control Group (g)
Heart	0.37±0.01	0.375±0.12
Liver	2.69±0.07	2.601±0.05
Spleen	0.20±0.00	0.188±0.01
Kidneys	0.71±0.01	0.713±0.01
Stomach	0.30±0.01	0.304±0.01
Body Weight (g)	210.00±4.60	211.60±9.60

Note: Organ body index = (organ weight × 100)/body weight; dried mycelium of *G. neojaponicum* was given to rats at dosage of 2000 mg/kg. (Mean± S.E, n=5)

Throughout the 14-day observation period of oral acute toxicological test, all animals appeared active and healthy. For both groups, increase in physical weight of the animals was noted during the 14-day experiment. No significant changes in behaviour, skin effects, breathing, impairment in food intake and water consumption as well as

postural abnormalities. There were no signs of toxicity or death observed in the animals, upon administration of 2000 mg/kg of dried mycelium of *G. neojaponicum*.

4.9.1 Haematology analysis

The blood of the Sprague Dawley rats treated with dried mycelium of *G. neojaponicum* were analysed for haematology and biochemical parameters including full blood count, renal function test, liver function test and glucose metabolism test. Table 4.15 shows the blood profile (RBCs, Hb concentration, PCV, MCV, MCH, MCHC and TLC) was not significantly affected by oral administration of dried mycelium *G. neojaponicum* compared with the untreated control group. In terms of concentration of serum urea nitrogen, no differences were found between the test and control group of rats after being treated with *G. neojaponicum*. The liver function test and renal function test were conducted to observe any abnormalities in the organs caused by oral administration of dried mycelium of *G. neojaponicum*. Serum creatinine was not affected by the oral administration of extract; this indicates that the kidney function was not affected after administration of *G. neojaponicum*. The liver function test included Aspartate transaminase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), total bilirubin and Gamma GT (gamma-glutamyltranspeptidase). Nevertheless, oral administration of *G. neojaponicum* did not cause any significant difference ($P<0.05$) for both ALT and ALP levels in the serum compared with the control group. The amount of ALT, ALP and AST were determined at 49.4±5.2, 108.8±4.1, 117.9±8.5, respectively. In this study, there was no significant difference in the treatment and control group considering the amount of total cholesterol and triglycerides with 2.1±0.1 and

2.0±0.0 mmol/L, respectively. This result indicated no increase or decrease of blood fats level in consumption of the dried mycelium of *G. neojaponicum*.

Table 4.15: Haematology and biochemical analysis of untreated group (control) and treated group (dried mycelium) for *G. neojaponicum*.

Biochemical Test	Abbreviation	Unit	Untreated group (control)	*G. neojaponicum extract*
Full Blood Count				
Red Blood count	RBC	$x10^{12}$/L	7.2±0.3	6.9±0.2
White Blood Count	WBC	$x10^{5}$/L	7.5±1.6	7.2±1.8
Hemoglobin	Hb	g/L	140.4±4.8	135.4±4.3
Hematocrit (packed cell volume)	PCV	L/L	0.4±0.0	0.4±0.0
Mean corpuscular volume	MCV	fL	48.0±12.0	61.4±0.5**
Mean corpuscular hemoglobin concentration	MCHC	g/L	322.6±6.1	319.2±1.5
Thrombocytes-platelets	Thromb	$x10^{9}$/L	622.8±30.0	818.6±61.0**
Lysis Index	I. Index	Unit	Lysed	Lysed
Renal Function Test				
Total protein	T. Prot	g/L	66.2±1.6	62.5±1.7
Albumin	Alb	g/L	40.6±1.2	41.4±0.9
Creatinine	Crea	umol/L	72.4±1.5	69.6±2.8
Urea (Blood Urea Nitrogen)	Urea	mmol/L	8.2±0.7	6.4±0.9
phorus	Phos	mmol/L	3.7±0.3	3.7±0.4
Calcium	Ca	mmol/L	2.8±0.0	2.8±0.0
Sodium	Na	mmol/L	146.8±0.8	146.5±0.9
Potassium	K	mmol/L	6.3±0.4	6.0±0.6
Chloride	Cl	mmol/L	97.0±0.3	96.4±0.8
Liver Function Test				
Alanine aminotransferase	ALT	U/L	55.9±2.8	49.4±5.2
Alkaline phosphatise	ALP	U/L	105.5±11.2	108.8±4.
Aspartate aminotransferase	AST	U/L	140.0±12.5	117.9±8.5**
Cholesterol	Cholt	mmol/L	2.2±0.1	2.1±0.1
Triglyceride	Trig	mmol/L	0.3±0.1	0.2±0.0
Gamma glutamyltranspeptidase	GGT	U/L	<0.3	<0.3
Total bilirubin	T Bil	umol/L	0.6±0.2	0.9±0.1
Glucose Metabolism				
Glucose	Gluc	mmol/L	2.8±1.0	2.5±0.4
Urinanalysis	U.A	umol/L	99.7±19.2	80.5±19.0

Note: Values are mean ± SE (n = 5) at 5% level of significance ($P < 0.05$)**

CHAPTER 5.0 DISCUSSION

5.1 Formulation of low-cost materials for optimum growth of *G. neojaponicum*

The *G. neojaponicum* can be potentially cultivated as an alternative source for *G. lucidum* commercial production in Malaysia. The application of waste or low cost materials in cultivation medium can be highly effective to reduce cost in mushroom cultivation in large scale production. According to Miller & Churchill, (1986), the fermentation medium represented almost 30% of the cost for microbial fermentation.

Four types of low cost materials (molasses, spent yeast, corn steep liquor and brown sugar) were investigated in this study for the cultivation of *G. neojaponicum*. These low cost materials were selected due to their easy availability from sugar cane and brewery industry by-product in Malaysia. The amount of carbon and nitrogen in the raw materials were preliminary screened using AOAC (1980). The spent yeast and corn steep liquor are suitable as nitrogen sources while; brown sugar and molasses are suitable as carbon sources for *G. neojaponicum* cultivation.

The combination of spent yeast-brown sugar medium was the most successful cultivation medium for growth of *G. neojaponicum* in plate agar medium containing 0.06% (v/v) carbon and 6% (v/v) nitrogen, respectively, promoted the most effective mycelial growth of 20.86±0.02 mm/day. According to Hseih & Yang (2004), starch was found as the best carbon source for mycelial growth of *G. neojaponicum*. The Malt extract agar (MEA) and Potato dextrose agar (PDA) were used by Hseih *et al*., (2005) as cultivation medium of *G. neojaponicum* in petri dish. The optimum glucose for *G. neojaponicum* growth was at 40-80 g/L while, amount of nitrogen was optimum at 0.02%

(v/v) in ammonium nitrate (NH_4NO_3) for the *G. neojaponicum* mycelial growth (Hseih & Yang, 2004).

5.2 Further optimisation of spent yeast and brown sugar concentrations in the medium carried out in shake flask by RSM

Submerged culture is an alternative cultivation technique for consistency and reproducible harvest of bioactive compound and mycelial growth production of medicinal mushroom. The mycelial growth and bioactive compounds of mushroom production can reduce harvesting time 1-2 weeks compared to fruiting body which takes 3-4 months. The growth of *G. neojaponicum* was successfully cultivated using brown sugar-spent yeast medium in submerged culture in 6 days of cultivation time.

In this study, the spent yeast and brown sugar concentrations in the medium were selected based on the production of mycelial dry weight and polysaccharides of *G. neojaponicum*. The optimal medium composition for submerged fermentation of *G. neojaponicum* was predicted by RSM at 37.25 g/L of spent yeast, 91.3 g/L brown sugar with 0.5 g/L of KH_2PO_4, 0.5 g/L of K_2HPO_4 and 0.5 g/L $MgSO_4$.7H20 as basal medium. The combination of 37.25 g/L of spent yeast and 91.3 g/L brown sugar in cultivation medium for *G. neojaponicum* was produced 21.18±0.04 g/L mycelial dry weight.

