PRODUCTION AND BIOACTIVITY OF *Ganoderma lucidum* BCCM 31549 EXOPOLYSACCHARIDE USING SUBMERGED LIQUID FERMENTATION

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Wan Abd A. Q. I. Wan Mohtar

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LIST OF SYMBOLS AND ABBREVIATIONS

AM	Arbuscular mycorrhizal
BCCM	Belgian Coordinated Collections of Microorganisms
C	Carbon
DS	Degree of sulphation
DO	Dissolved Oxygen
EPS	Extracellular polysaccharide
FTIR-S	Flourier Transform Infrared Spectroscopy
Glu	Glucose
h	Hour
H	Hydrogen
mL	Millilitres
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
N_2	Nitrogen
O	Oxygen
RBF	Repeated-batch fermentation
RFBF	Repeated fed-batch fermentation
S	Sulphur
STR	Stirred tank reactor
PDA	Potato dextrose agar
P_{EPS}	Exopolysaccharide productivity
P_x	Biomass productivity
VVM	Volume of air per volume of culture per minute
v/v	Volume per volume
YE	Yeast extract
$Yield_{EPS/GLU}$	Yield of EPS on sugar consumed
$Yield_{DCW/GLU}$	Yield of biomass on sugar consumed

ABSTRACT

The RBF strategy has successfully produced fungal mycelial biomass and EPS in a very strictly regulated manner at high productivity rates compared to batch fermentation. The problematic lag phase and seed culture preparation were reduced in length; harvesting volume doubled, yield of product increased, and medium consumption was reduced in an RBF relative to batch. 80% broth replacement volume and transition phase were optimised. Dispersed mycelial filaments with ovoid-shaped pellets are the typical morphological characteristics associated with EPS production. N-limiting medium in an unbaffled 2.5-L bioreactor stimulated EPS formation during RBF compared to in baffled condition. The current study has managed to alter the molecule's hydrophobicity thus making it water-soluble as proved by compositional analysis and spectroscopy. The sulphated derivative of native glucan was identified as (1, 3)-β-D-glucan. Sulphation was an effective approach to improve antibacterial, antifungal, antiproliferative and immunomodulatory (NO stimulation) activity of the sulphated (1,3)-β-D-glucan or GS. GS maybe safe in *in vitro* trials due to its demonstrated lack of toxicity towards a normal human prostate cell line (PN2TA). GS also showed antimicrobial-antifungal-immunomodulatory activities derived from a single compound. Fungal cells tended to grow well in the porous structure of PUF cubes and the RBF using immobilised fungal cells was an efficient method for production of β-glucan with a high yield. This study could be beneficial for other medicinal mushroom fermentation.

CHAPTER 1

1.0 INTRODUCTION

For many years, fungi have been used to treat various health conditions due to their therapeutic properties. This application of fungi has been common in traditional medicine, but has recently also attracted increased attention in conventional medicine. With the rise of fungal-based drugs in the pharmaceutical industry, their therapeutic potential is clear.

The Eumycota represents a group of fungi that shares a common ancestor or a monophyletic group. This fungal group is distinct from the structurally similar slime moulds and water moulds. According to the 2007 classification of Kingdom Fungi, seven phyla are recognised by their sexual reproductive structures: Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Ascomycota and Basidiomycota (Hibbett et al., 2007). Amongst these, the Ascomycota and the Basidiomycota are contained within a branch signifying subkingdom Dikarya.

Ascomycota constitutes the largest taxonomic group within the Eumycota, together with the Basidiomycota, which are often referred to as the 'higher fungi' (Hibbett et al., 2007; McLaughlin et al., 2009). The most recent classification by Hibbett et al., (2007) recognised many types of subphyla and class level taxa among the Basidiomycota. Of these, one genus which has received particular attention in the present context is *Ganoderma*, a woody Basidiomycotina which belongs to the family of Ganodermaceae of Polyporales. Although inedible, *Ganoderma* has been

used in Chinese medicine as a remedy for longevity and health promotion since prehistoric times (Leskosek-Cukalovic et al., 2010). The most common species associated with therapeutic use is *Ganoderma lucidum* (Fr.) Karst (Polyporaceae) (Yang et al., 2000).

G. lucidum has been prescribed as an effective medicine to treat various human diseases, such as chronic hepatitis, nephritis, hepatopathy, hypertension, hyperlipemia, hypercholesterolemia, insomnia, arthritis, bronchitis, asthma, gastric ulcer, arteriosclerosis, leucopenia, diabetes, anorexia, neurasthenia, and gastric cancer, and also is reported to boost the immune system (Fazenda et al., 2008; Leskosek-Cukalovic et al., 2010; Liu et al., 2012b; Paterson, 2006; Yang et al., 2000).

Furthermore, *G. lucidum* produces several functional metabolites with biological activity, such as polysaccharides (Wagner et al., 2003), which might explain some of the observed medicinal properties. The polysaccharide fractions appear to be the primary source of the biological activity of *G. lucidum* (Hsieh et al., 2006). Traditional medical usage has stimulated the interest of scientists worldwide to undertake mass cultivation and production of the bioactive metabolites of these medicines (Li et al., 2013).

G. lucidum cultivation has traditionally been prevalent mostly in the regions of China, Japan, Korea, and Taiwan (Yang et al., 2000). During cultivation, the majority of active compounds are recovered from extracts from the fruiting body (Fazenda et al., 2008), which are ordinarily produced using solid-state fermentation (SSF). Nonetheless, the formation of the fruiting body takes six months and is

associated with many operational challenges. For this reason, much interest has focused on overcoming these challenges using submerged liquid fermentation (SLF).

SLF may be a better option than SSF, especially for obtaining useful cellular materials or for producing effective substances from *Ganoderma* (Liu et al., 2012b; Yang et al., 2000). Furthermore, SLF is capable of manipulating the fungal culture to optimise mycelial growth, reduce cultivation time, and minimise contamination (Chien et al., 2011; Wagner et al., 2003). However, Paterson (2006) observed that there have been fewer studies to date on extracts from the liquid-cultivated mycelium of *G. lucidum*, and reports on improvements to SLF are limited. This is because modern techniques (using bioreactors) are much easier to control, especially with respect to critical environment conditions such as temperature, dissolved oxygen, and pH. In fact, the reason that some of the *Ganoderma* preparations are not yet available as commercial medicines might stem from complications related to their large-scale production (Paterson, 2006; Smith et al., 2002).

To produce effective *G. lucidum* cultivation with reduced process time, more work using SLF fermenters is necessary (Paterson, 2006; Wagner et al., 2003). Therefore, the aim of this work was to improve the fermentation production of *G. lucidum* using a repeated-batch fermentation (RBF) strategy, with exopolysaccharide (EPS) as the primary target from the shake flask and bioreactor. Quantitative research methodology was used for the design of the RBF strategy. The second aspect of the study was concerned with mycelia-extracted EPS, and included structural characterisation using Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), and Elemental Analysis (EA) to obtain the β-glucan linkages with the sulphated derivative. The compound solubility was tested

and later screened for potential antimicrobial, antifungal, cytotoxicity, and immunomodulatory activities. The third element of the work was to improve RBF production using an immobilisation strategy. Taken together, this approach ensured a structuralised study of up- and down-stream processing for *G. lucidum* fermentation.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Macrofungi

In recent years, investigations have been carried out to distinguish between two groups of organisms, namely 'the lower fungi' (microfungi) and 'the higher fungi' (macrofungi) (Fazenda et al., 2008). Macrofungi are those filamentous fungi that form visible and fleshy fruiting bodies, comprising a macroscopic spore-bearing structure; microfungi are defined as fungi that habitually have a distinct filament and no fruiting bodies or macroscopic dimensions. Gow and Gadd (1995) have proposed that the potential of the macrofungi is undervalued, possible due to the complexity of their life cycles compared with the better-understood microfungi, and mainly with respect to their mycelial organisation and evolution (Carlisle et al., 2001). They can be tested for correlation using DNA metabarcoding as reported by Geml et al., (2014).

The macrofungi are eukaryotic, spore reproducers which are characteristically mycelial organisms, with chitin-based cell walls that have absorptive nutritional properties (Fazenda et al., 2008). The edible macrofungi are an established food source, but have also been used to improve human health and ensure longevity. For instance, *Lentinus edodes* (Shiitake), *Pleurotus ostreatus* (Oyster Mushroom), *Flammulina velutipes* (Enokitake), Chanterelle, and *Agaricus* spp are commonly consumed. Based on the macrofungi reproductive structures (sexual and asexual), five phyla divisions have been established: Zygomycota, Chytridiomycota, Ascomycota, Basidiomycota, and a new phylum, the Glomeromycota (Walker and Schüßler, 2004), whose members were previously included in the Zygomycota.

2.2 Phyla

2.2.1 Chytridiomycota

The first phylum to be discussed is the chytrids (Chytridiomycota), morphologically simple aquatic fungi which are characterised by zoospores that typically have a single, posterior-directed flagellum (James et al., 2000). These microorganisms, which are classified as macrofungi, are globally distributed and include approximately 1,000 designated species (Bartnicki-Garcia, 1970; James et al., 2006; McLaughlin et al., 2009). From a biochemical perspective, chytrids possess chitin in their cell walls, favour the α-aminoadipic acid lysine synthetic pathway, and use glycogen as their carbohydrate storage form.

2.2.2 Zygomycota

The phylum Zygomycota (Mucorales) represents ubiquitously-distributed, morphologically simple terrestrial fungi that are unified taxonomically by the possession of coenocytic mycelium (O'Donnell et al., 2001). Composed of ten morphologically and ecologically distinct orders, they include endo- and ectomycorrhizal symbionts of vascular plants. For reproduction, non-motile mitotic spores are produced asexually in uni- to multispored sporangia, while zygospores are formed following the fusion of sexually compatible hyphae (Hibbett et al., 2007; O'Donnell et al., 2001).

2.2.3 Glomeromycota

As described in the literature, a phylum was split from the polyphyletic Zygomycota and placed into a new monophyletic phylum, entitled the

Glomeromycota (Schüßler et al., 2001). The informal glomeromycetes of Glomeromycota is one of the seven presently-recognised phyla within the Kingdom Fungi (Hibbett et al., 2007), with approximately 230 designated species (Walker and Schüßler, 2004). This group is referred to as arbuscular mycorrhizal (AM) fungi and identified as obligate symbionts which favour asexual reproduction (Walker and Schüßler, 2004). The AM species is terrestrial and broadly scattered in soils worldwide, where they form symbioses with the roots of common plant species.

2.2.4 Ascomycota

The Ascomycota consists of 60,000 described species, representing almost 75% of all known fungi globally (Kirk et al., 2001). As a member of the subkingdom Dikarya, Ascomycota form croziers for their dikaryotic hyphae cells. Consequently, they typically have mycelia with simple tapered septa, and cells customarily exist as diploids. In other words, they favour sexual reproduction that later produces spores called ascospores inside an ascus (Hibbett et al., 2007).

2.2.5 Basidiomycota

The phylum selected for this work is Basidiomycota, and its differences from its closest counterpart (Ascomycota) are rigorously described by Fazenda et al. (2008). Kirk et al., (2001) has determined that Basidiomycota encompasses about 30,000 species; that is, 37% of the defined species of fungi. Hibbett et al., (2007) has reported that Basidiomycota is grouped under the subkingdom Dikarya, which develops clamp connections, has a complex dolipore septum, and whose mycelium is the dominant form throughout the life cycle. All fungi that produce clamp connections are members of the Basidiomycota, but not all Basidiomycota form

clamp connections (Fazenda et al., 2008). For growth, Basidiomycota develop sexual spores (exogenous basidiospores) on a particular sporogenous cell called a basidium (Carlisle et al., 2001). Their cells exist as dikaryons, in which two genetically compatible nuclei exist disjointedly, dividing in a coordinated manner.

In SLF, this feature has implications such as the complex process of clamp connections, maintenance of the dikaryotic state, and modest growth rates compared with other fungi. Typically, Basidiomycota have lengthier fermentation processes than Ascomycota for the above reasons. The microorganism used in this thesis was a Basidiomycete, *G. lucidum* (section 2.6.2), so this species alone will be further discussed.

2.3 Habitat

The described phyla can exist in several types of habitat; Zygomycota is found in soil or on decaying plant or animal materials, Chytridiomycota in fresh water, and Glomeromycota in plant roots (Hibbett et al., 2007; McLaughlin et al., 2009). Meanwhile, Ascomycota are found in all land ecosystems worldwide, occurring on all continents including Antarctica (Laybourn-Parry, 2009), the atmosphere and freshwater environments. Basidiomycota, however, prefer vegetable matter as their host, especially decaying cellulose-based plants (Lindequist et al., 2005; Smith et al., 2002; Stamets, 1993).

2.4 Functional properties

Many interesting compounds that possess antibacterial, phytotoxic, nematocidal, cytostatic, antiviral, antifungal, and other pharmacological properties have been isolated from macrofungi (Fazenda et al., 2008). In general, most of these

bioactivities are associated with the phylum Basidiomycota, or so-called 'medicinal mushrooms'; as selected for investigation in this study.

2.5 Mushrooms

Mushrooms have been consumed throughout history, and the most studied mushrooms for the treatment of disease are the medicinal mushrooms (Lindequist et al., 2005; Liu et al., 2012a). The Japanese are believed to have been the first to grow medicinal mushrooms, cultivating Shiitake (*Lentinus edodes*) two thousand years ago; the practice was followed by other Oriental countries such as Taiwan and China. Medicinal mushrooms are high in demand because research has shown that numerous species of these mushrooms produce anticancer, antihyperglycaemic, antiviral, antimicrobial, cardioprotective and anti-inflammatory compounds (Lindequist et al., 2005; Paterson, 2006). The use of these mushrooms has a long tradition in Asian countries, whereas their use in the Western hemisphere has marginally increased, only in recent decades. As described previously, the most prevalent medicinal mushrooms fall under the division of Basidiomycetes (*Ganoderma* families) (Leskosek-Cukalovic et al., 2010; Paterson, 2006), so improvements in the cultivation of these species may be beneficial.

2.5.1 *Ganoderma*

The genus *Ganoderma* includes mushrooms that grow on wood, both coniferous and hardwood species, and typically on Blume logs (*Quercus variabilis*) (Dennis and Ainsworth, 1962). *Ganoderma* comprises about 80 species, mainly from the humid regions of China, Korea, Japan and Malaysia (Leskosek-Cukalovic et al., 2010). These species also have a worldwide distribution in both tropical and temperate

geographical regions, including North and South America, Europe, Africa, and Asia. In part, *Ganoderma* is a white-rot fungus containing enzymes that permit them to break down wood components, such as cellulose and lignin. In traditional Asian medicines, *Ganoderma* is an economically important genus, denoted as bracket fungi or shelf mushrooms. Amongst the species that are popular due to their medicinal properties are *G. applanatum*, *G. atrum*, *G. tsugae*, and *G. lucidum.* Since the 1980s, *Ganoderma* species have been the subject of much research interest (Fazenda et al., 2008; Figlas and Curvetto, 2000; Liu et al., 2012b).

2.5.2 *Ganoderma lucidum*

G. lucidum (Basidiomycota) was chosen for the current study based on its reported value in the literature (Chen et al., 2014; Fraga et al., 2014; Liu et al., 2014; Shi et al., 2013; Tung et al., 2013). This well-known species is also termed 'Lingzhi' in China, and is widely used in traditional medicine. The fruiting body part of this species contains numerous chemical substances, including triterpenes and many types of polysaccharides. The polysaccharides of Lingzhi are the core source of its natural activity and healing properties such as anti-inflammatory and antitumour effects and cytotoxicity to hepatoma cells (Hsieh, 2004).

Since 1988, Lingzhi has gained widespread use as a fitness and health food, mostly in tropical countries (Huie and Di, 2004; Lorenzen and Anke, 1998). Likewise, it has been used in Taiwan and Korea as a popular medicine for treating various illnesses such as hypertension, hepatitis, hypercholesterolemia and gastric cancer, encouraging longevity, lowering the risk of cancer, heart disease and increasing the immune system (Figlas and Curvetto, 2000).

2.5.3 Appearance of *G. lucidum*

The main mycological or observable characteristics of *G. lucidum* are its hymenium pores, bare stipe, brown spore print, offset cat, and parasitic or saprotrophic behaviour (Stamets, 1993). *G. lucidum* arises in six different colours, but the red variety is most common (Nasreen et al., 2005). This species are typically found as flat, soft, corky, kidney-shaped caps (Fig. 2.1) with white to dull brown pores underneath the cap (Arora and Shepard, 2008).