Ganoderma neojaponicum was successfully grown in both medium containing commercial medium (glucose-yeast extract) in Experiment 3.2 and spent yeast-brown sugar in Experiment 3.6 as shown in Table 5.1. The optimum formulation for *G. neojaponicum* using glucose-yeast extract medium was at 10% (v/v) carbon and 0.1% (v/v) nitrogen to produce 30.12±0.02 g/L of mycelial dry weight (Experiment 3.2), while, low cost medium consisting of spent yeast-brown sugar medium was optimized at only

5.74% (v/v) carbon and 0.06% (v/v) nitrogen (Experiment 3.7) to produce 25.32 g/L of *G. neojaponicum*. The combination of minimum amount of brown sugar and spent yeast were proven suitable for replacement of expensive synthetic mediums such as glucose, yeast extract, malt extract and potato dextrose for cultivation of *G. neojaponicum*. The highest values of mycelial dry weight, total carbohydrate, and β-glucan production are 25.32 g/L, 115.89% (v/v), and 23.56 (g/L), respectively.

Our study recorded higher production of mycelial dry weight and polysaccharides in *G. neojaponicum* compared to Hseih & Yang (2004) that used 60% (v/v) brewery spent yeast (thin stillage) to produce 7.8 g/L mycelial dry weight and 7.5 g/L polysaccharides of *G. lucidum* in shake flask fermentation. Besides this, the use of 71.4 g/L brown sugar and 2.28 g/L yeast extract with other medium (malt extract, skim milk, sunflower oil and olive oil) produced 18.70 g/L mycelial dry weight and 0.420 g/L of polysaccharide by *G. lucidum* (Yang *et al*., 2000). Mizuno (1999) also reported the medium formulation consist of 50 g/L yeast extract and 20 g/L glucose with other mineral salt/ trace element produced 5.5 g/L mycelial dry weight and 1.71 g/L polysaccharides by *G. lucidum* in a shake flask culture.

Table 5.1: Summary of mycelial dry weight, total carbohydrate and β-glucan in non-optimized and optimum condition of shake flask and 2-L STR reactor fermentation of *G. neojaponicum*

Fermentation Mode	Parameter studied	Parameter optimized	Mycelial dry weight (g/L)	Total carbohydrate (%)	β-glucan (%)
Shake Flask (Experiment 3.2)	1.Glucose concentration: 0- 20% (w/v) 2. Yeast extract concentration: 0-1.0% (w/v)	Glucose and yeast extract concentrations	30.12±0.02	ND	ND
Shake Flask (Experiment 3.3)	1. Brown sugar concentration : 2-10% (w/v) 2. Spent yeast concentration: 0.02-1.0% (w/v)	Brown sugar and spent yeast concentrations	21.18±0.04	ND	ND
Shake flask (Experiment 3.4)	1. Tween 80: 0-1% (v/v) 2. Vegetable oil: 0-1.2% (v/v)	Effect of surfactant types	12.14±0.8	47.7	12.55
Shake Flask (Experiment 3.5)	1. Tween 80: 0.1-0.5% (v/v) 2. Temperature: 22-30 ^{o}C	Tween 80 and temperature	22.21	ND	20.56
Bioreactor (Experiemnt 3.6)	1. Temperature: 22-29 ^{o}C 2. Aeration: 0.5-2 vvm	Optimization of physical condition	25.31	73.56	21.34
Bioreactor (Experiment 3.7)	Profiles for reducing sugar, pH, β-glucan and mycelial dry weight	Production at optimum condition	25.32	115.89	23.56

Note: All medium prepared in the same amount of basal medium (0.5 g/L $MgSO_4 \cdot 7H_20$, 0.5 g/L KH_2PO_4 and 0.5 g/L K_2HPO_4) at constant pH 6 and agitation speed of 160 rpm

In this study, the ratio of carbon to nitrogen (C/N ratio) was studied at the range of 0.1 to 9.84 for combination of spent yeast and brown sugar as growth medium for *G. neojaponicum.* The optimal C/N ratio of 2.9 was predicted in spent yeast-brown sugar medium to produce 20.23±0.02 g/L mycelial dry weight for *G. neojaponicum*. According to Baojing *et al*., (2012), the medium cultivation of *G. lucidum* at C/N ratio of 5 produced mycelial dry weight and extracellular polysaccharide (EPS) at 7.24 g/L and 1.72 g/L, respectively. Besides that, Babitskatya *et al*., (2005) suggested C/N ratio of 25 was suitable for *G. lucidum* cultivation medium. Thus, it can be concluded that the different growth medium sources and *Ganoderma* species were influenced by the suitable ratio of C/N in submerged cultivation medium (Miller, 2000).

5.3 Effect of surfactant for the growth of *G. neojaponicum* in shake flask

In this study, a selection of surfactant was made between Tween 80 and vegetable oil for the growth of *G. neojaponicum* in shake flask. The study of surfactant was conducted to disperse mycelial growth in cultivation medium; consequently this increases homogeneity and facilitates sampling of mycelium or broth of *G. neojaponicum*. The addition of Tween 80 in the cultivation medium increased the total carbohydrate and β-glucan content as opposed to the addition of vegetable oil in the medium for *G. neojaponicum*. The addition of vegetable oil showed significant amounts of mycelial dry weight compared to the medium containing Tween 80. Medium containing vegetable oil produced high viscosity due to the formation of emulsion.

The polysaccharides extracted from the medium containing vegetable oil cannot be considered as pure carbohydrates due to the presence of proteins and fats in the medium precipitate. Steve & Yolanda (2006) proposed the use of hexane to remove the fat

content for extraction of polysaccharides. Therefore, the yield of mycelial dry weight was higher for medium containing vegetable oil compared with that the medium with Tween 80. 0.5% (v/v) of Tween 80 was applied for the production of polysaccharides in *Trametes sp* (Bolla *et al*., 2011).

The comparison between the addition of vegetable oil and Tween 80 to the medium of *G. neojaponicum* showed higher amounts of β-glucan and polysaccharides content in the medium containing Tween 80 compared with that containing vegetable oil. According to Cassiano *et al*., (2007), the addition of vegetable oil and fatty acid aided mycelial dispersion, mycelial dry weight and polysaccharides production in *Botryosphaeria rhodina* growth. Furthermore, the medium containing vegetable oil was cloudy. This might be due to the formation of emulsion at higher concentrations of vegetable oil. This characteristic has resulted in vegetable oil not being suitable as an additive for mycelial production from *G. neojaponicum*. Meanwhile, the addition of Tween 80, interacted hyphae with each other resulting in reduction of pellet formations. The hyphae were either joined to one another by adhesive forces determined by their surface properties, or repulsed and formed free mycelia. This effect may be due to depression, induction or stimulation of secretion of the mycelial cell (Yang *et al*., 2000).

It can be concluded that the suitable surfactant to be added to growth medium of *G. neojaponicum* was Tween 80 at 0.46% (v/v). The optimisation of Tween 80 and temperature was predicted by RSM at 0.46% (v/v) of Tween 80 at 26 ^{o}C and these conditions yielded mycelial dry weight of 22.21 g/L and β-glucan content of 20.56% (w/v).

It has been suggested that the effect of surfactants is on the permeability of cell membrane (Casssiano *et al*., 2007). According to Cassiano *et al*., (2007), several features

of the cell surface, which include surface antigens, hormone binding, cell recognition, adhesiveness and adsorption, play a role in the production of polysaccharides. The yield of intracellular and extracellular polysaccharides increased the mycelial growth, which correlated with the decrease of pellet size in the cultivation medium.

5.4 Optimum physical parameters for IPS and EPS production in shake flask and 2-L stirred tank reactor (STR) in spent yeast-brown sugar medium

The physical parameters such as pH, temperature, aeration and agitation were important factors to obtain optimum mycelial growth and bioactive substance in submerged cultivation. In this study, the optimum temperature for *G. neojaponicum* was successfully predicted by RSM at 26 ^{o}C in shake flask and 26.71 ^{o}C in 2-L STR reactor to produce mycelial dry weight of 22.21 g/L and 26.57 g/L, respectively. This finding is in agreement with Hseih (2004) who reported that the optimum temperature for the mycelial growth of *Ganoderma* spp. were at 24–28 ^{o}C. According to Wagner (2003), mycelial dry weight of *G. lucidum* produced at amount of 25 kg at temperature of 26 ^{o}C in 2-L of STR bioreactor. It is the simplest mode of operation in bioreactor systems. The use of bioreactor would be able to control the cultivation parameters easily and simultaneously. These parameters include medium composition, inoculation density, pH, temperature, aeration and agitation (Tang & Zhong, 2002b and Fang & Zhong, 2002b).