2.5.4 Natural habitat of *G. lucidum* and its life cycle

Naturally, *G. lucidum* is observed as a shelf-like mushroom that grows on dead and dying trees (Nasreen et al., 2005). It is typically found with its nearby relative, *G. tsugae*, in the northern Eastern Hemlock forests of Canada. In the wild, Lingzhi matures on old logs and at the base and stumps of deciduous trees, mainly oak and maple (Stamets, 1993). Cold weather causes their irregular shape while high concentrations of carbon dioxide in these locations stimulate the fruiting body stage.

Figure 2.1 Fruit body of *G. lucidum* (adapted from http://www.stridvall.se/fungi/gallery/album45/AAAA2241)

The life cycle of *G. lucidum* starts with the germination of haploid basidiospores to form monokaryotic mycelia, where each cell contains a haploid nucleus (primary mycelium). Later, when two genetically compatible monokaryons are joined, they undergo somatogamy to produce secondary mycelium-containing dikaryotic cells. The two haploid nuclei divide synchronously by a mechanism comprising clamp connections, which appear to ensure that the dikaryotic state of the cells is sustained (Deacon, 2005). The key feature of the life cycle of *G. lucidum* is the production of fruiting bodies called ‘mushrooms’ or ‘toadstools’, as shown in Fig. 2.2.

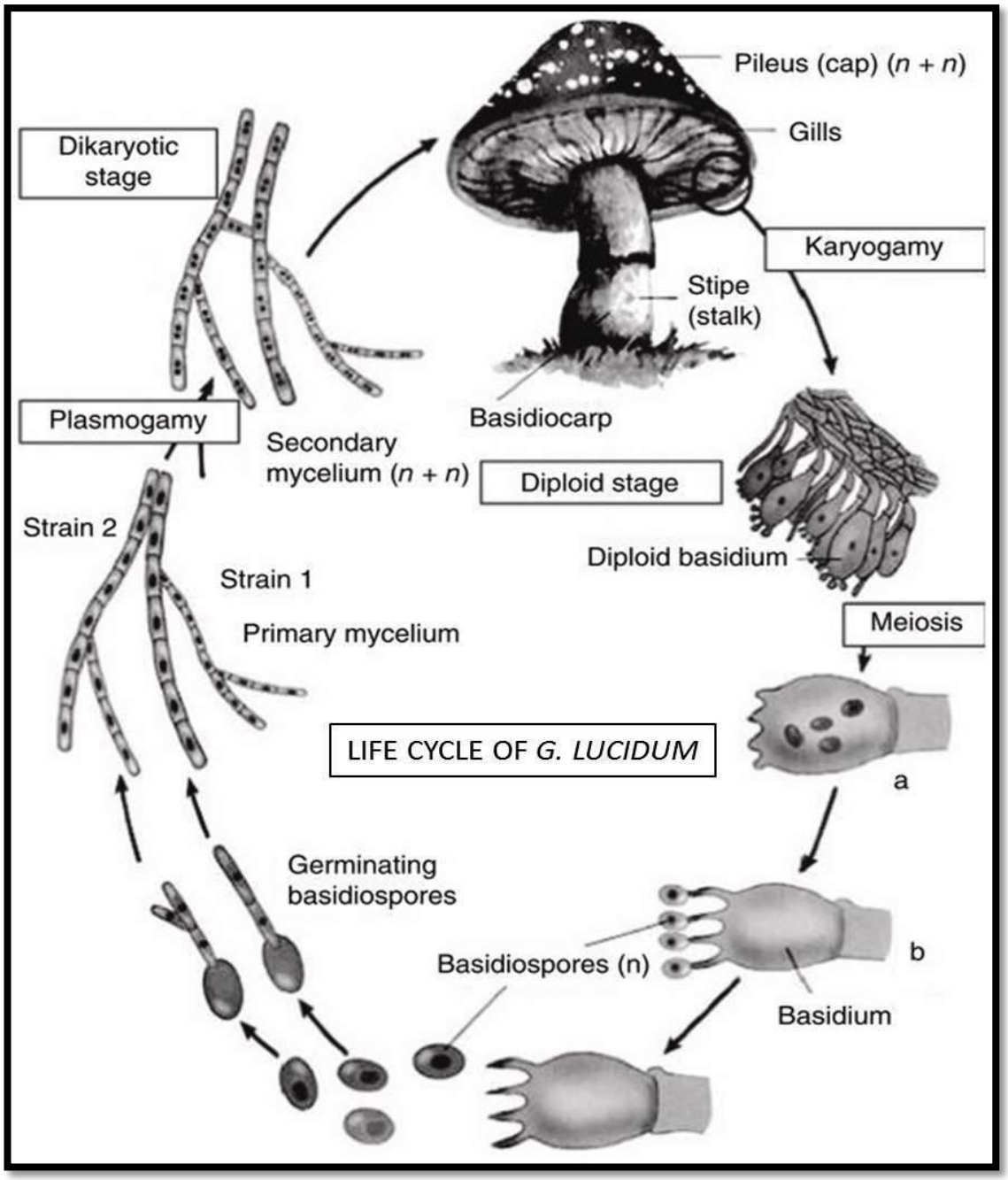


Figure 2.2 Life cycle of *G. lucidum*. Basidium development: (a) Formation of basidiospores, where the four nuclei have not migrated into spores; (b) Nuclear migration into basidiospores (adapted from Fazenda et al., (2010)).

2.5.5 Toxicity of *G. lucidum*

G. lucidum does not exhibit cytotoxicity and is this considered a safe food, due to its long history of oral administration which has not been associated with toxicity (Figlas and Curvetto, 2000; Kim et al., 1986). Moreover, extracts of *G. lucidum* that have been used in animal trials exhibited very low levels of toxicity, indicating that the organism is safe for human consumption (Figlas and Curvetto, 2000).

2.5.6 Role of *G. lucidum*

The use of *G. lucidum* is common in Japan, China and other countries as a source material for the development of natural drugs (Nasreen et al., 2005). Typically, the fruiting body of *G. lucidum* is employed medicinally. Section 2.6 describes the roles of this species, particularly in terms of its pharmacological properties.

2.5.7 Natural cultivation of *G. lucidum*

The global production of *G. lucidum* was valued at 4900–5000 tonnes in 2002, and at least 100 brands of this fungal product are available on the market (Nasreen et al., 2005). Until the nineteenth century, the techniques used for the early cultivation of this mushroom were significantly different than those employed today. The first cultivation of this species involved gathering the fruit bodies from their natural habitat and taking them to a region of ‘fresh’ substrate. Eventually, spores would germinate and colonise the substrate, thus giving rise to new fruit bodies (Fazenda et al., 2008). There has been significant progress made in biochemical fermentation methods related to the cultivation of *G. lucidum* in recent years

(Pandey, 2003) through the implementation of kinetic analysis and mathematical modelling (Suryanarayan, 2003). *G. lucidum* mycelium has traditionally been grown in waste from the processing of citrus fruits and molasses, and in sulphite waste liquor from the pulp and paper industries (Nasreen et al., 2005). This significant improvement of an ancient technology is termed solid-state fermentation (SSF).

SSF is defined as when the growth of *G. lucidum* on a solid substrate proceeds in the near absence of free water ($a_w \approx 0$), but with sufficient presence to sustain fungal growth and metabolism (Fazenda et al., 2008; Hsieh, 2004). SSF has been tested in the solid fermentation of pasteurised substrates, inoculated with suitable spawn that progresses under controlled temperature and humidity. In other words, it is the cultivation of fungi under controlled conditions for the production of bioproducts. Much work had been done to cultivate a complete fruiting body of *G. lucidum* using SSF (Hsieh, 2004; Hsieh et al., 2005; Hsieh et al., 2006).

SSF utilises agro-industrial residues such as grain, sawdust and wood, and cheap raw materials as substrates for growth. During the SSF upstream process, these substrates are broken down by the extracellular enzymes of the fungus, permitting further microbial processing to yield valuable foodstuffs. These SSF-produced foodstuffs are products of an ancient technology that is similar to the Koji processes that produce soy sauce, tempeh miso, and sake (Dennis and Ainsworth, 1962; Fazenda et al., 2008).

SSF is a process that is challenging to control, monitor, and scale-up, and is restricted to processes that do not require ‘containment’ (meaning that operatives can come into contact with the product during cultivation). Furthermore, in SSF, the

fruiting body composition is highly variable, particularly in the content of their bioactive substances, and this traditional method makes extraction (of polysaccharides) from fruiting bodies challenging (Lo et al., 2006). Thus, the production of fungal bioproducts in established agro-industries had been widely replaced by solid-liquid fermentation (SLF) processes, chiefly with respect to *Ganoderma* species.

2.6 Pharmacological properties of *G. lucidum*

2.6.1 Antimicrobial activity

Over the last three decades, the problem of antibiotic resistance has increased. This resistance is due to the behaviour of bacterial and fungal pathogens that have developed numerous defence mechanisms against antimicrobial agents. The present necessity to discover novel and more potent natural' drugs as alternatives to (or in association with) antibiotic therapy using synthetic drugs is substantial (Kim et al., 2006b). Newly-discovered natural compounds are the focus of several biotechnological companies that are searching for new antimicrobial drugs (Heleno et al., 2013), typically from mushrooms. Antimicrobial mushrooms, typically from the phylum of Basidiomycota, are rich sources of bioactive compounds with a vast variety of chemical structures. In this respect, the isolated Basidiomycota compounds may be of value in the search for new potent antimicrobial agents (Ariyajaroenwong et al., 2012). In previous work, Fazenda et al., (2008) reported that Basidiomycota (from *Ganoderma* species) was effective in treating a range of microbial infectious diseases in humans, yet these species have not been further investigated.

Extracts of *G. lucidum* are used as functional food additives and in preventative remedies, with an annual international market revenue of over USD $1.5 billion (Pala and Wani, 2011). However, there are few on antimicrobial activities of this species (typically with respect to polysaccharide extracts) as natural antibacterial agents (Gao et al., 2003; Li et al., 2012). This fungus exerts its antibacterial activity by inhibiting the growth of bacteria and killing pathogenic bacteria (Skalicka-Wozniak et al., 2012).

Positive outcomes were reported against Gram-positive bacteria from using extracts of *G. lucidum* (Kim et al., 1993), while Yoon et al., (1994) has explored the additive effect observed using an aqueous extract of *G. lucidum* with four known antibiotics; the antibacterial activity was shown to increase. Furthermore, a recent study by Prasad and Wesely (2008) assessed *G. lucidum* for its antimicrobial properties on Multidrug resistant *Staphylococcus aureus* (MRSA). However, most active extracts in that study were sourced from the fruiting bodies and not from the mycelium. Mycelium-based extracts are considered superior, yet the amounts available of a standardised quality are scarce.

Chang et al., (2006) and Prasad and Wesely (2008) recommended further research on suitable quality parameters and analytical methods to produce antimicrobial-mycelium products. To date, there are relatively few studies on antimicrobial or antifungal extracts (as discussed in Chapter 5) using SLF (Paterson, 2006). The use of screening assays is also presented in Chapter 5.

2.6.2 Antiviral activity

During the 1920s, Gao et al., (2003) studied the 'antiviral value' of members of the genus *Ganoderma*, including major biologically active constituents, effects and mode of action. An acidic protein-bound polysaccharide isolated from water-soluble substances of *G. lucidum* was shown to possess antiviral activity against herpes simplex viruses (HSVs) (Huie and Di, 2004).

2.6.3 Antitumour activity

Eastern societies have traditionally cultivated the species of Basidiomycota, especially *G. lucidum*, for formulating traditional medicines. Zaidman et al., (2005) proved that medicinal effects were mainly due to β-glucans (polysaccharides) and their derivatives. For example, the antitumour activity noted against transplanted Sarcoma 180 in mice, both from *G. applanatum* (Sasaki et al., 1971; Wasser, 2002) and *G. lucidum* (Sone et al., 1985; Wang et al., 2009), was mainly attributed to β-glucans.

Wang (2009) reported amplified interleukin and tumour necrosis factor production by human macrophages and T-lymphocytes after incubation with polysaccharides extracted from the fresh fruiting bodies of *G. lucidum* (Dudhgaonkar et al., 2009; Ibelgaufts, 1995). Of the various extracts of *G. lucidum*, 150 types of triterpenes have been isolated and have received substantial attention due to their ability to inhibit tumour growth. These observations indicate the therapeutic potential of *G. lucidum* for the clinical prevention and treatment of tumours (Liu et al., 2012a).

2.6.4 Anticancer activity

The current interest in anticancer properties of *G. lucidum* is explained in more detail in this section. In general, anticancer or antineoplastic drugs are used to treat malignancies or cancerous growth, hence controlling the growth of the malignant cells (Ren et al., 2013). Routinely, drug therapy might be used to achieve this alone, or in combination with other treatments such as surgery or radiation therapy. Cancer is typically defined as the uncontrolled growth of cells, with the loss of differentiation and progression to metastasis, the spread of cancer to other organs and tissues. To date, several classes of drugs are used in cancer or malignant growth treatment, depending on the affected organ.

According to Kao (2013), cancer cells have a continuous cell cycle leading to uncontrolled cell proliferation. Extracted anticancer agents can arrest the cell cycle, thus diminishing the rate of proliferation (Patra et al., 2011); research has shown that extracts from Basidiomycetes can stop the cell cycle. As an example, ganodermanontriol, a *G. lucidum* mushroom triterpene extract, inhibited the proliferation of colon cancer cell lines HCT116 and HT-29 by inhibiting the expression of β-catenin (Jedinak et al., 2011). Studies have shown that treatment with *G. lucidum* increases the ratio of Bax/Bcl-2 in human cancer cells by increasing Bax expression while downregulating Bcl-2 expression (eg. inhibits cell death, suppress chemotherapy-induced apoptosis, and regulate tumour suppressor p53) (Liu and Zhong, 2011). This finding is a primary reason for the substantial development in *G. lucidum* bioproduct research and mycelium cultivation, due to the possibility that these agents may address the high demand for natural anticancer therapies over modern synthetic drugs.

Synthetic drugs are used to treat the common forms of cancer, such as prostate and breast cancer, by disrupting cell growth (Ren et al., 2013). Since cancerous cells grow more rapidly than other cells, synthetic drugs target those cells that are in the process of reproduction. As a result, these drugs affect not only the cancerous cells, but other healthy cells that also replicate quickly, including blood-forming organs, ovaries and testes, and the hair follicles. Therefore, researchers have screened more than 23,000 natural compounds to identify those that help fight cancer while leaving healthy cells unharmed (Chen et al., 2010).

Extracts of *G. lucidum* are popular natural drugs and have been widely used for the joint promotion of health, particularly for the prevention of several types of cancer (Wasser, 2010). Reports show that the isolated compounds displayed cytotoxicity against cancer cells (Min et al., 2000) and inhibited growth and cancer metastases (Kimura et al., 2002; Wasser, 2002). Therefore, *G. lucidum*, in the form of a dietary supplement or as a naturally-derivative drug, can be used as an additional therapeutic aid in cancer patients (Fraga et al., 2014).

The most reactive anticancer compounds are polysaccharides, which have shown beneficial properties such as the capacity for inhibiting carcinogenesis, inducing apoptosis, and suppressing the migration of cancer cells (Fraga et al., 2014). These properties frequently lead to the cytotoxicity of the extracted *G. lucidum* polysaccharide against cancerous cells. EPS produced by this species have been investigated for their potential therapeutic activities (Paterson, 2006; Sone and Misaki, 1985) which have been linked to their structural features, mainly the branched glucan core involving β-(1-3) and β-(1-6) linkages. These glucans have been investigated using SLF techniques (Berovic et al., 2003). The anticancer and

cytotoxic properties of glucans from *G. lucidum* are discussed in Chapter 5, and are sourced from their underutilised mycelial extracts and not from sporing bodies.

2.6.5 Immunomodulation

The mechanisms of action of fungal polysaccharides and their chemical derivatives as powerful immune modulators are complex. These compounds may affect both the innate and adaptive immune systems via a stimulation reaction. This reaction recognises β-glucan as a foreign or non-self molecule (Chen and Seviour, 2007), thus promoting immunomodulation. One report has stated that *G. lucidum* may have a potential immunomodulating effect in patients with advanced colorectal cancer (Chen et al., 2006). Nevertheless, more work is required in this area.

2.7 Bioactive compounds extracted from *G. lucidum*

The valuable health properties of *G. lucidum* are exhibited across a broad range of bioactive components present in the fruiting body, spore, and mycelium (Leskosek-Cukalovic et al., 2010). As bioactive compounds, polysaccharides and triterpenes are the two major groups, followed by phenols, mycins, vitamins, nucleosides, steroids, amino acids, lignins, and nucleotides, as listed in Table 2.1.

Table 2.1 The effects of bioactive compounds derived from *Ganoderma*

Extract	Bioactivity	Reference
Adenosine	Anti-platelet aggregation	(Kawagishi et al., 1997)
Lectins	Mitogenic	(Ngai and Ng, 2004)
Protein (LZ-8)	Immunomodulatory	(van der Hem et al., 1995)

	Immunosuppressive	(van der Hem et al., 1995)
Polysaccharides	Antifibrotic	(Park et al., 1997)
	Antiherpetic	(Oh et al., 2000)
	Antidiabetic	(Pan et al., 2014)
	Anti-inflammatory	(Ukai et al., 1983)
	Antioxidant	(Chen et al., 2014)
	Hepatoprotective	(Zhang et al., 2002)
	Hypoglycemic	(Zhang and Lin, 2004)
	Immunostimulatory	(Pi et al., 2014)
	Antitumour	(Zhang et al., 2013)
	Miscellaneous (radiation protection, DNA damage)	(Lee et al., 2001)
Terpenoids and related compounds	Antibacterial	(Smania et al., 1999)
	Anticancer	(Lin et al., 2003)
	Anticomplement	(Min et al., 2001)
	Anti-inflammatory	(Ko et al., 2008)
	Immunomodulatory – antitumour	(Nakamura et al., 2007)
	Immunotherapeutic activities	(Chan et al., 2005)
	ACE inhibitory	(Liu et al., 2006)
	Hypotensive	(Morigiwa et al., 1986)
	Antineoplastic	(Zhao et al., 2011a)
	Antihepatitis B	(Li and Wang, 2006)
	Antimicrobial	(Moradali et al., 2006)
	Antifungal	(Wang and Ng, 2006)

Antiviral	(Paterson, 2006)
5α-reductase inhibition	(Liu et al., 2006)
Antioxidant	(Zhu et al., 1999)
Antiaging ergosterols	(Weng et al., 2010)
Antiplatelet aggregation	(Shiao, 1992)
Enzyme inhibitors	(Lee et al., 1998)
Hepatoprotective	(Shieh et al., 2001)
Hypolipidemic (cholesterol inhibitors)	(Hajjaj et al., 2005)

*Literature on therapeutic activities up until 2015 is included.

2.7.1 Polysaccharides and β-glucans

Polysaccharides from *Ganoderma* are a structurally diverse class of biological macromolecules with a broad range of physicochemical characteristics, of which the most-studied are those of the β-glucans (Paterson, 2006). β-glucans are made up of D-glucose monomers linked by β-glycosidic bonds and contain only glucose as a structural constituent. Several forms of β-glucan are valuable in human nutrition as soluble fibre supplements and texturing agents. These compounds are commonly found as cellulose in plants, the cell wall of baker's yeast, the bran of cereal grains, fungi, bacteria and mushrooms. To the best of our knowledge, medicinal mushroom-produced β-glucans are significant for their therapeutic activities, especially from the species of *G. lucidum*.

β-glucans of *G. lucidum* have gained wide attention as a health supplement due to their perceived health benefits (Hsieh et al., 2006). For instance, several glucans isolated in the early 1980s from alkali and water extracts were found to be

bioactive (Paterson, 2006). Although these extracts have been isolated from more than 200 basidiocarps (Zhang and Lin, 2004) and mycelial biomasses (Kim et al., 1993), only a few have been isolated from culture media (Kim et al., 2003). These highly valuable compounds have to date been inadequately researched, especially with regards to their production (Liu et al., 2012b; Zhang et al., 2012).

Many attempts, including the use of SLF, have been made to increase extracellular polysaccharide (EPS) content, especially β-glucans (Hsieh et al., 2006). The structure of β-glucans is β-1-3 D-glucopyronan with 1–15 units of β-1-6 monoglucosyl side chains; this constituent promises to be a novel type of carcinostatic agent, which may eventually be useful in immunotherapy (Mizuno et al., 1995).

The therapeutic activity of β-glucans depends on features such as chemical structure, conformation, molecular weight, and configuration of the glycosidic linkages. These in turn seem to be reliant on several growth factors such as morphology, fermentation time, temperature, pH, oxygen, stirring, use of baffled and unbaffled bioreactors, carbon (sugar choices), nitrogen, dissolved oxygen, catabolite repression and continuous culture method (Chen et al., 2008; Ferreira et al., 2015a; Liu et al., 2014; Ma et al., 2013; Papinutti, 2010; Skalicka-Wozniak et al., 2012; Wei et al., 2014; Zhang et al., 2012). These growing conditions may affect the biological activities of the extracted compounds. To date, there is no consensus on the optimal growth and glucan-forming conditions for *G. lucidum*, as parameter manipulation may lead to an increase in the desired target (either polysaccharide or biomass).

2.7.1.1 Solubilisation of water-insoluble β-glucan

The biopolymers extracted from *G. lucidum* are mostly water-insoluble (1-3)-β-D-glucans; for pharmaceutical study, this may cause challenges due to their poor water solubility. Depending on their degree of polymerisation, branching, and chemical derivation, the solubility of the extracted β-glucan can vary (Wang et al., 2005b). β-glucan depolymerisation by acidic or alkali hydrolysis, enzymatic degradation, or ultrasonic treatment, along with sulphation and phosphation, have been evaluated to overcome this problem by improving solubility in aqueous solution (Han et al., 2008). Of these, sulphation is the preferred solubilisation technique due to its positive impact on biological function and formation of sulphated β-glucan.

2.7.1.2 Sulphated β-glucan

Previously, chemically-derived polysaccharides including sulphated and carboxymethylated polysaccharides have received much attention due to their biological properties (Wang et al., 2009). These polysaccharide derivatives showed improved water solubility and altered chain conformation, resulting in the enhancement of their biological activities (Wang et al., 2009).

These bioactive polysaccharides may be developed and improved using chemical modifications, thus widening their application and ease of experimental use. Of these, sulphated chemical agents and modification may help to generate increased bioactivity, functional properties and polysaccharide by-products (Zhang et al., 2012). By definition, sulphation is a method where the extracted β-glucan is treated as another state or structural organisation of β-glucan through the introduction of a sulphate group. Popularly-utilised methods for the sulphated modification of

polysaccharides include sulphuric acid, sulphur trioxide-pyridine, chlorosulphonic acid-carboxamide, chlorosulphonic acid-pyridine and sulphur trioxide-dimethylformamide (Chen et al., 2010). Further explanations and observations are presented in Chapter 4.

2.8 Artificial cultivation of *G. lucidum*

G. lucidum fruiting body production (SSF) or so-called the artificial cultivation is an extended procedure that usually takes several months for the first product to appear. Product quality from this route can be variable (Liu et al., 2012a). The development of SLF methods for these organisms permits hastening of the growth reactions, resulting in a biomass yield in several days (Nasreen et al., 2005). Therefore, researchers have focused on studying SLF conditions to accelerate *G. lucidum* growth and formation of bioproducts (Liu et al., 2012a; Yang et al., 2000). In this context, derivatives of SLF, batch or fed-batch fermentation, continuous batch fermentation, and repeated-batch fermentation (RBF) have been attempted to boost the cultivation of this species.

2.8.1 The fermentation strategies of *G. lucidum*

SLF is a process which utilises free-flowing liquid substrates such as soluble sugars, liquid media, molasses, plant-based juices, and waste water (Subramaniyam and Vimala, 2012), mainly for the extraction of bioactive metabolites. SLF has become the method of choice for *G. lucidum* cultivation, principally due to the superior characteristics of products compared with SSF (Fazenda et al., 2008). SLF is far less problematic (e.g., it exhibits better heat and oxygen mass transfer and improved mixing) than SSF, making it more consistent, flexible, reliable, and easier

to monitor and control the key parameters (Fazenda et al., 2008). Furthermore, SLF yields bioactive compounds more rapidly and hygienically (sterile).

G. lucidum growth in SLF has implications, particularly for intricate and synchronised processes like the formation of clamp connections and maintenance of the dikaryotic state, resulting in moderate growth rates compared with other Basidiomycetes (Fazenda et al., 2008). The reason for this is that the clamp connections joining the hyphae may deteriorate in a long culture period which can affect longevity.

2.8.2 Batch and fed-batch fermentation

Batch fermentation technology is the most ancient and simplest technique for cultivating *G. lucidum*. Although the process can endure only from 2 to 5 days of cultivation, due to an extended lag phase the entire process can be prolonged by up to 18 days (Oh et al., 2007). Unfortunately, this method has a growth rate that is ten times lower than that of normal filamentous fungi, a major restricting factor to the commercial cultivation of *G. lucidum*. Hence, to develop the most efficient approach using SLF, all information associated with batch cultures requires careful examination to identify better methods for accelerating production.

Fed-batch fermentation technology has also been introduced in order to improve the traditional batch fermentation process. This method incorporates feeding according to different strategies and depending on the desired product. As an example, by using initial low glucose (10 g/L) and three different glucose levels (10, 25, and 50 g/L), this strategy was shown to enhance process efficiency for *G.*

resinaceum (Kim et al., 2006a). Here, the fed-batch mode is a useful tool to explore substrate limitation effects and possible inhibition characteristics (Papagianni, 2004).

The fed-batch method is a cost-efficient procedure for the profitable production of bioproducts because, throughout this process, nutrients are provided to the reactor while cells and products remained in the reactor until the end of fermentation. This method has been broadly applied to the production of numerous by-products of interest (Qu et al., 2013). Implementation of this technique in the cultivation of *Ganoderma* species is therefore worth researching in more detail.

2.8.3 Continuous batch fermentation

In recent years, Rau (2004) reported an improvement on the reported batch fermentation into a continuous culture system, thus demonstrating the potential of such regimes. In contrast to the batch fermentation process that periodically adds nutrients, continuous fermentation adds nutrient continuously. When the continuous technique is used as a chemostat, it is preferably suited to growth-related products such as cell biomass or intracellular-polysaccharide (IPS). In other words, continuous culture avoids the prolonged downtime of batch cultures and eliminates long lag phases as all cells are growing exponentially (Fazenda et al., 2008). However, no research to date has been done on *G. lucidum* using continuous batch fermentation due to the high possibility of contamination, lower conversion, harvesting limitations, and inconsistent productivity during the fermentation process (Feng et al., 2012).

2.8.4 Repeated batch fermentation

In this study, a strategy that can avoid the limitations of the batch process as well as accommodate the physiology of *G. lucidum* fungal cells is required. This strategy formed one of the goals of this work. Amongst the constraints of batch fermentation which needed to be addressed are nutrient exhaustion, unattainability of harvesting at the initial operation stage, catabolite repression from the excreted compounds, incapability to control process operation and the level of substrate uptake. Furthermore, the insertion of freshly prepared nutrient and inoculum into the primary batch fermentation occurs only once, thus saving the use of medium.

Additionally, the high quantity of nutrient required in the batch fermentation strategy can trigger catabolite repression. Based on these drawbacks, an underutilised method was proposed for the improvement of SLF specifically for *G. lucidum*, namely, repeated-batch fermentation (RBF). RBF is an alternative process of fermentation in which the medium or part of the medium is withdrawn, and fresh medium is added periodically (Birhanli and Yesilada, 2010). RBF also allows the possibility of storing and reusing fungal pellets with maintenance of long-term activity (Birhanli and Yesilada, 2006). As a result, no fresh inocula are required at each repeated batch cycle.

Use of the proposed RBF technique to culture *G. lucidum* may overcome the limitations and restrictions attributed to the batch process itself. In RBF, nutrient exhaustion can be overcome by both fresh media and nutrient insertion, which also lessens catabolite repression. Besides, the harvesting procedure will occur at earlier stages (during the exponential phase), thus improving production effectiveness while reducing growing time. One other feature of RBF is the high-quality data that may be

generated from cultivated *G. lucidum* which can later undeniably link cause and effect relationships between culture parameters and product quality.

Recent research by Moeller et al., (2010) and Ganjali Dashti et al., (2014) has indicated that RBF reduced the bioreactor preparation time to a minimum in comparison with batch fermentation. Furthermore, the fermentation time was reduced due to the loss of the lag phase of the microorganisms studied (*Yarrowia lipolytica* and *Cunninghamella bainieri*) in successive cycles. Birhanli and Yesilada (2010) showed that both white rot fungi *Funalia trogii* and *Trametes versicolor* experienced enhanced product formation (laccase) with RBF compared with batch processes.

Table 2.2 Comparison of batch, fed-batch, and repeated-batch fermentation for *G. lucidum* cultivation

	Technique		
Detail	Batch	Fed-batch	RBF
Suitability	Microorganisms	Microorganisms	Ascomycota, Zygomycota, Basidiomycota
Lag phase	Present	Present	Absent
Harvesting percentage (v/v) from the total working volume	≤ 20%	≤ 20%	> 50%
Cell (pellet) reuse	No	No	Yes
Fresh media insertion	No	No (only specific substrates)	Yes
Fermentation period	Long	Long	Short

Cells and products	Remain in the reactor until the end of fermentation	Remain in the reactor until the end of fermentation	Harvest more than 50% at each cycle
Process cost - raw material - power - staff	High	High	Reduced
Seed culture cost	High	High	Low
Metabolite productivity	Short-term	Short-term	Long-term
Cleaning time	High	High	Reduced
Sterilization time	High	High	Reduced
Working volume	Maintain	Increase	Maintain

References: (Ariyajaroenwong et al., 2012; Feng et al., 2012; Ganjali Dashti et al., 2014; Huang et al., 2008; Ji et al., 2014; Mirończuk et al., 2014; Moeller et al., 2011; Qu et al., 2013; Wenyan et al., 2014; Zhang et al., 2014a)

Thus, this strategy is a possible technique for gaining insights into organism physiology and longevity. To the best of our knowledge, RBF has not yet been applied to the cultivation of *G. lucidum*, so more effort should be made to progress this approach, at least on a research scale (using a shake flask and bioreactor).

From Table 2.2, it can be seen that RBF is superior to both batch and fed-batch fermentation. However, Ariyajaroenwong (2012) showed that RBF can cause a reduction in fungal cell growth. Therefore, an immobilisation cell system is needed to overcome this problem. Fortunately, *Ganoderma* species have the ability to undergo self-immobilisation in liquid culture by forming a structure called a pellet; this feature may be useful in an RBF strategy. The entangled mycelium is shaped

into fungal pellets that later protect and self-immobilise themselves in the liquid environment.

With RBF implementation, the lag phase is eliminated while the fermentation period in the bioreactor is shortened at the end of cultivation (Ganjali Dashti et al., 2014). When compared with batch and fed-batch strategies, RBF operates at lower production costs, including raw materials, power, and staff. Ariyajaroenwong (2012) also reported that operational control for RBF is easier than that of continuous fermentation. Furthermore, the working volume of RBF is maintained compared with the fed-batch approach, thus leading to energy savings.

Both cleaning and sterilisation time were significantly reduced with RBF. Even though metabolite productivities for both fed-batch and RBF methods are the same, RBF is preferred for being more economical. The key principle is that RBF can harvest 50% more (50–90%) of the produced cells and bioproducts, while both batch and fed-batch methods retain these in the bioreactor until the end of fermentation or until harvesting has occurred. Batch and fed-batch approaches can also substantially reduce the quality of bioproducts produced (Fraga et al., 2014) by holding fungal cells in the bioreactor for an extended period.

RBF has been shown to increase cell productivity, thus ensuring a high cell growth rate (Dashti and Abdeshahian, 2015; Huang et al., 2008); this is due to operating factors, harvesting time (broth replacement times) and harvesting volume (broth replacement ratios) of the culture broth (Dashti and Abdeshahian, 2015; Yang et al., 2005).

A further factor in RBF strategy is the agitation speed. Based on work done on *Mortierella alpina*, a short-time high impeller speed was used before reducing it to the optimised value that favoured cell growth (Ji et al., 2014). These factors can maximise the number of growing points, which are the hyphal tips. In principle, these approaches can lead to pellet break-up or large mycelial aggregation, generating smaller pellets or dispersed mycelial forms and thus increasing the oxygen transfer rate. These criteria are vital for the application of RBF to the culture of *G. lucidum*.

Future optimisation is needed with respect to the two pivotal factors of RBF, harvesting time and harvesting volumes. Accordingly, the research in this study focused on these factors.