Temperature and aeration rates are the most crucial parameters of STR reactor employed for scale-up process (Felse & Panda, 2000). In this study, the use of batch

fermentation was carried out using a 2-L STR system (Biostat® A plus, B. Braun International, Germany Biostat A plus) to study the optimum conditions of temperature and aeration rate for *G. neojaponicum* polysaccharide production. The optimum conditions for polysaccharide production by *G. neojaponicum* was found to be an aeration rate of 1.339 vvm and temperature of 26.71 ^{o}C with a mycelial growth (26.57 g/L), total carbohydrate (75.56% w/v) and β-glucan content (21.57% w/v) in 2-L STR reactor.

Yang & Liau (1998b) noted that 22 g/L mycelial dry weight and 1.25 g/L extracellular polysaccharide of *G. lucidum* was produced at a temperature of 30 ^{o}C in bioreactor. Tang & Zhong (2002b) reported that 21.9 g/L mycelial dry weight and 0.87 g/L extracellular polysaccharides of *G. lucidum* at an aeration rate of 0.25-0.5 vvm in an agitated 2-L bioreactor. It is proven that different species of *Ganoderma* and different medium composition as well as different physical conditions producing different amounts of mycelial dry weight and polysaccharides in submerged fermentation (Fang & Zhong, 2002b).

5.5 Growth profiles of *G. neojaponicum* at optimum medium composition and physical conditions in a 2-L bioreactor

A trend of maximum yield of mycelial dry weight, β-glucan content and total polysaccharide were observed on day 4 of *G. neojaponicum* cultivation in submerged fermentation in 2-L STR reactor. The highest value was observed on day 4 based on mycelial dry weight, β-glucan and total polysaccharides, 25.32 g/L, 115.89% (w/v) and 23.56% (w/v) respectively. It can be concluded that the production of polysaccharides and β-glucan was associated with cell growth of *G. neojaponicum*. As comparison, the

mycelial growth of *Hericium erinaceus* was obtained at 14.021 g/L on 9th day of culture in a 5 L bioreactor, while, the polysaccharide produced by *H. erinaceus* was found to be 0.994 g/L at the 7th day of culture (Malinowska *et al.*, 2009). This showed that higher amount of mycelial dry weight and polysccharide content was produced in *G. neojaponicum* in shorter cultivation time compared to *H. erinaceus*.

The profiling of *G. neojaponicum* showed a declining pattern in the pH 6 to 4.2, and this might be caused by the production of organic acid from carbon sources during consumption and cell growth (Shih, 2006). Profiling of *G. neojaponicum* was revealed that higher amount of total carbohydrate content was obtained for IPS and EPS as compared to dried mycelium and broth. Meanwhile, the amount of IPS and EPS had low mycelia weight as compared to dried mycelium and dried broth of *G. neojaponicum*. This might be caused by the losses of nutrients or components in dried mycelium and broth during partial purification and extraction procedures.

The usage of ethanol is to eliminate the smaller molecules such as oligosaccharides or monosaccharides by interfering with the quantification of the polysaccharides. In this study, precipitation using ethanol had successfully developed a method of using concentration of 1:1 (v/v) of broth: 95 – 96% (v/v). This method was can reduce cost and time in polysaccharides production as compared to method suggested by Wagner (2003) at concentrations ratio of 1:4 (v/v) of broth: 95 – 96% (v/v). Table 5.1 shows the summary of mycelial dry weight, total carbohydrates and β-glucan in non-optimized and optimum condition of shake flask and 2-L STR reactor fermentation of *G. neojaponicum*.

As a conclusion, the mycelial dry weight and β-glucan content of *G. neojaponicum* in submerged cultivation had increased up to 13.42% (w/v) and 12% (w/v), respectively by using bioreactor system as compared to shake flask fermentation system. The increment of mycelial dry weight might be due to the efficiency in controlling simultaneously among operational factors in bioreactor rather than in shake flasks. The use of bioreactor had increased mycelial growth up to 20% compared to shake flask culture.

5.6 Immunomodulatory properties of polysaccharides from *G. neojaponicum*

In the present study, dried mycelium, dried broth, intracellular polysaccharides and polysaccharides extracellular of *G. neojaponicum* extracts were tested further for *in-vitro* immune response activity. This was done by studying the activities of proliferation, phagocytosis and inhibiting NF-κB on macrophages (RAW264.7) and human colon cancer cell. The defense mechanism of macrophages against pathogens includes secreting cytokines, such as the tumor necrosis factor-α (TNF-α), and inflammation mediators, (NF-kB). The highest proliferation ability of macrophages exhibited at a concentration of 1000 ug/mL by IPS of *G. neojaponicum* with an increment of 226% compared with negative control samples.

It can be concluded that no significant cytotoxic effects were observed in macrophages treated with *G. neojaponicum* extract as compared with the negative control ($P<0.05$). All *G. neojaponicum* extracts were positively stimulated to RAW264.7 of phagocytosis assay. The mycelium of *G. lucidum* at 50 and 100 mg/kg inhibited the growth of RAW264.7 of S-180 in Balb/c mice with inhibitory rates of 37.8% - 78.1% (Hu & Lin, 1999). It was interesting to note that the highest level of phagocytosis activity in this study exhibited at 460% using 1000 µg/mL of IPS from *G. neojaponicum*. To

support the data, the range of 2.5-25 g/mL of *G. lucidum* stimulated RAW264.7 cells proliferation in a dose-dependent manner and with low associated cytotoxic effect (Guan, 2011). At the range of concentrations tested (0-100 g/mL), the 25 g/mL concentration produced a maximum 205.8% of the stimulation effect on macrophages (Guan, 2011).

Similar stimulating effect on macrophages was also observed in a study by Lee (2008) using polysaccharides from *Lentines edodes*. This result implies that *G. lucidum* triggers macrophage secretion of inflammatory cytokines, which is also promoted by the existence of lipopolysaccharide. This is possible by binding to dectin-1 and toll-like TLR-2/6 receptors, which activate NF-kappa B and trigger secretion of cytokines (Batbayar *et al*., 2011). However, nitric oxide production is not enhanced by *G. lucidum* mycelium in RAW264.7 cells (Kuo *et al*., 2006). Ryu (2010) had reported the inhibition of human colon cancer cell line (HT29) using polysaccharides extract from *Ostaracys japonicas* at a concentration of 2 mg/mL decreased dramatically to 28.43% as compared to the control.

In comparison, the inhibition of Nf-kB was reported on human breast cancer cell by Muller (2006) and prostate cancer cell (Sliva, 2003) of the extract of *G. lucidum*. Taken together, the antitumor activity of *Ganoderma* spp.are caused by the inhibition or activation of specific mechanisms and pathways. The reduction of proliferation of cancer cells might be due to the down-regulation of estrogen receptor and NF-κB signalling. Some of the effects on cancer cells are indirect and are caused by stimulation of the immune system by polysaccharides and the release of cytokines from activated macrophages and T lymphocytes. Other effects of *Ganoderma* spp. are targeted directly to the cancer cells by modulating their intracellular signaling and can affect the behavior of cancer cells.

Based on the findings on RAW264.7 cells, it could be deduced that *G. neojaponicum* has positive immune response. This is an indication that this wild Malaysian strain can be potentially used as immunomodulating agent. Further investigations on cytokines need to be conducted to confirm that this wild Malaysian strain of *G. neojaponicum* has immunomodulating properties. The inhibition trend of human colon cancer cell lines was observed of IPS and EPS of *G. neojaponicum*. Lowest inhibition of proliferation of IPS of *G. neojaponicum* was observed to be at a reduction of 28.8% as compared with negative control.