2.8.5 Repeated fed-batch fermentation

The two methodologies described previously (fed-batch and RBF) can be potentially merged into one strategy. The fed-batch fermentation can be implemented and combined with RBF to produce an improved technique called repeated fed-batch fermentation (RFBF). In RFBF, both the elongation of the production period (idiophase) by substrate feeding and the shortening of the bioreactor and inoculums preparation are merged (Moeller et al., 2010). These techniques are compared in Section 2.8.6.

2.8.6 Studies using RBF and RFBF

Table 2.3 Current research that uses repeated-batch fermentation (RBF) and repeated fed-batch fermentation (RFBF) technique in Dikarya cultivation

Mode of operation	Phylum /Fungi	Hypothesis	Conclusion	References
RBF	(Ascomycota) *S. cerevisiae*	Self-cycling fermentation (SCF) improves glucose utilisation and ethanol production under very high gravity (VHG) conditions	RBF ethanol productivity is greater than in batch and continuous batch fermentation	(Feng et al., 2012)
	(Ascomycota) *Y. lipolytica*	Development and comparison of various process strategies (batch, repeated batch, fed-batch and repeated fed-batch) for the bioprocessing of citric acid production from glucose.	RBF is the most suitable technique compared with others, by increasing citric acid production due to the elongation of the production phase	(Moeller et al., 2010)
	(Basidiomycota) *F. trogii* *T. versicolor*	Enhancement of laccase production in the repeated batch cultures	RBF process with pellets is the most suitable method for high yield and sustained laccase production	(Birhanli and Yesilada, 2006; Birhanli and Yesilada, 2010)
	(Zygomycota) *C. bainieri*	Investigation of lipid production in the batch culture and the repeated batch culture as a comparative study	RBF culture was dependent on harvesting time and volume, and more reliable than the batch method	(Dashti and Abdeshahian, 2015)
RFBF	(Ascomycota) *Y. lipolytica*	Biosensor-controlled substrate feeding facilitates the performance of long-time repeated fed-batch process	RFBF is a highly efficient fermentation methodology for the citric acid production process	(Moeller et al., 2011)
	(Ascomycota) *P. chrysogenum*	Substrate (phosphorus) feeding technique improves productivity of penicillin G fermentation	Supplying an optimal amount of (substrate) phosphorus enhanced mycelium growth, economic value and improved industrial penicillin production for RFBF	(Li et al., 2005)
	(Zygomycota) *M. alpina*	RFBF method was designed to shorten the fermentation period, and thus enhanced fungal ARA-rich oil productivity which was suitable for commercial use	RFBF process has a reduce fermentation time and significantly improved productivity	(Ji et al., 2014)

RFBF requires more media usage than RBF alone; therefore, RBF was prioritised in this work. The latest findings for both methods are shown in Table 2.3. Nevertheless, RFBF remains uncharacterised for the production of *G. lucidum* in the bioreactor. Thus, more work is needed to determine whether or not RFBF is worth implementing for this type of organism and product.

2.9 Physical factors in *G. lucidum* cultivation

Commonly, cultivating *G. lucidum* mycelium on the chosen substrates does not include a sporulation step during inoculum preparation since vegetative inocula are employed (Eyal, 1991). Growing *G. lucidum* in liquid media involves the consideration of four main factors, chemical, biological, physical, and morphology. Biological factors describe its behaviour, while physical and chemical factors define the environment of the biocatalyst. The optimal fermentation conditions depend on the nature of the desired product and the strain of fungus used. Moreover, the growth rate and fungal morphology depend on the culture conditions, as discussed later (section 2.12).

Factors such as aeration, temperature, agitation rate or shear force, fermenter design and culture time can affect the fermentation macro-environment, as reported by Vaidyanathan et al., (1999). These factors can contribute to the morphological and physiological behaviour of the fungus, and thus affect the performance of the bioprocess. Fungal physical conditions can influence the growth and productivity of the investigated strains (Petre et al., 2010). Factors affecting *G. lucidum* fermentations are discussed in the following sections.

2.9.1 Temperature

During *G. lucidum* liquid cultivation, temperature is easy to control. Process temperature affects growth rate, medium evaporation, dissolved oxygen tension (DOT), pellet development, and product formation in filamentous fungal cultures (Fazenda et al., 2008). The growth of *G. lucidum* has typically been studied in SLF at temperatures between 25 and 36 °C, with most established at 30 °C (Fazenda et al., 2008; Wagner et al., 2003).

2.9.2 Agitation

Rushton turbines chiefly provide the necessary agitation in *G. lucidum* bioreactor systems (Wagner et al., 2003). Throughout growth and bioproduct development, agitation rate is equivalent to the impeller speed of a bioreactor, which plays a significant role in determining the fungal growth rate through mixing, heat transfer and mass. In such fungal cultures, especially at scale, there can exist noticeable mass transfer gradients throughout the bioreactor vessel, triggering both mycelial morphology and product spectrum changes (Fazenda et al., 2008; Tang et al., 2011)

Agitation can also facilitate the removal of waste gases and other by-products (Fazenda et al., 2008). Due to the oxygen-dependency of *G. lucidum* (Tang and Zhong, 2003), adequate mixing speed is required to ensure sufficient oxygen supply to the fungal cells throughout the vessel, which is vital for cell growth, metabolite biosynthesis and cellular morphology.

The shear force generated by agitation can affect the mycelium in numerous ways such as by damaging the cell structure, stimulating morphological change, and

causing variations in growth rate and bioproduct formation (Papagianni, 2004). For these reasons, optimum agitation is crucial to achieving adequate oxygen transfer into the medium while avoiding shear stress (Fazenda et al., 2008; Wagner et al., 2003). McNeil and Harvey (2008) have shown that high agitation conditions can influence culture morphology, such as in the growth of Basidiomycetes; studies in *G. lucidum* are limited, however (Papagianni, 2004).

According to the literature, EPS production is favoured by a moderate agitation rate (166 rpm) using *Grifola frondosa* grown in a 5 litre bioreactor (Lee et al., 2004), whereby an agitation rate increment gave lower EPS yields. Moreover, changing the impeller speed (from 50 to 250 rpm) in shake flask cultures of *G. lucidum* favoured EPS release (at 150 rpm) into the medium, instead of it adhering to the mycelial pellet, thus stimulating the secretion of further EPS (Yang and Liau, 1998a).

Conversely, at an agitation speed of 400 rpm in a bioreactor, EPS rapidly developed in the early stages of the fermentation but mycelial growth fell swiftly after three days. This was due to the harmful effects of shear force at high agitation speeds. Berovic et al., (2003) confirmed that the high agitation speeds had damaged mycelial agglomerates and EPS (Rau, 2004). Hence, for *G. lucidum* fermentation, the agitation rate must be a compromise between attaining efficient mixing and mass transfer in a highly viscous, pseudoplastic suspension, and avoiding shear damage to the fungus and EPS.

2.9.3 Aeration

For any Basidiomycete culture, aeration is one of the critical parameters for fermentation. Typically, aeration is connected with dissolved oxygen (DO) concentrations (Seviour et al., 2011), making it the most influential physiological variable to control and optimise in aerobic fermentations (Fazenda et al., 2008). This aeration level can be linked to the desired agitation speed via any fermentor controller set-up. Furthermore, air supply affects growth, morphology, metabolite biosynthesis rates, and nutrient uptake rate in *G. lucidum*.

2.9.4 Fermenter design (baffle effects)

Continuous stirred tank reactors (CSTR) are commonly used in *G. lucidum* fermentation with standard radial flow Rushton impellers (Fazenda et al., 2008; Seviour et al., 2011). The manufacturers regularly supply these fermenter designs to ensure high oxygen transfer rates (OTRs) and rapid growth competency for aerobic fungi. The Rushton turbine itself delivers flexibility for the culture of various microbial and animal cell types; however, it imparts high shear stresses and may cause detrimental consequences to product yield from shear-sensitive cells (Fazenda et al., 2008; Liu et al., 2012a). As described by Seviour (2011), higher EPS yields were obtained in a low shear oscillatory baffled bioreactor (OBR) than with a CSTR under similar culture conditions, although no morphological differences were reported (Gaidhani et al., 2005).

Recent practice in using CSTR with an unbaffled bioreactor system (as described in Chapter 3) might generate the same effect as the OBR, and may help to overcome the fungal wall growth limitations of prolonged RBF cycles. Frequently,

the installation of baffles in the bioreactor is recommended for the purpose of mixing performance enhancement via increased turbulence in the region of the baffles, although cell damage in the baffle zone should generally not be of concern since the hydrodynamic force is lower than that in the impeller zone (Zhong, 2010). However, fungal build up and engulfment of the bioreactor baffles may disrupt mixing performance and cell growth in long continuous cultures, especially for *G. lucidum* (as tested in this work). Therefore, fermenter modification by baffle removal may warrant further investigation in the culture of this species, as they tend to adhere to baffles or top plate components. This might avoid the overgrowth issues previously observed, and affect pellet sizes through improved mixing. To date, these hypotheses remained untested.

2.9.5 Foaming

Fermenter medium, when aerated and agitated, may form a foam which can lead to significant problems. Foam formation is undesirable since it affects the ability to monitor fermentation conditions and can obscure product recovery (Fazenda et al., 2008). Additionally, microorganisms present in the liquid phase may preferentially partition into the foam phase. Foam may also block exit air filters and increase headspace pressure. Fortunately, the discovery of conventional chemical antifoam agents to overcome this problem, especially in large-scale cultivation, may avoid these issues but can evidently decrease OTRs, with subsequent effects on cell growth (Wagner et al., 2003).

2.9.6 Culture time

The harvesting time of *Ganoderma* species in SLF determines the quality of the desired yield (polysaccharide), usually due to carbon source exhaustion with increasing culture age (Shu and Wen, 2003). Consequently, culture age may have a profound effect on the product of interest, and therefore should be taken into consideration as an optimisation parameter. For this work on RBF, this parameter was considered vital as it required an extended cultivation of *G. lucidum*.

2.10 Chemical factors for *G. lucidum* cultivation

Chemical factors such as metals, ions, special additives, medium pH, medium composition, carbon source, complex media, by-products and nitrogen source strongly influence the evolution of biotechnological processes (Petre et al., 2010). Therefore, optimisation of these factors is necessary, especially for implementing an RBF strategy in *G. lucidum*; thus, certain chemical factors that reflect this are examined in the present study.

2.10.1 Medium pH

Many parameters may affect *G. lucidum* during SLF, including the initial pH of the medium, salt solubility, ionic state of the substrates, cell membrane function, cell morphology, substrate uptake rates (such as glucose consumption), all of which are strongly influenced by pH (Kurosumi et al., 2006) and bioproduct formation (Fazenda et al., 2008; Papagianni, 2004). Typically, the ability of most *Ganoderma* species to function at pH 5.0 or lower can reduce the risk of potential bacterial contamination, chiefly when culturing on waste materials under non-aseptic

conditions. Overall, the selected *G. lucidum* culture functions best at a pH below 5.0 (Papinutti, 2010; Yang et al., 2000; Yang et al., 2013).

2.10.2 Medium composition

The correct medium composition is vital for growing *G. lucidum*, and a range of complex, synthetic media or chemically-defined media and waste substrates have been used (Fazenda et al., 2008). In other words, the optimum medium must promote both growth and bioproduct formation (Papagianni, 2004; Revankar and Lele, 2007). As an example, complex media can produce a higher biomass concentrations compared with synthetic chemically-defined media (Fan et al., 2007). A common approach to medium composition is explored by controlling the carbon:nitrogen (C:N) ratio source, crucial for mycelium efficiency and bioproduct formation in *G. lucidum* (Hsieh, 2004).

2.10.3 Complex media

Complex media have been used in the large-scale cultivation of *Ganoderma* species through the use of waste materials. Compared with synthetic media, complex media are cheaper and contain poorly understood growth enhancers, either because of the presence of unknown growth factors or of trace elements (Fazenda et al., 2008), which are applied in the current RBF strategy. Furthermore, the carbon or nitrogen sources of complex media are broken down slowly so that concentrations of their metabolised products always remain low, thus influencing bioproduct production. Research suggests that the optimal conditions for *G. lucidum* are complex media in liquid culture (Wei et al., 2014).

2.10.4 Carbon source

The supply of a suitable and sufficient carbon source is vital for *G. lucidum* development, growth, and product formation, and to uphold the primary components of the cell cytoskeleton. Some excellent carbon sources are simple sugars like glucose, maltose, sucrose, and complex sources such as starch, molasses, malt syrup, and bagasse (Fazenda et al., 2008). These sources usually support fungal growth and exopolysaccharide (EPS) formation (Wymelenberg et al., 2006).

From the literature, carbon sources such as disaccharides (sucrose, lactose, and maltose) are superior to monosaccharides (glucose, fructose, and galactose) for EPS production (Shih et al., 2006). However, the use of complex carbon sources like molasses has yielded higher mycelial growth and EPS production than a simple carbon source (Xiao et al., 2006). Hence, carbon source concentrations are more affected by EPS production (Kim et al., 2006a). Typically, higher levels of the carbon source (> 35 g/L) lead to greater production of fungal EPS, implying that a high C:N ratio is necessary, although good mycelial growth does not always ensure a high yield of EPS (Shih et al., 2008; Shih et al., 2006). Furthermore, Lee et al., (2007) reported that both mycelial and EPS production by *G. applanatum* increased with increasing concentration of glucose. Kim et al., (2006b) also reported that the optimum glucose levels for EPS production in *G. lucidum* are between 35 and 70 g/L. The range of glucose used in this study was 30 to 50 g/L.

2.10.5 Nitrogen source

Broadly speaking, the common sources of N are inorganic (nitrite, nitrate, and ammonium salts) and organic (casein, peptone, and amino acids) in *G. lucidum*

cultures. Other nitrogen-rich compounds (corn-steep liquor, Pharma-media, yeast extract, beet or cane molasses, whey powder, and soy flour) can also be utilised (Fazenda et al., 2008). In nature, *G. lucidum* lives on substrates that are low in most nutrients except carbon (Carlisle et al., 2001), due to the extremely low N content in wood; most *G. lucidum* in the wild are thus N-limited. Therefore, by understanding the mechanisms by which this fungus copes with N limitation, strategies for their repeated-batch fermentation may be developed.

However, inorganic N sources support lower biomass production but improved yields of EPS, while organic N sources increased biomass yields in submerged culture fermentation (Fang and Zhong, 2002b). Therefore, it can be seen that type and concentration of N source strongly influences cell growth and EPS production in *G. lucidum*.

2.10.6 Metals and ions

Appropriate levels of key metals and trace elements are required for the growth and product formation of *G. lucidum*. These salts (e.g., magnesium sulphate and potassium phosphate) are required for fungal development, while trace elements (manganese, zinc, iron, copper, and molybdenum) act as co-factors for enzymes (Fazenda et al., 2008). Other ions like magnesium (Mg^+) and potassium (K^+) are also vital for Basidiomycete growth, a Mg^+ is responsible for enzymatic reactions while K^+ promotes hyphal tip extension (Fazenda et al., 2008). Magnesium sulphate (in the form of Epsom salts) is commonly used in *G. lucidum* fermentation.

2.11 Biological factors for *G. lucidum* cultivation

Biological factors, including inoculum age and volume, prominently affect the growth and product formation of *G. lucidum*. Therefore, a proper characterisation of the inoculum is vital for potential industrial applications such as the isolation, successive selection and maintenance of cultures (Seviour et al., 2011; Stanbury et al., 1995). Additionally, these evaluations need to be performed on the selected inoculum as they are vulnerable to contamination, spontaneous mutation, deterioration, and death (Fazenda et al., 2008). For that reason, inoculum preservation and maintenance is crucial, and it is advisable to prepare frozen stock cultures as soon as possible after isolation (Walser et al., 2001).

2.11.1 Inoculum

There are many kinds of inoculum variables that can exert a major influence on the *G. lucidum* fermentation profile, such as type, age, concentration, amount, and viability (Fazenda et al., 2008). These also have an effect on fungal morphology, especially in pellet production and the types of pellets produced (Gibbs et al., 2000). The inoculum for *G. lucidum* can only be standardised with mycelial-based inocula, since sexual spores are formed once the fruiting body has matured (Wagner et al., 2003), compared with most readily-produced asexual spores (e.g., conidia) of other filamentous fungi which can be used as a source of standardised inoculum.