These findings suggest that the most active *G. neojaponicum* extract with the possession of anti-cancer activity of human colon cancer cell (HT29) is IPS. For comparison, the anti-cancer effect of *G. neojaponicum* was in agreement with the findings of *G. lucidum* reported by Yuen & Gohel, 2005. According to Wasser & Weis (1999), the polysaccharides do not respond directly to cytotoxic effects on tumour cells. Instead, they strengthen the host mediated immunomodulatory response by stimulating the immune system in curing cancer. The results related to inhibition of cancer cell proliferation were also supported by increment level of NF-κB activity on HT29. The highest inhibition rate (32.88%) of NF-κB activity of HT29 was determined of IPS as compared with the negative control.

Similar results were reported in a study by Anne *et al*., (2011), who showed that lipopolysaccharides inhibited the NF-κB activity. Inhibitory effects on proliferation on human breast cancer cell were also displayed through the use of *G. lucidum* extract. The reduction of proliferation of cancer cells might be due to the down-regulation of NF-κB signalling and estrogen receptor (Muller, 2006). The trend of dose dependent results can be seen clearly in proliferation on RAW264.7 as well as phagocytotic ability.

Meanwhile, in the present study, the concentrations of the cell line of HT29 were not dose dependent as seen in the NF-κB assay results. The Nf-kB assay had significant inhibitory activity of HT29 of mycelium and broth of *G. neojaponicum*. All *G. neojaponicum* extracts were further tested on human immune response *in-vitro* and showed immunostimulating effects by enhancement of proliferation and phagocytotic ability of RAW264.7 macrophages as well as decreasing proliferation of human colon cancer cell (HT29) in a dose dependent manner ($P<0.05$). Meanwhile, the IPS of *G. neojaponicum* showed the highest potential to stimulate the immune function and to act as anti-cancer agent against human colon cancer cells. It showed promising results on immune response of IPS and EPS compared with dried mycelium and broth of *G. neojaponicum*. Besides that, dried mycelium was the lowest cytotoxic effect on human colon cancer cell and also had positive immunomodulatory effect on macrophages cell *G. neojaponicum*.

5.7 Evaluation of *in-vivo* toxicity on dried mycelium of *G. neojaponicum*

The dried mycelium of *G. neojaponicum* was evaluated for its safety by analysis of *in-vivo* toxicology study. This is a method to ascertain the safety for consumption of polysaccharides extract of *G. neojaponicum* either as a drug or functional food. In this study, the dried mycelium of *G. neojaponicum* was evaluated for its safety and efficacy using a single dose testing for acute oral toxicity. This method is one of the most widely used in toxicology tests as according to the Organization of Economic Cooperation and Development (OECD) Guidelines (OECD, 1987). The adverse effects occurring within a short period of time after oral administration of a single dose of the substance was determined according to Chan & Hayes (1994). Acute toxicity study conducted revealed

that the administration of polysaccharide extract (up to a dose of 2000 mg/kg) of *G. neojaponicum* did not cause any significant changes in behaviour of the animals. No death was observed with doses up to 2000 mg/kg body weight. The rats were physically active. These effects were observed during the experimental period (14 days).

The results showed that in single dose, the polysaccharide extract had no adverse effect. This indicates that the medium lethal dose (LD50) could be greater than 2000 mg/kg body weight in rats. No toxic symptoms were observed for doses up to 2 g/kg body weight. All animals behaved normally. No neurological or behavioural effects were noted. No mortality was found throughout the 14-day study. Similar findings were reported in the aqueous extract of *G. lucidum* that was administered to mice (5 g/kg during 30 days); no differences in physical weight, organ weight or hematological parameters were observed (Wasser, 2005). In comparison with literature, the experiments of oral administration of hot water extract of *G. lucidum* (5,000 mg/kg) to mice for 30 days showed that there were no changes in body weight, haematological features and organ weight (Kim, 1986). Furthermore, an alcoholic extract of *G. lucidum* was given to young rats (1.2 and 12 g/kg daily during 30 days) by Wasser (2005) also showed no signs of toxicity in major organs as well as growth or development of the rats.

In this study, haematology test carried out on dried mycelium of *G. neojaponicum* including full blood count, renal function test, liver function test and glucose metabolism test did not show any significant abnormalities as compared to the control ($P<0.05$). In this study, no significant effect on total protein and albumin level were observed ($P<0.05$). This indicated that protein catabolism was not affected. Similar observation was noted with no changes in the blood test conducted after administration of *G. lucidum* (McKenna, 2002).

Therefore, in this study, no adverse effect level for polysaccharide extract was observed in the results. It can be concluded that doses of 2000 mg/kg body weight/day of dried mycelium of *G. neojaponicum* is safe to be used as functional food or medicinal application.

CHAPTER 6.0: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study has demonstrated that mycelium of *G. neojaponicum* can be cultivated in submerged fermentation on low cost medium. A mixture of spent brewery yeast and brown sugar was the best combination among four sources (molasses, corn steep liquor, spent yeast and brown sugar) usable as cultivation medium. Optimal growth conditions as predicted by the Design Expert Software via RSM (Minitab Ver 16) were achieved (28.7 g/L of spent yeast, 50.6 g/L brown sugar, 0.5 g/L of KH_2PO_4, 0.5 g/L of K_2HPO_4 and 0.5 g/L $MgSO_4.7H_20$). The C/N ratio of 2.9 was predicted as optimal in the use of spent yeast and brown sugar to produce 20.23±0.02 g/L mycelial dry weight for *G. neojaponicum*. The physical parameters were also predicted by RSM and were found optimal at temperature of 26.72 ^oC, aeration of 1.33 vvm, constant pH 6 and agitation of 160 rpm. The addition of selected surfactant (0.465 (v/v) Tween 80) was optimised the β-glucan production at 47.7% (w/v) in shake flask, and 26.39% (w/v) in 2-L-stirred tank reactor.

By fermenting *G. neojaponicum* according to the predicted growth medium requirements and physical parameters, mycelial dry weight obtained was at 25.32 g/L. The cultivation period for *G. neojaponicum* was discovered to be at optimal conditions on day 4 using 2-L STR. The amount of β-glucan and total carbohydrate content dried mycelium of *G. neojaponicum* were obtained on day 4 of cultivation time at 23.56±0.01% (w/v) and 115.89±2.78 g/L, respectively. *Ganoderma neojaponicum* showed positive immunostimulating effects by human immune response *in-vitro* test.

They enhanced the macrophages (RAW264.7) proliferation, increased phagocytosis activity and decreased human colon cancer cell (HT29) proliferation in dose dependent manner ($P<0.05$).

The findings of this study also indicate that dried mycelium, dried broth and IPS and EPS of *G. neojaponicum* have the potential to be used as immunomodulating agents to stimulate the innate immune system for fighting infectious diseases. Dried mycelium of *G. neojaponicum* had lowest cytotoxic effect on human colon cancer cell and also had positive immunomodulatory effect on macrophages *G. neojaponicum*. Thus, the dried mycelium at doses up to 2000 mg/kg body weight/day for 14 days did not cause either mortality or have any toxic effects on the rats. No adverse effects were observed for dried mycelium of *G. neojaponicum* based on the experimental results. Hence, dosage of 2000 mg/kg body weight/day has established its safety in regard to potential immunomodulatory properties.

Further research may focus on additional purification and identification of the specific polysaccharide(s) involved in the immunomodulatory action of these fungi to better understand its immunomodulatory properties for the benefits pharmaceutical industries and medicinal products for the public.

6.2 Recommendation

In this study, the production of polysaccharides and β-glucan have been successfully optimised using shake flask and 2-L stirred tank reactor. This application will also benefit the nutraceutical and pharmaceutical industry. This production on a large scale has to be validated further with regard to the application of polysaccharides of *G. neojaponicum* as immunomodulating agent. It is vital to purify the polysaccharides

further, focusing on water soluble polysaccharides with high molecular weight that would exert higher effect of immunomodulatory property.

However, limited studies have reported on the effects of polysaccharides from *G. neojaponicum* on immune system using *in-vivo* and *in-vitro* trials. Furthermore, lack of translational approach of β-glucan to animal studies or clinical trials need to be identified to apply knowledge of receptor and signal pathways. Therefore, it is of great interest in regard to the discovery of polysaccharides by *G. neojaponicum* which can either modulate positively or negatively the biologic response of immune system. The exact immunological actions and signalling pathway induced by β -glucan are still unclear and need to be defined further.

REFERENCES

Adachi, Y., Ohno, N. & Ohsawa, M. (1990). Change of biological activities of β-1,3-D-glucan from *Grifola frondosa* upon molecular weight reduction by heat treatment. *Chemical and Pharmaceutical Bulletin*, 38, 477-481.

Alice, W.C. & Philip, G.M. (1996). Biomedical Research and the Application of Mushroom Nutriceuticals from *Ganoderma lucidum*. *Mushroom Biology and Mushroom Products*, Royse (ed.) Penn State University, 161-169.

Amran, M. (2006). Biomass production and formulation of *Bacillus subtilis* for biological control Indonesian. *Journal of Agricultural Sciences*, 7(2), 51-56.

Anne, B.S., Anne, R., Stine, G., Terje, E.M. & Svein, H.K. (2011). Immunomodulatory activity of dietary fiber: arabinoxylan and mixed-linked beta-glucan isolated from barley show modest activities *in vitro*. *International Journal of Molecular Science*, 12(1), 570–587.

Association of Official Analytical Chemists. (1980). *Official Methods of Analysis* (AOAC 998.18 & AOAC 928.08, 13th ed.). Washingthon, D.C: U.S Federal Government Printing Office.

Babitskatya, U.G., Sherba, V.V., Mitropolskaya, N.Y. & Bisko, N.A. (2000). Expolysaccharides of some medicinal mushrooms: production and composition. *International Journal of Medicinal Mushrooms*, 2, 51-54.

Baabitskaia, G. (2005). Polysaccharides of *Ganoderma lucidum*: factors affecting their production. *Prikladnaia Biokhimiia Mikrobiologiia*, 41 (2), 194-199.

Bae, J.T., Sinha, J., Park, J.P., Song, C.H. & Yun, J.W. (2000). Optimization of submerged culture for exo-biopolymer production by *Paecilomyces japonica*. *Journal of Microbiology and Biotechnology*, 10, 482-487.

Bao, X.F., Zhen, Y., Ruan, L. & Fang, J.N. (2002b). Purification, characterization, and modification of T lymphocyte-stimulating polysaccharide from spores of *Ganoderma lucidum*. *Chemical and. Pharmaceutical Bulletin*, 50, 623–629.

Baojing, Y., Xiaoyan, C. & Rijun, Z. (2012). Optimization of exopolysaccharides production from a novel strain of *Ganoderma lucidum* CAU5501 in submerged fermentation. *Brazilian Journal of Microbiology*, 490-497.

Batbayar, S., Kim, M.J. & Kim, H.W. (2011). Medicinal mushroom lingzhi or reishi, *Ganoderma lucidum* (W.Curt.:Fr.) P. Karst., beta-glucan induces Toll-like receptors and fails to induce inflammatory cytokines in NF-kappaB inhibitor-treated macrophages. *International Journal of Medicinal Mushrooms*, 13 (3), 213-225.

Baumer, J. D., Diego, M., Maria, S., Pacheco, Sabrina, M. V., Morgado, Ayres, F. M., Furigo. & Agenor, F. (2008). Comparative study of mycelia growth and production of Cinnabarin by different strains of *Pycnoporus Sanguineus*, 2,1-5.

Bilgrami, K.S. & Verma, R.N. (1992). *Physiology of fungi*. Villkas publishing House Hvt Ltd, London. 70-71.

Bluhm, T.L. & Sarco, A. (1977). The triple helical structure of lentinan, a β-(1-3)-D-glucan. *Canadian Journal of Chemistry*, 55, 293-299.

Bohn, J. & BeMiller, J. (1995). (1-3)-ß-Glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydrate Polymers*, 28(1), 3-14.

Bolla, K., Hima, B.N., Samatha, B. & Singara, C.M.A. (2011). Effect of plant oils, surfactants and organic acids on the production of mycelial biomass and exopolysaccharides of *Trametes* spp. *Journal of Agricultural Technology*, 7(4), 957-965.

Brancato, P. & Golding, N.S. (1953). The Influence of Concentrated Substrates on the Diameter Attained by the Fungus Colony F. Mycologia. *Mycological Society of America*, 46 (4), 442-456.

Brendan, J.S. & Sivasithamparam, K. (2003). Morphological studies of *Ganoderma* (Ganodermataceae) from the Australasian and Pacific regions. *Australian Systematic Botany*, 16(4), 487 – 503.

Cao, L.Z. & Lin, Z.B. (2002).Regulation on maturation and function of dendritic cells by *Ganoderma lucidum* polysaccharides. *Immunological Letters*, 83,163–169.

Cao, L.Z. & Lin, Z.B. (2003). Comparison of the effects of polysaccharides from wood-cultured and bag-cultured *Ganoderma lucidum* on murine spleen lymphocyte proliferation in vitro. *Acta Pharmalogica Sinica*, 38, 92–97.

Cassiano, C.S., Robert, F.H.D., Rui, S.S.F.S., Maria, L.C.S. & Aneli, M.B. (2007). Effect of soybean oil and Tween 80 on the production of botryosphaeran by *Botryosphaeria rhodina* MAMB-05. *Process Biochemistry* , 42 (8), 1254–1258.

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

Chang, S.T. & Philip, G.M. (2004). *Mushrooms: Cultivation, Nutritional value, medicinal effect and environmental impact* (2nd ed.). United States: CRC Press, 357-364

Chan, P.K. & Hayes, A.W. (1994). Acute toxicity and eye irritancy. In Hayes, A.W. (Eds.), *Principles and Methods of Toxicology* (3rd ed). New York: Raven Press, 579–647.

Chau, C.F. & Wu, S.H. (2006). The development of regulations of Chinese herbal medicines for both medicinal and food uses. *Trends in Food Science and Technology*, 17, 313– 323.

Chen, R.Y. & Yu, D.Q. (1993). Studies on the triterpenoid constituents of the spores from *Ganoderma lucidum. Journal of China Pharmaceutical Society*, 2 (82), 91-96.

Chien, C.M. (2004). Polysaccharides of *Ganoderma lucidum* alter cell immunophenotypic expression and enhance CD56+ NK-cell cytotoxicity in cord blood. *Bioorganic and Medicinal Chemistry*,12, 5603– 5609.

Corner, E. J. H. (1983). Ad *Polyporaceas* I. *Amauroderma* and *Ganoderma. Nova Hedwigia*, 75, 181-182.

Cox, P. W., Paul ,G.C & Thomas, C. R. (1988). Image analysis of the morphology of filamentous microorganisms. *Microbiology*, 144, 817-827.

Dacera, D.D.M., Babel, S., Parkpian, P. (2009). Potential for land application of contaminated sewage sludge treated with fermented liquid from pineapple wastes. *Journal of Hazard Mater*, 167, 866-872.

De Souza, W. (1989). Components of the Cell Surface of Trypanosomatids. *Progress Protistology*, 3, 87-184.

Dhampure Specialty Sugars Ltd (2003) *Natural brown sugar* . Retrieved on 6th July 2010 from http://www.sugarindia.com

Dominguesa, F.C., Queiroza, J.A., Cabralb, J.M.S & Fonseca, L.P. (2000). The influence of culture conditions on mycelial structure and cellulose production by *Trichoderma reesei* Rut C-30. *Enzyme and Microbial Technology*, 26, 394–401.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350-356.

Duta, F.P., Da Costa, A.C.A., Lopes, L.M.D.A., Barros, A., Servulo, E.F.C. & De, F.F.P.(2004). Effect of Process Parameters on Production of a Biopolymer by *Rhizobium* sp. *Applied Biochemistry and Biotechnology*, 114 , 639-652.

El-Nawwi, S. A. & El-Kader, A. A. (1996). Production of single-cell protein and cellulase from sugarcane bagasse: effect of culture factors. *Biomass and bioenergy*, 11(4), 361-364.

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, 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, 51-54.

Fang, Q.H. & Zhong, J.J. (2002c). Submerged fermentation of higher fungus G*anooderma lucidum* for production of valuable bioactive metabolites-ganoderic acid and polysaccharides. *Biochemical Engineering Journal*, 10, 61-65.