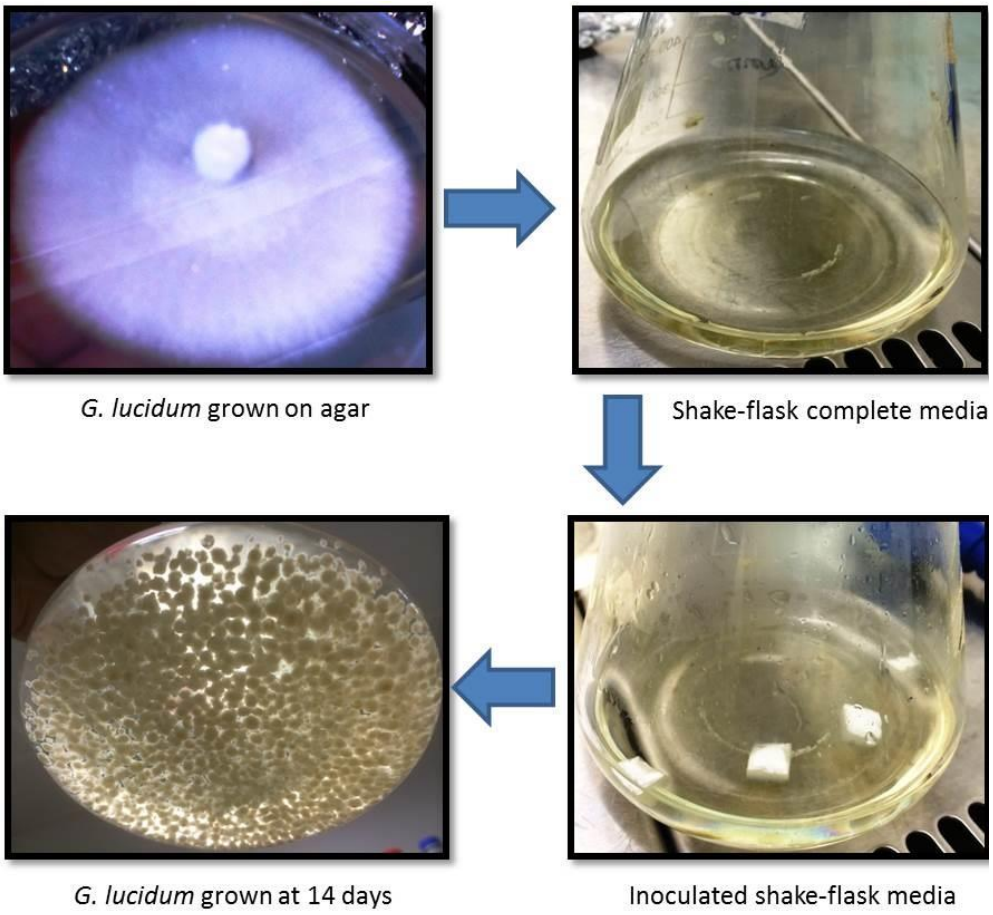


Figure 2.3 Preparation of *G. lucidum* inoculum from agar plate to shake flask (adapted from Chapter 3)

Most mushroom scientists use small pieces of mycelium that are still attached to the agar plates on which the fungi were grown, and inoculate these directly into the fermentation broth when using small scale shake flasks or agar cultures. Fig. 2.3 shows the inoculum preparation process from the standardised square-cut of *G. lucidum* mycelium into the shake flasks used in this study. This seed culture strategy is used to inoculate a bioreactor so that the mycelium can adapt from a solid to a liquid environment, thus reducing the lag phase and lowering inoculum densities (Fazenda et al., 2010).

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

For *G. lucidum*, the mycelium can be homogenised aseptically to increase the number of active hyphal tips (Fazenda et al., 2010; Stanbury et al., 1995), using a short blending time with a sterile stainless steel Warring Blender (Hsieh et al., 2006; Rogalski et al., 2006). The inoculum blending time should be no more than 20 seconds at a low-speed setting (Chapter 3), in order to avoid possible mycelial damage from the treatment.

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

2.11.2 Morphology and rheology

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

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

The metabolism of *Ganoderma* is connected with its morphology (Wagner et al., 2004). Pellet growth is usually triggered by variables such as sugar concentration

in the medium, agitation regime and inoculum density. Pellet morphology is measured in terms of shape, surface area, hairiness or roughness (R), circularity or diameter, and compactness (Fazenda et al., 2008; Riley et al., 2000). The filamentous form is usually characterised by the total hyphal length and the number of actively growing tips. Pellet morphology can influence the bioproducts produced, although the relationship between *G. lucidum* pellet morphology and bioproduct production remains poorly understood (Wagner et al., 2004).

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

2.12 Morphological analysis of *G. lucidum*

A detailed quantitative structural analysis of fungal morphology is required if a better understanding of the relationship between morphology and desired bioproducts is sought. This is an example of where the application of methods such as image analysis (IA) would permit the measurement of relevant morphological parameters (Riley et al., 2000), and IA is thus suggested for any work where the development of fungal hyphae is significant (Tucker et al., 1992). Therefore, IA is sometimes used for the microscopic characterisation of Basidiomycetes (Park et al., 2002; Tepwong et al., 2012; Treskatis et al., 2000) and has also been developed as a fast and precise method for quantitative morphological characterisation, since parameters of an observed object such as size, total, shape, position and intensity can easily be evaluated in this way (Znidarsic and Pavko, 2001).

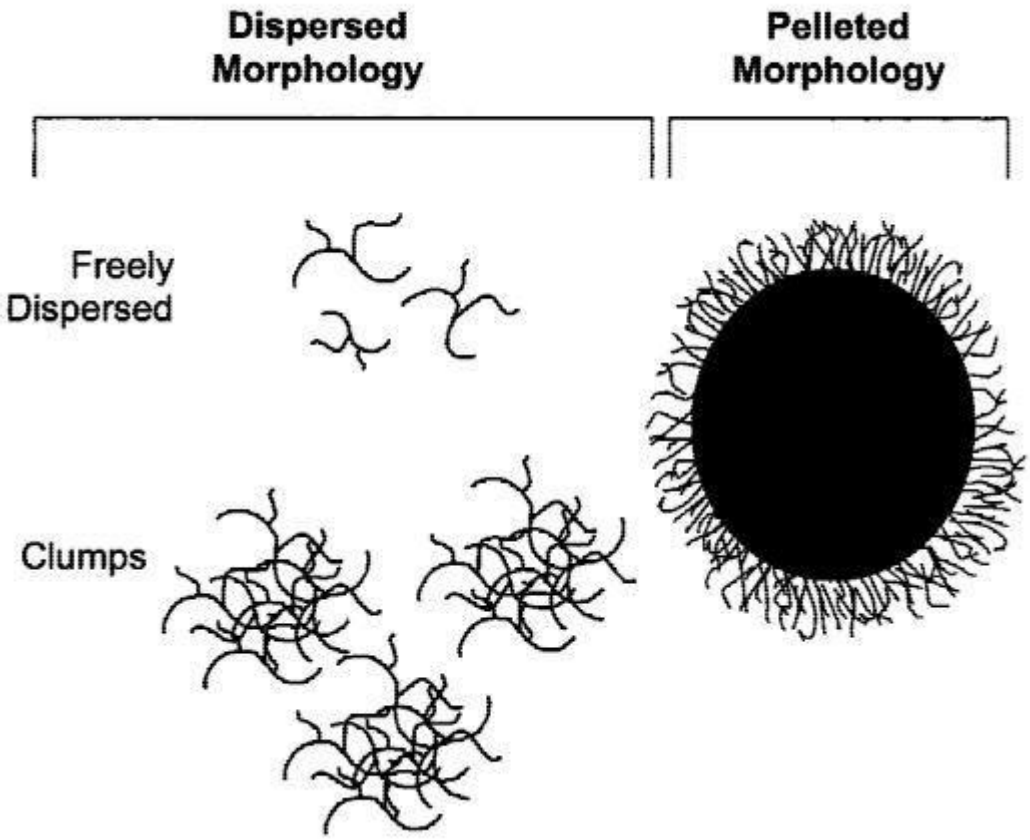


Figure 2.4 Schematic representation of fungal morphologies in suspension culture; adapted from Li et al., (2000)

The filamentous fungi (mushrooms) are typically characterised by light microscopy (IA) during fermentation (Fazenda et al., 2010). IA aims to qualitatively describe the most prominent morphology and recognise any relevant morphological changes associated with any change in the process such as growth phase, nitrogen limitation, repeated-batch cycle, harvesting time, starvation, nutrient depletion, and catabolite repression. In suspension culture, morphology is usually classified as either dispersed or pelleted, and the dispersed form can be further divided into freely dispersed and clumps (Tepwong et al., 2012; Thomas and Paul, 1996).

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

2.12.1 *G. lucidum* morphology in EPS production

Understanding, and, if possible, controlling morphology is crucial to the proposed RBF applications. Therefore, *G. lucidum* studies which evaluate this feature, both in the shake flask and bioreactor, are listed in Table 2.4, particularly with respect to their EPS-morphology relationship. It can be observed that smaller and dispersed pellets are associated with EPS production (Fang and Zhong, 2002b; Wagner et al., 2003).

Table 2.4 *G. lucidum* morphologies reported to favour EPS production

Morphology	Study details	Reference
0.8–2.5 mm pellets	500 mL shake flasks (Effect of medium composition)	(Ding et al., 2012)
Feather-like pellets	10 L stirred-tank bioreactor (Effect of controlled dissolved oxygen)	(Fazenda et al., 2010)
Small pellets	2 L agitated-tank bioreactor (Fed-batch effects)	(Tang and Zhong, 2002)
Dispersed pellets	5 L stirred-tank bioreactor (Controlled pH effects)	(Kim et al., 2006b)

Ovoid pellets	500 mL shake flask (Glucose effects)	(Wagner et al., 2004)
Small-sized pellets	Review work	(Wagner et al., 2003)
Hairy pellets	250 mL shake flasks (Oxygen effects)	(Hsieh et al., 2006)
Small-diameter pellets	250 mL shake flasks (Initial glucose effects)	(Fang and Zhong, 2002a)
Small-sized pellets	250 mL shake flasks (Inoculation density effects)	(Fang et al., 2002)

2.13 Project aims

Overall, the chosen RBF strategy was considered likely to be compatible with the chosen fungus (*G. lucidum*), with optimised fermentation conditions and characteristics (Chapter 3). The resulting biopolymer was structurally characterised (Chapter 4), and its biological activities assessed (Chapter 5). RBF methodology was also tested using an immobilisation strategy at the shake flask level (Chapter 6).

CHAPTER 3

INTENSIFICATION OF EXOPOLYSACCHARIDE PRODUCTION FROM *Ganoderma lucidum* (Fr.) Karst, BCCM 31549, USING REPEATED-BATCH FERMENTATION

3.0 Introduction

The perceived health benefits of *G. lucidum* have resulted in significant research being carried out to produce polysaccharides from this medicinal mushroom by cultivating the organism on grain, sawdust or wood, producing mature fruiting bodies and spores (Liu et al., 2012b; Ruan and Popovich, 2012). This technique, which is called solid-state fermentation (SSF), requires a long cultivation, and is thus time-consuming, economically unviable and potentially prone to contamination (Leskosek-Cukalovic et al., 2010). Scientists are trying to solve this problem by replacing the mature fruiting bodies with fungal mycelia using submerged liquid culture techniques which allow the potential for higher cell growth and metabolite production. The submerged liquid fermentation (SLF) uses a compact space with optimised culture environment which leads to shorter cultivation time, higher yields and decreased contamination. Therefore, a more efficient production of the desired products particularly mycelial biomass and polysaccharides can be obtained (Yang et al., 2013; Yang et al., 2004).

The use of SLF by *G. lucidum* for the purpose of accelerating its mycelial growth and metabolite production is affected by several factors. To date, the effects of medium composition (Chang et al., 2006), inoculation concentration (Fang et al., 2002), pH (Fang and Zhong, 2002b; Kim et al., 2006b), environmental conditions (Yang et al., 2009), dissolved oxygen (Fazenda et al., 2010), two-stage culture process (Fang and Zhong, 2002c), pH-shift and dissolved-oxygen transfer (DOT)-shift integrated fed-batch fermentation (Tang et al., 2009), and oxygen supply (Tang and Zhong, 2003) have been studied. Most papers published previously focus on improving the fermentation process to increase metabolite activity. However, little

work on increasing metabolite productivity by reducing the fermentation time, cost for seed culture and extending the fermentation cycle has been carried out. It is known that reducing the time for cell growth by decreasing the lag phase of fermentation is crucial for obtaining higher productivities of any bioactive metabolites from the desired organism. To enhance the metabolite production efficiency, modification of fermentation techniques would be vital in the submerged culture of *G. lucidum*. Thus, it might also be influenced by different cultivation conditions such as the potential of RBF to increase the productivity of metabolites.

RBF is an adaptation of an existing technique or an alternative strategy in which the medium or a portion of the medium is removed and fresh medium is introduced periodically or repeatedly without changing the old culture (Birhanli and Yesilada, 2010). Based on these, RBF is different from fed-batch techniques as it has the advantage of reducing the cost for seed culture and the need for inoculation between each fermentation cycle (Qu et al., 2013). RBF has been shown to enhance the productivity of microbial fermentations (Birhanli and Yesilada, 2010; Zhang et al., 2014b), as it requires less time for washing and sterilising the fermentor (Qu et al., 2013). These advantages have the potential to lead to significant savings in terms of both time and labour (Naritomi et al., 2002). Consequently, with RBF implementation, it is possible to reuse and store fungal pellets and maintain their long-term cell activity (Birhanli and Yesilada, 2006), especially for bioactive metabolites production (Birhanli and Yesilada, 2006; Birhanli and Yesilada, 2010; Yang et al., 2005).

In a few cases of filamentous fungi, nitrogen (N) limitation is an efficient strategy for secondary metabolite formation, including polysaccharides (Zhou and

Zhong, 2009). Ammonium, one of the primary N sources in fungi, is a key compound involved in N regulation of both primary and secondary metabolism (Marzluf, 1997). As mentioned by Kohut et al., (2009), the biosynthesis of polysaccharides by fungi is transcriptionally regulated by the N status of the cells. Nevertheless, it is currently not known if and how N supply influences the secondary metabolite production in mushrooms in a long continuous/extended batch cycle (RBF).

Previous studies using *G. lucidum* have mainly concentrated on bioactive metabolite (mainly EPS) production using batch (Kim et al., 2006b), fed-batch (Zhu et al., 2010) and two-stage strategy fermentation (Xu et al., 2010), but there are limited studies on EPS using RBF, which is a different cultivation condition from the batch system (Birhanli and Yesilada, 2006). The RBF system has shown numerous advantages in the long-term fermentation process, such as uncomplicated operating conditions (Birhanli and Yesilada, 2006), self-immobilisation pellet system (Wang et al., 2005a), long-term activity maintenance of sustained secretion of bioactive metabolites (Birhanli and Yesilada, 2006), increased economic efficiency (Birhanli and Yesilada, 2006), and metabolite mass production (Paterson, 2006; Smith et al., 2002). Birhanli and Yesilada (2006) have shown that the maximum metabolite production (laccase enzyme) obtained by RBF of *F. trogii* was about nine times greater than agitated batch culture, and 13 times higher than static batch culture.

As stated by Gadd and Sariaslani (2014), the role of medicinal mushrooms in metabolic regulation is gaining greater attention. Their use in the development and application of beneficial biological activities offers an advantage in that the active principle (chemical effects) is considered safe and can be tolerated by humans.

Traditional cultivation of such mushrooms would provide an adequate supply, but is unnecessary if simpler mycelial cultures grown in large-scale fermentations can produce the same active compounds. Therefore, the aim of the current study is to investigate whether or not mycelial cultures from large-scale fermentations (RBF system) are effective in producing EPS.

Based on reported work on other fungi (Mirończuk et al., 2014; Moeller et al., 2011), metabolites of *G. lucidum* particularly EPS could be induced by the RBF system. It has been shown that *G. lucidum* produces pellets during the fermentation process (Fazenda et al., 2010; Zhao et al., 2011b) which may be useful for RBF systems due to the ability of pellets to survive in long term fermentation processes (Birhanli and Yesilada, 2006). There is little information, however on the fungal behaviour (e.g. relationship of morphology with EPS) of *G. lucidum* in RBF. The objective of this work was to compare the kinetic parameters; particularly EPS productivity of RBF from the batch strategy. These results could provide useful information for the industrial fermentation of *G. lucidum* producing EPS.