Felse, P. A. & Panda, T. (2000). Submerged culture production of chitinase by *Trichoderma harzianum* in stirred-tank bioreactors-the influence of agitator speed. *Biochemical Engineering Journal*, 4, 115-120.

Forage, R.G., Harisson, D.E.F. & Pitt, D.E. (1985). Effect of environment on microbial activity. In Bull, A. & Dalton, H. (Eds.), *Comprehensive Biotechnology*. England: Pergamon Press, 251-280.

Gao, Y., Chan, E. & Zhou, S. (2004). Immunomodulating activities of *Ganoderma*, a mushroom with medicinal properties. *Food Reviews International,* 20 (2), 123 –161.

Gao, Y., Shufeng, Z., Jianbo, W., Min, H. & Anlong, X. (2002a). Mechanism of the antiulcerogenic effect of *Ganoderma lucidum* polysaccharides on indomethacin-induced lesions in the rat. *Life Science,* 72, 731-745.

Ghosh, S., May, M.J., & Kopp, E.B. (1998). NFκB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual Review of Immunology*, 16, 225-260.

Gibbs, P.A., Seviour, R.J. & Schmid, F. (2000). Growth of Filamentous Fungi in Submerged Culture: Problems and Possible Solutions. *Critical Reviews in Biotechnology*, 20 (1), 17-48.

Godfrey, C. F., Chan, W. K., & Daniel, M.Y. (2009). The effects of β-glucan on human immune and cancer cells. *Journal of Hematology & Oncology*, 2 (25), 1-11.

Grace, G.L., Yue, K.P, Gary, M.K., Tse, P.L. & B.S.L. (2006). Comparative studies of various ganoderma species and their different parts with regard to their antitumor and immunomodulating activities *in vitro*. *The Journal of Alternative and Complementary Medicine,* 12 (8), 777-789.

Guan, D., Zhang, Z., Yang. Y., Xing, G. & Lin, J. (2011) Immunomodulatory activity of polysaccharide from the roots of *Actinidia kolomikta* on macrophages. *International Journal of Biology*, 3(2),15.

Habijanic, J., Berovic, M., Wraber, B., Hodzar, D. & Boh, B. (2001). Immunomodulatory effects of fungal polysaccharides from *Ganoderma lucidum* submerged biomass cultivation. *Food Technology and Biotechnology*, 39, 327-331.

Han, M.D., Lee, E.S., Kim, Y.K., Lee, J.W., Jeong, H. & Yoon, K.H. (1998). Production of nitric oxide in RAW264.7 macrophages treated with ganoderan, the β-glucan of *Ganoderma lucidum*. *Korean Journal of Myocology,* 26, 246-255.

Hansen, S.K., Baeuerle, P.A. & Blasi, F. (1994). Purification, reconstitution and IκB association of the c-Rel-p65 (RelA) complex, a strong activator of transcription. *Molecular and Cellular Biology,* 14 (4), 2593-2603.

Hatcher, G.E & Lambrecht, R.S (1993). Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid producing bacteria. *Journal Dairy Sciences,* 76, 2485-2492.

Hochstenbach, F., Klis, F.M., Van Den, En. H., Van Donselaar, E., Peters, P.J. & Klausner, R.D. (1998), Identification of a putative α-glucan synthase essential for cell wall construction and morphogenesis in fission yeast. *Proceedings of National Academy of Sciences. USA*, 95, 9161- 9166.

Hseih, C., Hsu, T.H. & Yang, F.C. (2005). Production of polysaccharides of *Ganoderma lucidum* (CCRC36021) by reusing thin stillage. *Process Biochemistry,* 909-916.

Hseih, C. & Yang, F.C. (2004). Reusing soy residue for the solid –state fermentation of *Ganoderma lucidum. Bioresource Technology,* 91, 105-109.

Hsu, M.F., Lu, M.C., Tsao, L.T., Kuan, Y.H., Chen, C.C., Wang, J.P. (2004). Mechanisms of the influence of magnolol on eicosanoid metabolism in neutrophils. *Biochemical Pharmacology,* 67 (5), 831–840.

Hubert, O. (2006). *The Molasses*. Biotechnologie-Kempe. GmbH Germany.1-3

Hu, Y. H. & Lin, Z. B. (1999) Effects of polysaccharides isolated from mycelia of *Ganoderma lucidum* on HL-60 cell apoptosis. *Acta Pharmacologica Sinica* , 34, 268-271.

ICCVAM (Interagency coordination committee on the validation of alternative methods) test method evaluation. (2006). *Federal Register of America. America: ICCVAM Publishing*, 73 (58), 15757-15758.

Ishibashi, K., Miura, N. N., Adachi, Y., Ohno, N., & Yadomae, T. (2001). Relationship between solubility of grifolan, a fungal 1,3-β-D-glucan, and production of tumor necrosis factor by macrophages *in vitro*. *Bioscience, Biotechnology and Biochemical*, 65(9), 1993-2000.

Ji, H.J., Jong, M.L., Hyun, O.K., Sang, W.K., Hye, J.H., Jang, W.C. & Jong, W.Y. (2004). Optimization of submerged culture conditions for exopolysaccharide production in *Sarcodon aspratus* (Berk) S.lto TG-3. *World Journal of Microbiology & Biotechnology,* 20, 767-773.

Jong, S.C. & Birmingham, J. M. (1992). Medicinal benefits of the mushroom *Ganoderma. Advances in Applied Microbiology*, 37, 101-134.

Kanokarn, K., Pranee, R., Jean-Jacques, S. & Wai, P. (2010). Exobiopolymer production of *Ophiocordyceps dipterigena* BCC 2073: optimization, production in bioreactor and characterization. *BMC Biotechnology,* 10-51.

Kidd, P.M. (2000). The use of mushroom glucans and proteoglycans in cancer therapy. *Alternative Medicine Review*, 5, 4-27.

Kim, S.W., Hwang, H.J., Park, J.P., Cho, Y.J., Song, C.H. & Yun, J.W. (2002b). Mycelial growth and exo-biopolymer production by submerged culture of various edible mushrooms under different media. *Letters of Microbiology*, 34, 56-61.

Kim, M.J., Kim, H.W., Lee, Y.S., Shim, M.J., Choi, E.C. & Kim, B.K. (1986). Studies on safety of *Ganoderma lucidum*. *Korean Journal of Myocology*, 14 (1), 49-59.

Kim, H. K., Seo G. S. & Kim H. G. (2001). Comparison of characteristics of *Ganoderma lucidum* according to geographical origins (II): Consideration of morphological characteristics. *Mycobiolology*, 29, 80–84.

Kim, H. M., Park, M. K. & Yun, J.W. (2006). Culture pH affects exopolysaccharide production in submerged mycelial culture of *Ganoderma lucidum. Applied Biochemistry & Biotechnology*, 134 (3), 249-62.

Klis, F., Mol, P., Hellingwerf, K. & Brul, S. (2002). Dynamic of cell wall structure in Saccharomyces cerevisiae. *FEMS Microbiology Reviews*, 26, 239-256.

Kunsch, C., Ruben, S.M. & Rosen, C.A. (1992). Selection of optimal iκB/Rel DNA-binding motifs: interaction of both subunits of NF-κB with DNA is required for transcriptional activation. *Molecular and Cellular Biology*, 12(10), 4412-4421.

Kuo, M.C., Weng, C.Y., Ha, C.L., Wu, M.J. Ha & M.J. (2006) *Ganoderma lucidum* mycelia enhance innate immunity by activating NF-κB. *Journal of Ethnopharmacology*, 103 (2), 217–222.

Laskin, D.L. (2009). Macrophages and inflammatory mediators in chemical toxicity: A battle of forces. *Chemical Research in Toxicology*, 22,1376-1385.

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, 646-650.

Lee, S.S., Chang, Y.S. & Noraswati, M.N.R. (2008). Utilization of macrofungi by some indigenious communities for food and medicine in Peninsular Malaysia. *Forest Ecology and management*, 1-4.

Lee, S.S., Wei, Y.H., Chen, C.F., Wang, S.Y. & Chen, K.Y. (1995). Antitumor effects of *Ganoderma lucidum*. *Journal of Chinese Medicine*, 6, 1-12.