3.1 Materials and methods

3.1.1 Microorganism

A stock culture of the Basidiomycetes, *Ganoderma lucidum* BCCM 31549 was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL), [Agro] Industrial Fungi and Yeast Collection (Leuven, Belgium) in a well-preserved culture slant tube. The fungus was subcultured onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK) upon receipt from the supplier to avoid any contamination and ensure viability as suggested in the previous research (Fazenda et al., 2010). Plates were inoculated and incubated at 30 ^{o}C for seven days and stored at 4 ^{o}C. The strain was maintained on PDA slants every 2-3 months.

3.1.2 Chemicals

The chemicals for media preparation were all fresh and of analytical grade. Unless otherwise stated, ethanol used during EPS extraction and all other extraction chemicals were purchased from Sigma-Aldrich (Gillingham Dorset, UK).

3.1.3 Inoculum preparation

The inoculum preparation of *G. lucidum* BCCM 31549 involved two seed culture stages, both cultivated for 10 days at 30 ^{o}C and 100 rpm. Four mycelial agar squares (5 mm x 5 mm: extracted using a sterile scalpel) from a freshly grown 10 days old PDA plate was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of medium (first seed culture). To produce more active hyphal tips, the mycelium from the first seed culture was homogenised using a sterile Warring blender for 20 seconds. These are used as the inoculum for the second seed culture (500 mL Erlenmeyer flask containing 200 mL medium) and subsequently transferred to the

bioreactor. During the inoculum production, it is inoculated into the new fresh medium while they were in late exponential phase (from day 9 to day 11), which means the cells are biochemically at their most active and desired physiological state. Hence, total inoculum preparation prior inoculation procedure was approximately 30 days.

3.1.4 Culture conditions

The compositions of the medium used in all stages were at (g/L): [Yeast extract (YE) 1, KH_2PO_4 (mono-potassium phosphate) 0.5, K_2HPO_4 (dipotassium phosphate) 0.5, $MgSO_4$ 0.5, NH_4Cl (ammonium chloride) 4], unless specified. Different sets of conditions were set for optimisation [Condition A = 50 g/L of Glucose and 10% (v/v) inoculum, Condition B = 30 g/L of Glucose and 20% (v/v) inoculum, Condition C = 50 g/L of Glucose and 20% (v/v) inoculum and Condition D = 30 g/L Glucose and 10% (v/v) inoculum].

3.1.5 Mode of operation/ Bioreactor Use

All fermentations were carried out in batch or repeated-batch mode. The reactor used was a 2.5 L (total volume): 2 L working volume jacketed bioreactor vessel (New Brunswick Bioflow 3000, Edison L.N, USA) with two Rushton turbines. The pH was controlled at 4.0 by automatic addition of titrants (1 M NaOH and 1 M H_2SO_4). The temperature was kept at 30 ^oC throughout the runs and the agitation rate was set at 100 rpm. Uncontrolled dissolved oxygen (DO) for both batch and repeated-batch cultures were performed. These chosen culture conditions were based on previous work by Sanodiya et al., (2009), Wagner et al., (2004) and Fazenda et al., (2010) which prone for EPS production. The maximum air flow rate

used was 2.0 volume of air per volume of culture per minute (vvm) in both cultures or as indicated in the individual experiments. The values of pH, dissolved oxygen, agitation speed, temperature, airflow rate, and oxygen percentage during fermentations were recorded at the point of sampling at every 2 days.

3.2 Repeated-batch fermentation

The repeated batch fermentation (RBF) was performed for multiple cycles until the working culture became adequately viscous for growth or analysis of cells. At a certain time point in each cycle, the majority of the mature culture was removed and a known volume of the culture was held in the shake flask or bioreactor (depending on the procedure). A fresh newly prepared medium with the same composition was then added to the bioreactor and a new fermentation cycle was started until it achieved the highest growing point (the organism became too viscous or autolysis has occurred). Technically, the old fermentation broth was drained at an optimised broth replacement ratio (determined by percentages of 50% to 90% from the optimisation experiment) and replaced with a fresh preparation of medium to allow the *G. lucidum* cells in the shake flask or a bioreactor to continue to grow. The next cycle started once a stable, high cell density and high production rate of EPS was achieved. At this point, the old medium fermentation mixture was changed and the process was repeated for the following cycles.

3.2.1 Effect of broth replacement ratio

The batch fermentation was repeated (or extended by further nutrient solution addition to replace a specific fraction of the whole culture broth) according to the time when EPS concentration was the highest based on their fermentation growth curve. The experiment was examined using broth replacement ratios (v/v) of 50%, 70%, 80% and 90%, respectively. At the end of the optimisation procedure, RBF was compared to the batch fermentation for EPS productivity. During the entire process, biomass, residual sugar, EPS, and morphology were monitored every two days. Table 3.1 shows the details of the mode of operation.

Table 3.1 Mode of operation (broth replacement ratio) of repeated-batch processes by *G. lucidum* BCCM 31549 in the shake flask.

Experiments	Working culture (v/v)	Harvesting percentage (v/v)	Fresh broth replacement (v/v)
A	10%	90%	90%
B	20%	80%	80%
C	30%	70%	70%
D	50%	50%	50%

* Total working volume: 200 mL of 500 mL Erlenmeyer flask

3.2.2 Effect of broth replacement time point

During RBF, the broth replacement time point might be different from the three growth phases (at the end of the logarithmic phase, transition phase and stationary phase). These phases were obtained from the optimised shake flask or bioreactor batch fermentation designated as increasing EPS concentration (at the end of logarithmic growth phase), highest EPS concentration (transition phase) and stabilising EPS concentration (stationary phase). During the entire process, biomass,

residual sugar, EPS, morphology, N content and dissolved oxygen (bioreactor only) were monitored. In results and discussions, Table 3.4 (Section 3.4.5) showed the details of the experimental design in the shake flask. On the second approach, this design was optimised to produce the mode of operation for RBF in the bioreactor.

3.3 Analytical methods

3.3.1 Kinetic calculations

The *G. lucidum* fermentation kinetic parameters were calculated as follows (Stanbury, 1988; Stanbury et al., 2013):

$dx / dt = \mu$ max
R_{batch} = biomass concentration per hour (g L^{-1} day^{-1})
X_{max} = maximum cell concentration achieved at stationary phase
X_o = initial cell concentration at inoculation
t^i = time during which the organism grows at μ max
t^{ii} = time during which the organisms is not growing at μ max

Batch culture

- Biomass productivity, P_x (g L^{-1} day^{-1}), $P X = \frac{X max - X o}{ti + tii}$: Equation 1
- Yield ($_{biomass}$) = $\frac{\text{biomass produced}}{\text{glucose consumed}}$: Equation 2
- EPS productivity, P_{EPS} (g L^{-1} day^{-1}); $\frac{X max - X o}{ti + tii}$: Equation 3
- Yield ($_{EPS}$) = $\frac{\text{EPS produced}}{\text{glucose consumed}}$: Equation 4
- Specific production rate of EPS, $Q_{EPS/X,}$ [(g/g) day^{-1}] = $\frac{P EPS}{\text{EPS concentration}}$: Equation 5

Repeated-batch culture

- Biomass productivity, P_X = (g L^{-1} day^{-1}),
  ; $\frac{\text{X max} - \text{X o}}{\text{the time for product recovery at certain cycle in repeated batch culture (day)}}$: Equation 6

- Yield ($_{biomass}$) = $\frac{\text{X max} - \text{X } initial}{(\text{Initial glucose+glucose added}) - \text{R glucose end level}}$,
  [cycle time (day)] : Equation 7

- EPS productivity, P_{EPS} = (g L^{-1} day^{-1}),
  ; $\frac{\text{X max} - \text{X o}}{\text{the time for product recovery at certain cycle in repeated batch culture (day)}}$: Equation 8

- Yield ($_{EPS}$) =, $\frac{\text{EPS max} - \text{EPS } initial}{(\text{Initial glucose+glucose added}) - \text{R glucose end level}}$
  ,[cycle time (day)] : Equation 9

- Specific production rate of EPS,$Q_{EPS/X}$, [(g/g) day^{-1}] = $\frac{\text{P EPS}}{\text{EPS concentration}}$: Equation 10

The time for product recovery or cycle time was based on the actual time spent in this study.

3.3.2 Morphological analysis

The morphology details of the samples collected were evaluated using a light microscope (Nikon OPIPHOT-2, Japan) through a coupled camera (JVC, TK-C1381 Colour Video Camera). Five mL of culture sample was resuspended in 5 ml of a fixative solution according to the method described by Packer and Thomas (1990) and kept at 4 ^{o}C until measured. The fixative solution was prepared by mixing 13 mL of 40% (v/v) formaldehyde, 5 mL of glacial acetic acid, and 200 mL of 50% (v/v) ethanol. An aliquot (0.1 mL) of each fixed sample was transferred to a slide, air

dried, and then stained with methylene blue (0.3 g of methylene blue and 30 mL of 95% ethanol in 100 mL distilled water (Kim et al., 2006b). In contrast to human observation, which may be biassed and inconsistent, digital image analysis is an accurate, quick, and reproducible tool for evaluating microscopic images (Treskatis et al., 2000), thus the reason for its usage in the present study.

3.3.3 Dry cell weight determination (DCW)

The mycelial biomass or DCW from the harvested *G. lucidum* fermentation process was estimated by filtering a 10 mL sample through a pre-dried and weighed GF/C filter (Whatman Ltd., U.K.) using a Buchner funnel filter set, followed by repeated washing of the mycelial biomass with distilled water, Mili-Q® Advantage A10 (Millipore, Bedford, MA, USA). The filtrate was collected for EPS assays (Section 3.3.4). The mycelial or pellet filter cake was pre-dried for 2 minutes in a microwave oven (650 W) and cooled in a desiccator before weighing. Calculation of the DCW by subtraction of pre-weighed filter mass from the mass with the filtrate and multiplied by the dilution factor to get DCW in g/L. All values are averages of at least three independent trials.

3.3.4 Exopolysaccharide (EPS) determination

EPS was obtained from the centrifuged (8000 rpm for 15 to 20 minutes) supernatants of the harvested fermentation broth. The crude EPS was precipitated by the addition of four volumes of 95% (v/v) ethanol and left overnight at 4 ^{o}C to one volume of cell-free filtrate. The precipitate was then separated by centrifugation at 10,000 rpm for 15 min, which was repeated twice. The precipitate was then filtered through a pre-dried and weighted GF/C filter paper and washed twice with 5 mL of

95% (v/v) ethanol. It was then transferred to desiccators, left to dry to constant weight in a dryer, and the weight of EPS was then estimated. All assays were carried out in triplicates.

3.3.5 Concentration of residual glucose

An HPLC-RI determined the residual sugar in the fermentation broth with an ionic exchange column (Rezex ROA organic acid from Phenomenex, Cheshire, UK). The mycelial cells were recovered by centrifugation (HERMLE, model Z160M) at 10,000 rpm for 10 min, after which the supernatant was collected. The supernatant was filtered through a 0.45 µm sterile Corning syringe filter (Sigma-Aldrich, Gillingham, Dorset, and U.K). Later, a standard glucose dilution was done to obtain a glucose standard curve ($y = mx + c$, $R^2 = 0.99$). A filtrate sample (20 µL) was injected into the HPLC and onto an ionic exchange column (CARBO Sep COREGEL 87H3, TRANSGENOMIC), which was placed in a column oven (COLBOX) at 55 ºC. The 0.008 NH_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL / min at 55 ºC, and the effluent was monitored by an RI detector (RI-1530, Jasco, Japan) (Hsieh et al., 2006). The residual glucose in the fermentation broth was also determined by 2900D Biochemistry Analyser with a glucose biosensor (YSI, Xylem's Tunbridge Wells, U.K).

3.3.6 Concentration of residual nitrogen (N)

The residual nitrogen in the fermentation broth was determined using a High-Performance Ammonia Electrode model IS 570-NH_3 (Thermo Scientific, UK) coupled with Corning ISE pH/mV meter 240 with a BNC connector. Later, a standard ammonium chloride dilution was prepared to obtain an ammonia standard

curve (y=mx + c, R^2= 0.99). A 12.5 mL aliquot of the sample was mixed with 250 µL of pH-adjusting ISA (Thermo-fisher, UK) and stirred at a moderate, uniform rate. The ammonia electrode was rinsed in distilled water, blotted and placed into the mixture. When a stable reading was obtained the values were recorded and used to calculate the ammonia concentration. The mV values were recorded when a stable reading was displayed. The unknown concentrations of the samples were determined using the prepared calibration curve. The residual N in the fermentation broth was also determined by YSI-analyser (2950-D3, YSI, U.K).

3.3.7 Statistical analysis

All analysis were carried out in triplicate and the respective mean ± STD determined using the software, GraphPad Prism 5 (Version 5.01) and shown as error bars. If the error bars do not appear, it is assumed that they are smaller than the size of the symbol. A T-test was used for the plotting of fermentation graphs. One-way ANOVA and ad-hoc post-test (Bonferroni's Multiples Comparison Test) were used for kinetic parameters comparison.

3.4 Results and discussions

3.4.1 Macroscopic and microscopic observations

The mycelial characteristics were observed on agar plates after the mycelium activation process according to Stamets (1993) and Paterson (2006). The mycelium was non-aerial, rapid growing, becoming densely matted and longitudinally radial. The radial growth was defined when the hyphae germinated from the fungal spore in the middle of a petri plate and formed a circular growth. During their growth on the plate, the mycelium became difficult to cut and typically tore during transfer when colonised for 14 days. Fig. 3.1 below shows the characteristics of a *Ganoderma lucidum* BCCM 31549 plate culture over 14 days:

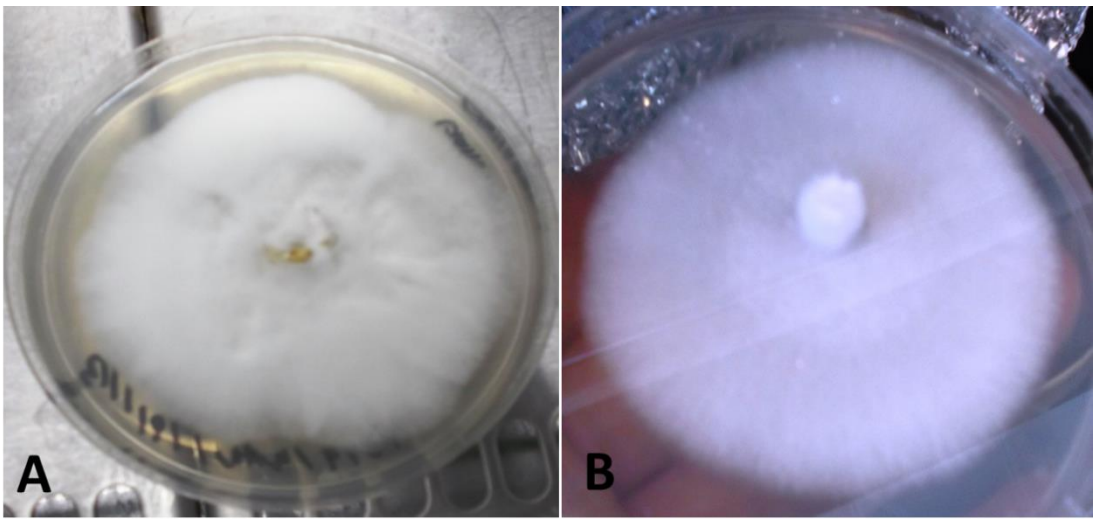


Figure 3.1 Macroscopic observations of *G. lucidum* BCCM 31549 (Leuven, Belgium) on PDA plate at (A) 14 days (B) 7 days.

The mycelium was white during the first 7 days and became yellow to golden-brown-like reaching day 14 onwards, indicating the ageing of the mycelium. Accordingly, the inoculation was done at day 10 to ensure the mycelium stays active.