Lei, L.S., Lin & Z.B. (1993). Antagonistic effect of *Ganoderma lucidum* polysaccharides on the immunosuppressive response induced by cicloporin A, hydrocortisone and antitumor agents. *Journal of Pharmacology and Toxicology* ,7, 183-185.

Li, M.C., Lei, L.S., Liang, D.S.X., Yuan, J.H., Yang, S.Q. & Sun, L.S. (2000). Effect of *Ganoderma lucidum* polysaccharides on oxygen free radicals in murine peritoneal macrophages.*Chinese Journal of Pharmacology and Toxicology,* 14, 65-68.

Lin, R., Gewert, D. & Hiscott, J. (1995). Differential transcriptional activation in vitro by NF-κB/Rel proteins. *The Journal of Biological Chemistry*, 270 (7), 3123-3131.

Lin, W.H., Yu, B., Lin, C.K., Hwang, W.Z. & Tsen, H.Y. (2007). Immune effect of heat-killed multistrain of *Lactobacillus acidophilus* against *Salmonella typhimurium* invasion to mice. *Journal of Applied Microbiology*, 102, 22-31.

Lin, C.P., Sun, A.T. & Liu, Y.A. (1973). Studies on the submerged cultivation of *Ganoderma* sp. *Acta Microbiologica Sinica*, 13,145-151.

Lin, Y.L., Liang, Y.C., Lee, S.S. & Chiang, B.L. (2005). Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathwaMys. *Journal of Leukocyte Biology*, 78(2), 533-543.

Loo, C. Y., Rosma, A., Mohd Azemi, M. N. & Wan Nadiah, W. A. (2002). Influence of agrowaste media on intracellular protein of *Saccharomyces cerevisiae* and *Candida utilis*. In Tanaka, R. and Cheng, L. H. (Ed.), *Lignocelullose: Materials for the Future from the Tropics* .139-143.

Maeda, Y.Y., Watanabe, S.T., Chihara, C. & Rokutanda, M. (1988). Denaturation and renaturation of a β-1,6-1,3-glucan, lentinan associated with expression of T-cell mediated responses. *Cancer Research*, 48, 671-675.

Malinowska, E., Krzyczkowski, W., Herold, F., Japienis, G., Ślusarczyk. J., Suchocki, P., Kuraś, M. & Turio, J. (2009). Biosynthesis of selenium-containing polysaccharides with antioxidant activity in liquid culture of *Hericium erinaceum*. *Enzyme and Microbial Technology,* 44, 334–343.

Matsuguchi, T., Takagi, A., Matsuzaki, T., Nagaoka, M., Ishikawa, K., Yokokura, T. & Yoshikai, Y. (2003). Lipoteichoic acids from *Lactobacillus* strains elicit strong tumor necrosis factor alpha-inducing activities in macrophages through Toll-like receptor 2. *Clinical Vaccine Immunology*, 10, 259-266.

McKenna, D.J., Jones, K. & Hughes, K. (2002). *Reishi Botanical Medicines: The Desk reference for Major Herbal Supplements* (2nd ed.). New York: The Haworth Herbal Press, 825-855.

Medici, M., Voderola, C.G., Weill, R. & Perdigon, G. (2005). Effect of fermented milk containing probiotic bacteria in the prevention of an enteroinvasive Escherichia coli infection in mice. *Journal of Dairy Resources*, 72, 243-249.

Mercurio, F., Zhu, Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbosa, M., Mann, H. M., Manning, A. & Rao A. (1997). IKK-1 and IKK-2: cytokine-activated IkB kinases essential for NF-kB activation. *Science*, 278, 860–866.

Miller, G.L. (1959). Use of DNS reagent for the measurement of reducing sugar. *Analytical Chemistry*, 31, 426-428.

Miller, R.N.G. & Holderness, M. (2000). Molecular and morphological characterization of *Ganoderma*. In Flood, J., Bridge, P.D. & Holderness, M. (Ed). *Oil-palm plantings Ganoderma diseases of perennial crops* Wallingford, UK: CABI Publishing, 159–182.

Miller, T.L. & Churchill, B.W. (1986). Substrates for large-scale fermentations. In: Demain, A.L., Solomon, L.A. (Ed). *Manual of Industrial Microbiology and Biotechnology*. Washington, D.C . American Society for Microbiology. 123-136.

Mizuno, T. *et al.* (1984). Fractionation, structural features and antitumour activity of water-soluble polysaccharides from "Reishi" the fruit body of *Ganoderma lucidum*. *Nippon Negeckagahu Kaish*, 58, 871-880.

Mizuno, T. (1995). *Ganoderma lucidum* and *Ganoderma tsugae*: bioactive substances and medicinal effects. *Food Reviews International*, 11(1), 151-166.

Mizuno, T. (1999). The extraction and development of antitumour-active polysaccharides from medicinal mushrooms in Japan. *International Journal of medicinal mushrooms* ,1, 9-29.

Mizuno, T., Yeohlui, P., Kinoshita, T., Zhuang, C., Ito, H. & Mayuzumi, Y. (1996) Antitumor activity and chemical modification of poly-saccharides from Niohshimeji mushroom, *Tricholoma giganteum*. *Bioscience, Biotechnology and Biochemistry*, 60, 30–33.

Mohamad-Fata, M., Hossein, M., Shirin, G. & Ghorban-Ali, H. (2007). Immunomodulating and anti cancer agents in the realm of macromycetes fungi (macrofungi). *International Immunopharmacology*, 7(6), 701-724.

Moncalvo, J. (2005). Molecular systematic of *Ganoderma:* what is reishi. *International Journal of Medicinal Mushrooms*, 7(3), 353-354.

Moncalvo, J.M. (2006). A cladistic approach to biodiversity in the Ganodermataceae. *Mushroom Biology and Mushroom products.* Royse (Ed) 1996. Penn State Univ,75-89

Moncalvo, J.M., Wang, G., H.F. & Hseu, R.S. (1995). Gene phylogeny of the *Ganoderma lucidum* complex based on ribosomal DNA sequences: comparison with traditional taxonomic character. *Mycological Research*, 99 (12), 1489-1499.

Montgomery, D.C. (1997). Response surface methods and other approaches to process optimization. In Montgomery (Ed.). *Design and analysis experiments*. New York: John Wiley and Sons, 427-510.

Moreira, A., Souza, D.A, & Vendruscolo, C.T. (1998). Determinação da composição de biopolímero por cromatografia em camada delgada: metodologia. *Revista Brasileira de Agrociência*, 4 (3), 222-224.

Mueller, A., Raptis, J., Rice, P.J., Kalbfleisch, J.H., Stout, R.D., Ensley, H.E., Browder, W. & Williams, D.L. (2000). The influence of glucan polymer structure and solution information on binding to (1,3)-beta-D-glucan receptors in a human monocyte like cell line. *Glycobiology*, 10, 339-346.

Muller, C.I., Takashi, K., James, O., Navindra, P., Seeram, D.H. & Koeffler, H.P. (2006). *Ganoderma lucidum* causes apoptosis in leukemia, lymphoma and multiple myeloma cells. *Leukocyte Research*, 30, 841-848.

Murrill, W.M. (1902). The Polyporaceae of North America. I. The genus *Ganoderma*. *Bulletin of the Torrey Botanical Club*, 29,599–608.

Nik Hafizah, N.U. (2009). *Cendawan senduk berpotensi menandingi kehebatan cendawan merah*. *Agromedia*, 29 (2). 28-29.

OECD (*Guidance Document on using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests).*(1987). Economic Co-operation and Development, (Series on Testing and Assessment No. 129). Paris: OECD Publishing.

Oh, J.Y., Cho, K.J., Chung, S.H., Kim, J.H., Lillehoj, H.S. & Chung, K.S. (1998). Activation of macrophages by GLB, a protein polysaccharide of the growing tips of *Ganoderma lucidum*. *Yakhak Hoeji (Korean)*, 42, 302-306.

Ohno, N. (2001). Anti-tumor β-glucan from the cultured fruit body of *Agaricus blazei*. *Biological and Pharmaceutical Bulletin*, 24, 820–828.

Ohno, N., Furukawa, M., Miura,N.N., Adachi, Y., Motoi, M., Yadomae, T., (2001) Antitumor β-Glucan from the Cultured Fruit Body of *Agaricus blazei*. *Biological and Pharmaceutical Bulletin*, 24 (7), 820-828.

Pang, X., Chen, Z., Gao, X., Liu, W., Slavin, M, & Yao, W. (2007). Potential of a novel polysaccharide preparation (GLPP) from Anhui-grown *Ganoderma lucidum* in tumor treatment and immunostimulation. *Journal of Food Science*,72, 435–442.

Park, J., Kim, S. W., Hwang, H. J., Cho, Y. J. & Yun, J. W. (2002). Stimulatory effect of plant oils and fatty acids on the exo-biopolymer production in *Cordyceps militaris Enzyme and Microbial Technology*, 31, 250–255.

Patrik, R.L., Päivi, Y., Christer, L., Lars, E. & Mohammad, J.T. (2012). Growth tolerance of Zygomycetes *Mucor indicus* in orange peel hydrolysate without detoxification. *Process Biochemistry*, 47 (5), 836-842.

Pegler, D. N., & Yao., Y.J. (1996). Oriental species of *Ganoderma* section *Ganoderma*. In Wasser S.P. (Ed). *Botany and Mycology for the Next Millenium: Collection of Scientific Articles Devoted to the 70th Anniversary of Academician Sytnik KM. Kyiv*: Kholodny NG Institute of Botany, National Academy of Sciences of Ukraine, 336–347.

Pegler, D.N. (2002). Useful fungi of the world:Ling-zhi - The mushroom of immortality. *Mycol*, 16, 100-101.

Perkins, N.D., Schnid, R.M., Duckett, C.S., Leung, K., Rice, N.R.&. Nabel, G.J. (1992). Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation. *Proceedings of the National Academy of Sciences of the United States of America*, 9 (5), 1529-1533.

Russell, R. & Paterson, M. (2006). Ganoderma- a therapeutic fungal biofactory. *Phytochemistry* , 67, 1985-2001.

Ryu, D.S., Baek, G.O., Kim, E.Y., Kim, K.H. & Lee, D.S. (2010). Effects of polysaccharides derived from *Orostachys japonicus* on induction of cell cycle arrest and apoptotic cell death in human colon cancer cells. BMB reports, 43 (11),750-755.

Sanodiya, Bhagwan S., Thakur Gulab, S., Baghel Rakesh, K., Prasad, G. B.K.S, & Bisen, P. S. (2009). *Ganoderma lucidum*: A potent pharmacological macrofungus. *Current Pharmaceutical Biotechnology*, 10(8), 717-742.

Shih, I. L., Tsai, K.L. & Hsieh, C. (2006). Effects of culture conditions on the mycelial growth and bioactive metabolite production in submerged culture of *Cordyceps militaris*. *Biochemical. Engineering Journal*, 33, 193-201.

Slaughter, J.C. (1988). Nitrogen metabolism In Berry D.R. (Ed.). *Physiology of industrial fungi*, Blackwell Scientific Publications , Oxford, 58-79.

Sliva, D. (2003) *Ganoderma lucidum* (Reishi) in cancer treatment. *Integrative Cancer Therapies*, 2 (4), 360-364.

Steckley, J.D., Grieve, D.G., Macleod G. K. & Moran, J.R. (1979). Brewer's Yeast slurry, I. composition as affected by length of storage, temperature, and chemical treatment. *Journal of Dairy Science*, 62, 941-946.

Standbury, P. F. & Whitaker, A. (1984). *Principles of Fermentation Technology*. New York: Pergamon Press.

Steve, W.C. & Yolanda, B. (2006). Detection and determination of polysaccharides. *In Foods in food polysaccharides and their applications*. CRC Press. 675–712.

Steyaert, R. L. (1972). Species of *Ganoderma* and related genera mainly of the Bogor and Lieden herbaria. *Persoonia*, 7, 55-118.

Su, C., Sun, C., Juan, S.W., Hu, C., K, W. & Sheu, M. (1997). Fungal mycelia as the sources of chitin and polysaccharides and their applications as skin subtitutes. *Biomaterials*, 18, 1169-1174.

Suzuki, I., Tanaka, H., Kinoshita, A., Oikawa, S., Osawa, M. & Yadomae, T. (1990). Effect of orally administered beta-glucan on macrophage function in mice. *International Journal of Immunopharmacology*, 12 (6), 675-684.

Takashi, N., Hiroyuki, N. & Kazuaki, K. (2009). Investigation on the evaluation method of fungi − polysaccharide marker substance for the identification by gel permeation chromatography. *Chromatography*, 30 (1). 25-39.

Tang, Y.J. & Zhong, J.J. (2002a). Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. *Enzyme and Microbial Technology*, 31, 20-28.

Tang, Y.J. & Zhong, J.J. (2002b). Exopolysaccharide biosynthesis and related enzyme activities of the medicinal fungus, *Ganoderma lucidum*, grown on lactose in a bioreactor. *Biotechnological Letters*, 24, 1023-1026.

Takei, T. (2006). Biologically active component in the mushroom. *Foods Food Ingredients Journal*, 211, 1–3.

Wagner, R. (2003). Submerged Cultivation of *Ganoderma lucidum*. *Food Technology and Biotechnology*, 41 (4), 371-382.

Wagner, R., Mitchell, D.A., Sassaki, G.L., Amazonas, M.A.L.D. & Berovic, M. (2003). Current techniques for the cultivation of *Ganoderma lucidum* for the production of biomass, ganoderic acid and polysaccharides. *Food Technology and Biotechnology*, 41, 371–382.

Wang, S.Y., Hsu, M.L., Hsu, H.C., Tzeng, C.H., Lee, S.S., Shiao, M.S. & Ho, C.K. (1997). The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *International Journal of Cancer*, 70, 699-705.

Wasser, S.P., Elisashvili, V.I. & Tan, K.K. (2003). Effect of carbon and nitrogen sources in the medium on *Tremella mesenterica* Retz.:Fr. (Heterobasidiomycetes) growth and polysaccharide production. *International Journal of Medicinal Mushroom*, 5, 49-56.

Wasser, S.P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, 60, 258-274.

Wasser, S.P. (2005). Reishi or Ling Zhi (*Ganoderma lucidum*). In *Encyclopedia of Dietary Supplement* . New York: Encyclopedia of Dietary Supplement. 603-622.

Wasser, S.P. & Weis, A.L. (1999). Medicinal properties of substances occurring in higher Basidiomycete mushrooms: current perspective. *International Journal of Medicinal Mushrooms* 1, 31-62.

Weeks, B.A., Keisler, A.S. & Myrvik, Q. N. (1987). Differential uptake of neutral red by macrophages from three species of estuarine fish. *Developmental & Comparative Immunology*, 11(1), 117–124.

Xie, F. & Yezeng, Z. (2004). Cultural and Physiological Studies of *Ganoderma Neo-japonicum* and *G. Zonatum*. *Bio Formosa*, 39(1), 23-32.

Yang, F.C., Ke, Y.F. & Kuo, S.S. (2000). Effect of fatty acids on the mycelial growth and polysaccharide formation by *Ganoderma lucidum* in shake flask cultures. *Enzyme and Microbial Technology*, 27, 295-301.

Yang, F.C. & Liau, C.B. (1998a). Effect of cultivation conditions on the mycelial growth of *Ganoderma lucidum* in submerged flask cultures. *Bioprocess Engineering,* 19, 233-236.

Yang, F.C. & Liau, C.B. (1998b). The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochemistry*, 33(5), 547-553.

Yuen, J. W.M. & Gohel, M. D. I. (2005). Anticancer effects of *Ganoderma lucidum*: A review of scientific evidence. *Nutrition and Cancer*, 53, 11-17.

Zabriskie, D.W., Armiger, W.B., Philips, D.H, & Albano, P.A. (1982). Traders Guide to fermentation media Formulation, In *Traders Protein*. Memphis, 1-2.

Zheng, Y. G., Chen, X. L. & Wang, Z. (2005). Microbial biomass production from rice straw hydrolysate in airlift bioreactors. *Journal of Biotechnology*, 118, 413-420.