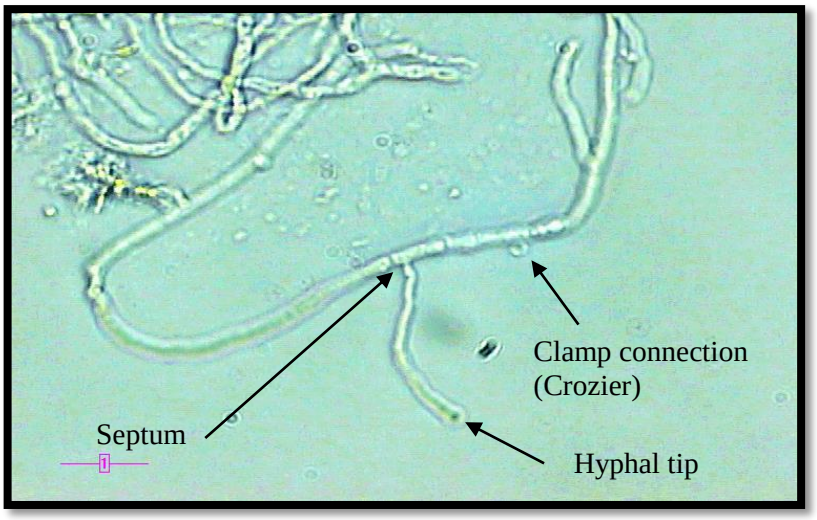


Figure 3.2 *G. lucidum* BCCM 31549 (Leuven, Belgium) hyphae showing the presence of clamp connections, septum and hyphal tip (typical features of Basidiomycetes). Bar = 15 µm.

G. lucidum showed different morphological characteristics throughout the various stages of a process. Figure 3.2 displays the typical morphology features of *G. lucidum* BCCM 31549 which is broadly in agreement with previous work by Fazenda et al., (2010) which used *G. lucidum* CCRC 36123. The strain that was grown in a shake flask (inoculum stage) showed a pelleted morphology with high hairiness (Fig. 3.3 below). These pellets are formed by the mycelia developing into a stable, spherical aggregates consisting of a branched, dense, and partially entangled network of hyphae, which can be investigated by a photomicrograph (Tepwong et al., 2012). When compared with the literature, Wagner et al., (2004) shown that *G. lucidum* grew with a pellet morphology that changed over time and produced long hyphae protruding from their regular sphere surface as observed in this work.

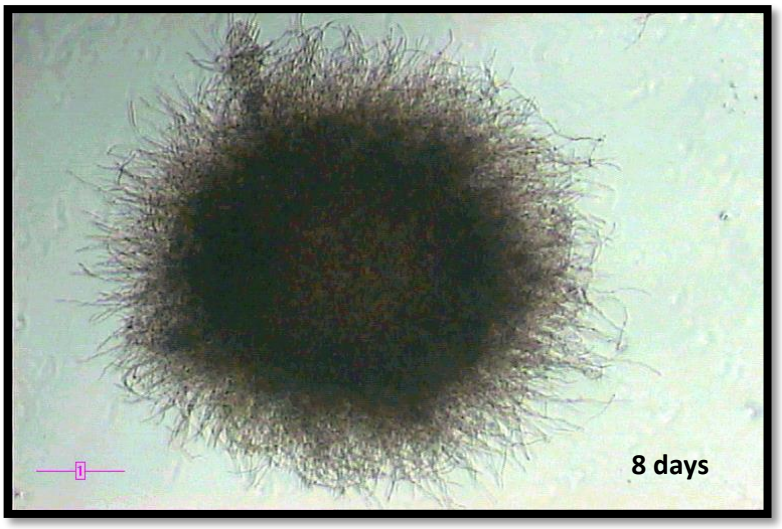


Figure 3.3 Photomicrograph of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) inoculum sample showing a pelleted morphology from an 8 days shake flask culture. Bar = 150 µm.

3.4.2 Shake flask batch fermentation profiles

To determine suitable fermentation parameters for EPS by *G. lucidum*, batch processes were carried out to attain EPS production rates under various conditions. Four different conditions were tested (Appendix 3.12A): Condition A [50 g/L of Glucose, 10% (v/v) inoculum], Condition B [30 g/L of Glucose, 20% (v/v) inoculum], Condition C [50 g/L of Glucose, 20% (v/v) inoculum], and Condition D [30 g/L of Glucose, 10% (v/v) inoculum]. Finally, only two conditions (A and B) were utilised and their morphological characteristics were discussed. Batch fermentation experiments with the desired culture conditions were implemented using shake flasks and the results are shown in Fig. 3.4 and the kinetic parameters were shown in Table 3.2.

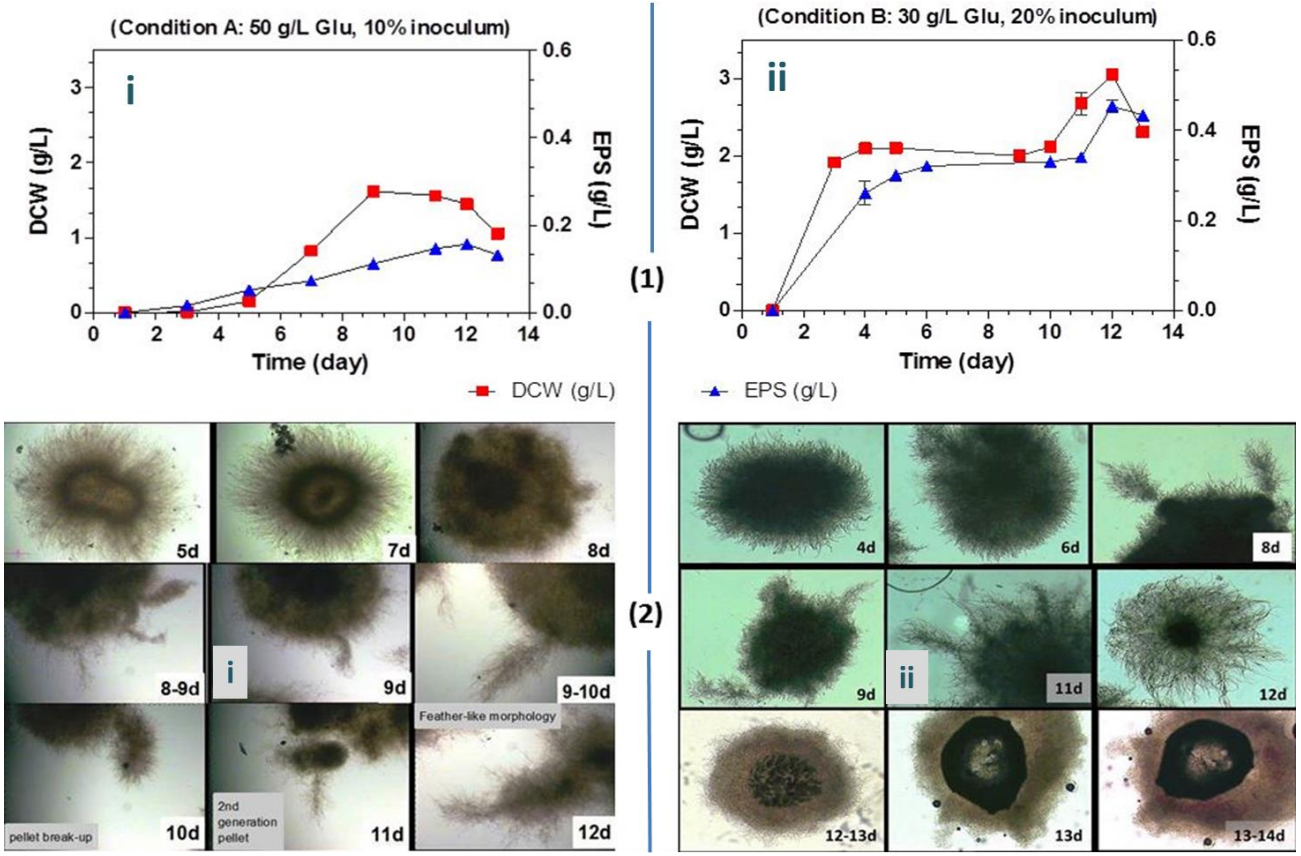


Figure 3.4 Time profile (1) and morphological changes (2) during shake-flask batch fermentation of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) using condition A: [50 g/L of Glucose, 10% (v/v) inoculum] and condition B: [30 g/L of Glucose, 20% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, and temperature 30 ^oC. (2) Images were taken at 4-fold magnification. Bar = 150 µm. 5-14 d means fermentation period in days.

(1) T-test assesses whether the means of two groups (DCW and EPS) are statistically different from each other. Therefore, paired T-test has been run for EPS and DCW for both conditions: Condition A, at 95% CI of discrepancy, the P value is 0.0135 [two-tailed P value] and Condition B, at 95% CI of discrepancy, the P value is 0.0014 [two-tailed P value]. Both means were significantly different ($P < 0.05$).

Figure 3.4 displays the relationship between time profile (1) and morphological changes (2) during shake-flask batch fermentation of *G. lucidum*. Based on the time profile in Fig. 3.4 (1), the fermentation process for both condition A and B have successfully followed the normal fungal growth pattern whereas the morphological changes in Fig. 3.4 (2) showed that *G. lucidum* grew with a pellet morphology that evolved over time, which proven by Wagner et al., (2004).

With condition A, mycelial biomass [Fig. 3.4 (1i)] started to increase at day 5 (0.44 g/L) and accelerated rapidly at day 8, reaching its highest value at day 9 (1.62 g/L). Meanwhile, EPS was low during the first 5 days because the mycelium was still in the growth stage (day 4 to day 9). Later on, the highest level of EPS was reached at day 12 (0.16 g/L). This result is comparable with Wagner et al., (2004), where the EPS production rate was low during the first generation pellet formation (day 5) and accelerated during the second stage (day 11). Besides, Yang and Liau (Yang and Liau, 1998c) have pointed out that the newly formed EPS would stick to the mycelial pellet and this probably slowed the secretion of more polysaccharide into the media.

The observed rapid EPS production was linked to the liberation of second generation pellets (day 11) shown by its morphological changes [Fig. 3.4 (2i)], as discussed below. According to Wagner et al., (2004) and Fazenda et al., (2010), second generation pellets were characterised as ovoid in shape but such cultures also still contained a few first generation pellets with protuberances (starburst appearance), which were then released (detached from its primary pellet surface) thus generating a feather-like morphology which can be observed from Fig. 3.4 (2i) at day 12.

The morphological changes for condition A are shown in Fig. 3.4 (2i). At their early stages (day 5 to 7), the mycelium showed a pellet ball with significant hairiness and this mycelial behaviour was due to possible self-immobilisation. These pellets functioned as an immobiliser which gives the fungus some protection in the liquid medium helping avoid any lethal shear effects (Yang et al., 2000). Later on, several long hyphal tips were protruding from the pellet surfaces and increasing in length to become protuberances at day 8. These protuberances were defined as a new-born pellet which protruding from their parent surfaces and this can be compared with the previous study by Kim et al., (2006b), where it was stated that the pellets originally produced protuberances during the liberation of the first generation to second generation pellets.

As growth progressed, the protuberances increased in length and width, giving the pellet a starburst appearance at day 9 and this morphology was associated with the starting point of the EPS acceleration phase on the same day. A day after (at day 10), the protuberances began to detach from the first generation pellet and this can be categorised as the early pellet break-up. These events continued until the protuberances were completely broken up on day 11 (full pellet break-up). These broke-up pellets transformed its shape into a feather-like morphology at the time of release on day 12 which seems to be the morphological form closely linked to EPS production. On this day, the EPS was the highest (0.16 g/L) while the biomass was reduced as the cells entered the death phase. Kim et al., (2006b) has also described this relationship between morphology and bioactive metabolite production, whereby the liberation of second generation pellets was proposed to be due to the pellet break-up thus initiating EPS production.

In comparison to the shake flask fermentation with condition A: Fig. 3.4 (1i), the same work with condition B: Fig. 3.4 (1ii) was more efficient in terms of growth and EPS production. The time profile for condition B: [Fig. 3.4 (1ii)] showed the biomass increased rapidly from day 3 (1.92 g/L) to day 9 (2.0 g/L). The highest biomass reached transition phase at day 12. This transition phase is defined as the transformation point from exponential growth phase (log phase) into the stationary phase (the new mycelia being produced is equal to the number of mycelia that are dying). Finally, at day 13, biomass slowly decreased into the death phase by day 14 which correspondingly can be called the autolysis period, with the mycelia experiencing colour changes from normal to yellowish or reddish appearance. Experimentally, the EPS production using condition B occurred at a steady rate starting at day 5 (0.30 g/L) until day 11 (0.34 g/L) and reached its highest concentration at day 12 (0.45 g/L).

Morphological variations during *G. lucidum* shake flask fermentation using condition B [Fig. 3.4 (2ii)] was generally in the same pattern as the condition A [Fig. 3.4 (2i)] though some of the pellet formation times were different. Pellet observation in Fig 3.4 showed that the formation of first generation pellets were less fluffy for condition B (2ii) at day 4 to 6 compared to condition A (2i) (at day 5 to 7), but the occurrence of protuberances were the same (at day 8) for both conditions. Also, the starburst appearance of condition B formed slower, which occurred on day 11 and the event of a pellet break-up was not completed at day 12. Later on, the protuberances which reformed into fluffier pellet engulfed the first generation pellet (dense black core) and fused around it at day 12 to 13. Following that, second

generation pellet was formed completely at day 13 and the biomass was triggered to decrease. Further growth has led to autolysis events starting at day 14.

Based on the morphology at day 12 [Fig. 3.4 (2ii)], condition B did not lead to production of the feather-like morphology compared to condition A [Fig. 3.4 (2i)] and this could be possibly due to the higher culture viscosity as mentioned by Fazenda et al., (2010). Increasing the inoculum percentage to 20% (v/v) has led to a higher number of hyphal growing tips in the culture, thus making it cloudier (more viscous) during the production of the first generation pellet. Treskatis et al., (2000) also mentioned that morphology influences the rheological properties of fermentation broth which affect viscosity. It is because dispersed filamentous growth leads to a highly viscous and pseudoplastic behaviour, and because of difficult mixing, inhomogeneities in the fermentation broth might arise. With the possibility of increasing viscosity, the mass and the heat transfer may be reduced and each can result in a lowered productivity.

During the batch, if the pellets became too large, oxygen restrictions and autolysis might arise, which could affect productivity (Sies, 1997; Treskatis et al., 2000). The viscosity problems from the packed hyphal tips of condition B [20% (v/v) inoculum] compared to condition A [10% (v/v) inoculum] might result in reduced growing space for the mycelia in the 500 mL Erlenmeyer flask and are probably the reason behind the lack of feather-like pellet productions. However, due to their ability to form fused-pellets (first generation pellet engulfed by second generation pellet) and avoid dispersing their mycelia in the system, condition B was suitable for bioreactor fermentation so that shear effects can be prevented in the bioreactor (more growing space).

The morphological properties of a filamentous fungus plays a major role in their metabolism during fermentation processes (Park et al., 2002), especially on EPS of *G. lucidum*. Wagner et al., (2003) reported that different pellet size would affect the relative productivity of the various metabolites, chiefly EPS concentration, which were higher in smaller pellets. If the pellets are smaller than the critical size for oxygen diffusion, less oxygen limitation could occur (good oxygenation), as a consequence the fermentation conditions can be worked to favour one product over another.

From these findings, *G. lucidum* shake flask fermentation using condition B produces higher fungal growth and EPS than using condition A. However, both conditions still can be applied in the upscaling work due to the fact that less than 20% (v/v) of inoculum was previously reported as optimal by some author (Berovic et al., 2003; Nasreen et al., 2005; Tang and Zhong, 2002). Besides, the pellet morphology of condition B (less dispersed) may suit the bioreactor environment compared to condition A (more dispersed). Table 3.2 summarises some kinetic parameters for these cultures.

3.4.2.1 Kinetics for shake flask batch fermentation

Table 3.2 shows fermentation characteristics of the final biomass (DCW) and EPS for condition A (1.62 g/L, 0.16 g/L) and condition B (3.05 g/L, 0.45 g/L), respectively. Biomass remarkably increased nearly 2-fold from condition A to condition B triggered by the reduced osmotic pressure (from 50 g/L of Glucose to 30 g/L of Glucose) and assisted by the increased in the inoculum percentage [from 10% to 20% (v/v)]. The concentration of glucose that is to be considered as suitable for

such processes was less than 35 g/L according to Wagner et al., (2003) and Fang and Zhong (2002b), thus the *G. lucidum* growth is less efficient at higher sugar concentrations which lead to higher osmotic pressure, and possibly due to increase in maintenance requirements.

Table 3.2 Kinetic parameters during shake flask batch-fermentation of *G. lucidum* BCCM 31549 (Leuven, Belgium) using condition A: [50 g/L of Glucose, 10% (v/v) inoculum] and condition B: [30 g/L of Glucose, 20% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, initial pH 4, and temperature 30 ^oC.

Kinetic parameters	Condition A*	Condition B*
Biomass concentration (g/L)	1.62 ± 0.1	3.05 ± 0.8
Biomass productivity, P_x (g/L day^{-1}) *Increase*	0.18 ± 0.02 1	0.24 ± 0.1 1.31
EPS concentration (g/L)	0.16 ± 0.11	0.45 ± 0.17
EPS productivity, P_{EPS} (g/L day^{-1}) *Increase*	0.013 ± 0.01 1	0.035 ± 0.04 2.69
Specific production of EPS, $Q_{EPS/X}$ [(g/g) day^{-1}]	0.009 ± 0.003	0.011 ± 0.004
Yield $_{DCW}$, (g_X/g_{GLU}) Yield $_{EPS}$, (g_{EPS}/g_{GLU})	0.19 ± 0.1 0.03 ± 0.01	0.38 ± 0.2 0.05 ± 0.01
Glucose consumed (g/L)	6.2 ± 1.9	8.0 ± 3.0
End N (ppm)	810.3 ± 9.7	747.6 ± 2.4
End ammonium chloride concentration (g/L)	3.09 ± 0.11	2.86 ± 0.14

*****The highest production of biomass and EPS were taken for each condition (End production value) according to their time (day) in the growth curve.

1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different ($P < 0.05$) vs End N, Biomass productivity vs End N, EPS concentration vs End N, EPS productivity vs End N, Specific production of EPS vs End N, Yield $_{DCW}$ vs End N, Yield $_{EPS}$ vs End N and, Glucose consumed vs End N and End N vs End NH_4Cl concentration, respectively.

Further on, the increasing inoculum percentage has improved the growing hyphal tips for condition B's initial inoculum in comparison to the lower initial inoculum of condition A, thus improving the biomass. These phenomena have also affected the EPS production, which increased nearly 3-fold for condition B (0.45 g/L) compared to condition A (0.16 g/L) in Table 3.2.

Enhanced productivity in EPS-fermentation was the main target in this work as this fuels the success of the future repeated-batch strategy. As observed in Table 3.2, the biomass productivity for condition A (0.18 g/L day^{-1}) was lower compared to condition B (0.24 g/L day^{-1}). Meanwhile, the EPS productivity of condition B (0.035 g/L day^{-1}) was greater under condition A (0.013 g/L day^{-1}) by more than 2-fold; thus the specific production of EPS was also improved. These phenomena may be due to the low N at the end of the process for condition B (747.6 ppm) compared to condition A (810.3 ppm) which creates a better environment (N-limitation) for EPS production. The results suggest that a relatively lower N concentration has led to a higher concentration, productivity and yield of EPS for *G. lucidum*, perhaps due to the organism using more N in the biosynthesis of cell mass.

Based on the glucose consumption during the process, the culture from condition B has consumed 8 g/L of glucose compared to condition A (6.2 g/L) at day 12. These have resulted in a higher yield for condition B (0.38 g_{X}/g_{GLU}, 0.05 g_{EPS}/g_{GLU}) for both biomass and EPS in contrast to condition A (0.19 g_{X}/g_{GLU}, 0.03 g_{EPS}/g_{GLU}). Next, an RBF process of *G. lucidum* under optimised condition was carried out in the shake flask to compare the relative efficiency of the two different fermentation techniques (batch and repeated-batch) which can enhance EPS productivity.

To gain insight into which of the changes in these culture conditions was causing the effects noted, further studies using condition C (50 g/L of Glucose, 20% inoculum) and condition D (30 g/L of Glucose, 10% inoculum) were examined in Appendix 3.13B. Broadly speaking, the results of these were roughly the same with the kinetics of condition A (50 g/L of Glucose, 10% inoculum) against condition B (30 g/L of Glucose, 20% inoculum) when the glucose concentration and inoculum percentage were swapped.

3.4.3 Repeated-batch fermentation and its optimisation

To investigate the long-term stability and performance of *G. lucidum*, RBF was implemented in this work. This technique was accomplished by substituting a certain portion of the mature fungal culture fluid with fresh medium. This approach has been shown to improve the productivity of fungal bioproducts as it saved time for growth of seed culture, inoculation, cleaning and sterilisation of the fermentor between each of the fermentation cycles (Qu et al., 2013). Therefore, in the present study, to develop an effective strategy for the RBF process, mainly for increasing EPS productivity, the optimisation experiment was carried out in shake flasks to find a suitable broth replacement ratio and the broth replacement time point for this process.

3.4.4 Effects of broth replacement ratio

The experiments with different broth replacement ratios were implemented for the repeated-batch culture and the different broth replacement ratios were set as 50% (100 mL), 70% (140 mL), and 90% (180 mL). During the broth replacement procedure, these volumes were withdrawn from three separate 500 mL

(200 mL of total working volume) flasks after 288 h (12 days) of fermentation, and then the same volume of fresh medium was added in into the flask. Samples were withdrawn at 144 h (6 days) intervals and analysed for mycelial biomass, EPS, residual sugar and mycelial morphology. These data were processed and compared to previous work by Yang et al., (2005) which also used RBF technique on the fungus *Rhizopus arrhizus*.

Based on the results displayed in Fig. 3.5 below, the three broth replacement ratios were able to tolerate continued production of biomass and EPS for up to 5 cycles (R5) during RBF. The cells of the *G. lucidum* were reused and this resulted in the elimination of lag phases during the subsequent culture and EPS productivity (g/L day^{-1}) was enhanced considerably. During the RBF cycles (R1-R5), there were adaptations of the cultures to their growth conditions starting from the second (R2) to third (R3) cycle for all broth replacement ratios. At these first two cycles, the fungus was adapting to the new conditions and some sub-populations may enter the death phase due to the stress of the new medium inserted. Later on, they were able to stabilise at the fourth (R4) and the fifth (R5) cycle and these cultures kept producing EPS until the environment became too viscous (turbid) for any further EPS production.

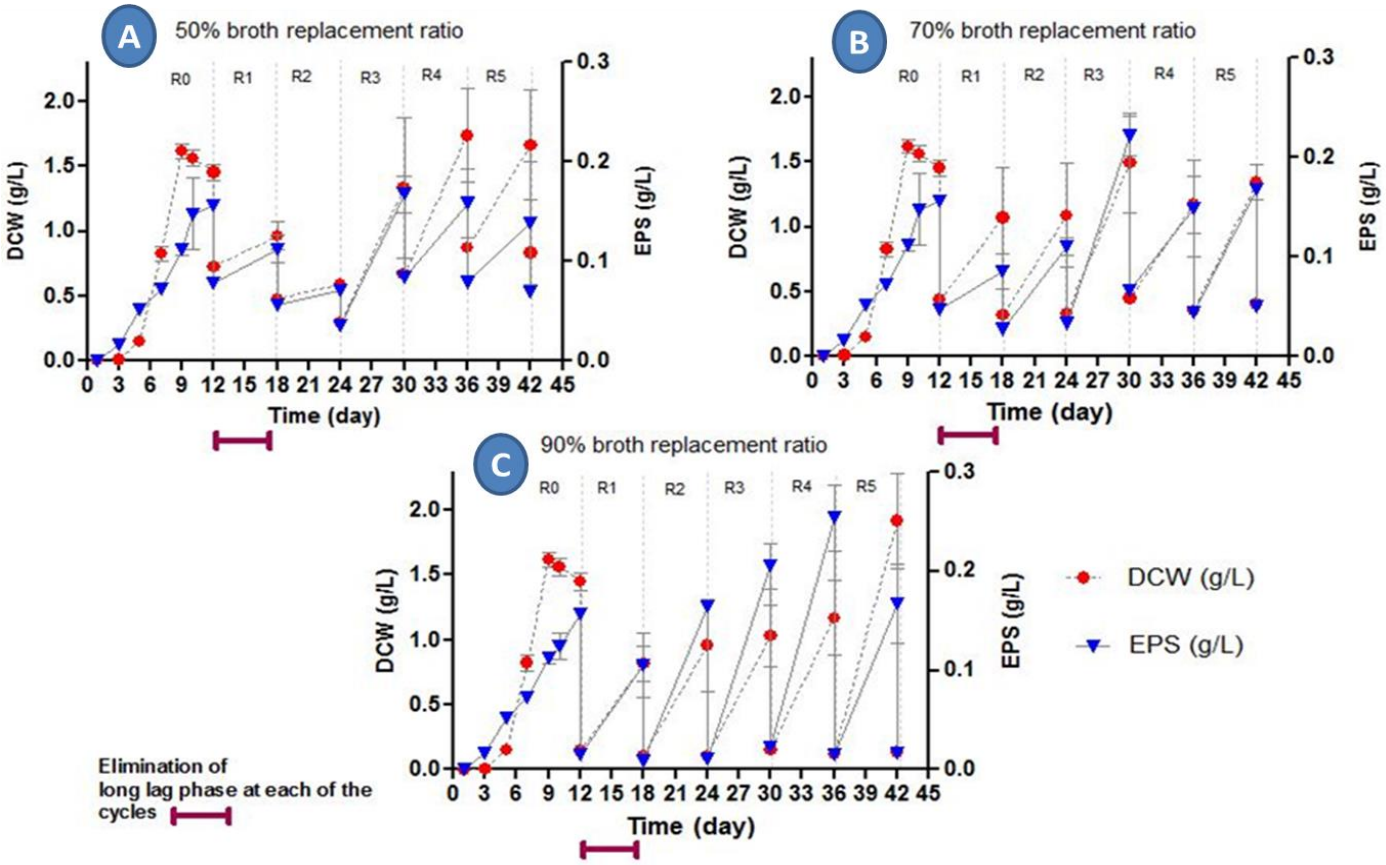


Figure 3.5 Effect of broth replacement ratio on DCW and EPS production during RBF of *G. lucidum* BCCM 31549 (Leuven, Belgium) in the shake flask at (v/v): (A) 50%, (B) 70% and (C) 90%. All other fermentation conditions were all the same [(g/L): Glucose 50, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_4 7H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, pH 4, 10% (v/v) inoculum and temperature 30 ^oC. R1-R5 means fermentation repetition in cycles.

In terms of time utilisation in batch cultures, it normally took 12 days to achieve the highest EPS production and approximately 6 days of lag phase based on the previous shake flask batch fermentation (Fig. 3.4). The RBF technique in Fig. 3.5 has successfully eradicated the extended lag phase for each of the cycles. This data is in agreement with the previous work by Zhang et al., (2014b) that used six continuous batches of RBF to improve lactic acid productivity of *Bacillus coagulans*. Thus, the implementation of RBF for the current work has reduced the fermentation time and eliminated the lag phase.

The DCW and EPS production of the three broth replacement ratios were compared to choose the optimal substitution value for further work. On the whole, between the first cycle (R1) until the fifth cycle (R5), EPS concentration was higher or the same compared to without RBF at R0 and this suggest a good survivability of the RBF culture. Overall, 50% broth replacement ratio was the least stable in both DCW and EPS productivity followed with 90% and 70% broth replacement ratios. These 90% and 70% strategies have shown a better EPS production throughout all cycles (R1, R2, R3, R4, and R5) compared to 50%. It can be observed that when the fermented broth had been replaced with 100 mL (50%), the growth of mycelium was too vigorous, making conditions for polymer synthesis poor; as a result EPS production was restrained. This may be due to the increased viscosity caused by the higher biomass and waste build-up in the culture, hence causing an undesirable growing environment for the fungus, thus signifying the requirements for increasing the broth replacement ratio.

Removing portions of old fermentation mixture in RBF has created a quasi-continuous production of inoculum for each cycle. Once the old medium had been

removed, the balance or residue in the system will indirectly act as an inoculum for the future cycles. This automatically saves much time in preparing a new set of fungal inoculum in the case of *G. lucidum*. It takes a total of approximately 30 days (Section 3.1.3) for a new inoculum to be prepared and matured while RBF in the shake flask has surprisingly required only 6 days. This can ensure the effectiveness of RBF as a substitute of normal batch fermentation both in time and labour.

3.4.4.1 Morphological characteristics at different broth replacement in RBF processes

With a light microscope and a coupled camera, the morphological features of the different broth replacement ratios on RBF were compared. Figure 3.6, 3.7, and 3.8 showed the evaluation of the morphological changes during the RBF of *G. lucidum* using 50%, 70% and 90% broth replacement ratios (v/v), respectively. For all broth replacements, the pellets were of starburst appearance with a mixture of feather-like pellet before the cycle had started at R0 and producing diverse morphology for each of the first cycles (R1). Throughout the process, significant autolysis (indicated by pellet colouration) was noticeable only in the third cycle (R3) for all broth replacement ratios.

For 50% broth replacement ratio (Fig. 3.6), following the batch fermentation (R0), the pellets have longer protuberances during the first RBF cycle (R1) and these protuberances (branch-points) kept increasing in length until it produced second generation protuberances by the second cycle (R2). By the third cycle (R3), coloured pellets were observed and increased passing the fourth cycle (R4) and ended at the fifth cycle (R5). Pellets turned yellow at R4 until they became brownish at R5 as observed in the flasks. Pellet colouration seemed to be associated with partial

disintegration of pellets, possibly indicating autolysis. This might be the reason for lowest EPS value in this strategy compared to 70% and 90% strategies.

Based on Fig. 3.7, 70% broth replacement started with feather-like pellets during R1 and pellets increased in hairiness for both R2 and R3. The increasing number of filaments have caused the feather-like pellets to disperse completely during R4 and terminated at R5. This strategy favoured EPS production for *G. lucidum* and showed the lowest presence of coloured (yellowish-brown) pellets. Pellets colouration was minimised at R4 and R5 compared to 50% broth replacement ratio.

By contrast (Fig. 3.8), 90% broth replacement ratio showed a larger pellet with a dense core at R1. Later on, this large pellet reduced in size (macroscopic observation), but increased in hairiness at R2. By the third cycle (R3), the mycelial filament was completely dispersed in the culture with an increasing number of coloured pellets, which related to the event of cell autolysis. These autolysing pellets were increased at R4 and completely reduced at R5. This broth replacement strategy was unable to produce a better morphology for EPS production compared to 70% broth strategy. Macroscopically, the pellet size was the largest compared to others at R4 and R5.

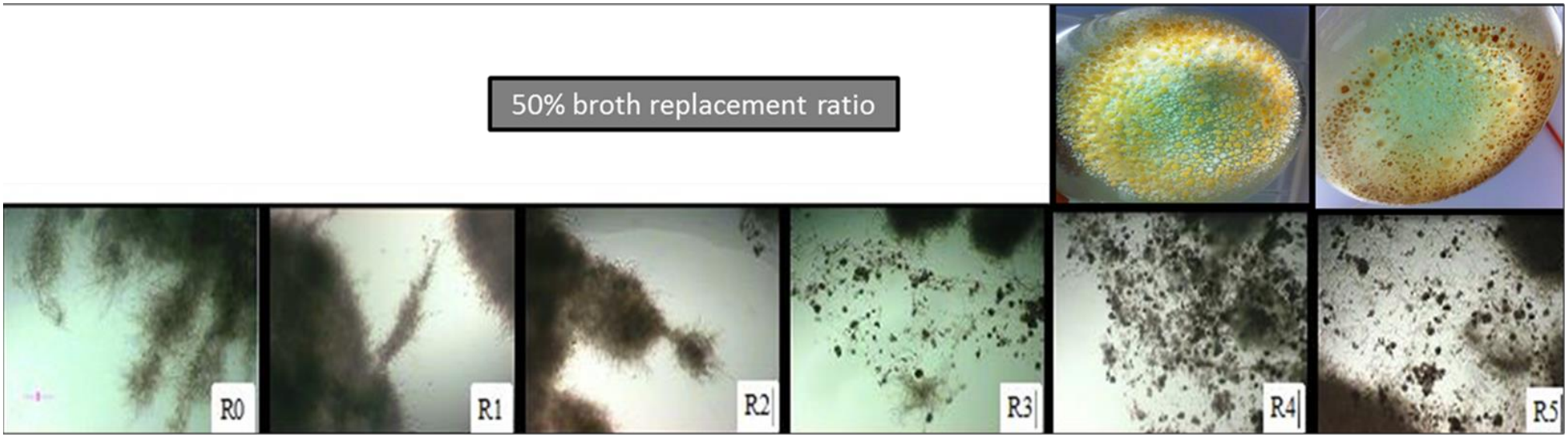


Figure 3.6 The comparison of morphological changes during shake flask RBF on *G. lucidum* BCCM 31549 (Leuven, Belgium) using 50% broth replacement ratio. Images were taken at 4-fold magnification. Bar = 150 µm. R0 – R5 means fermentation repetition in cycles. Macroscopic observations of the pellet colour changes were shown at R4 and R5, respectively.

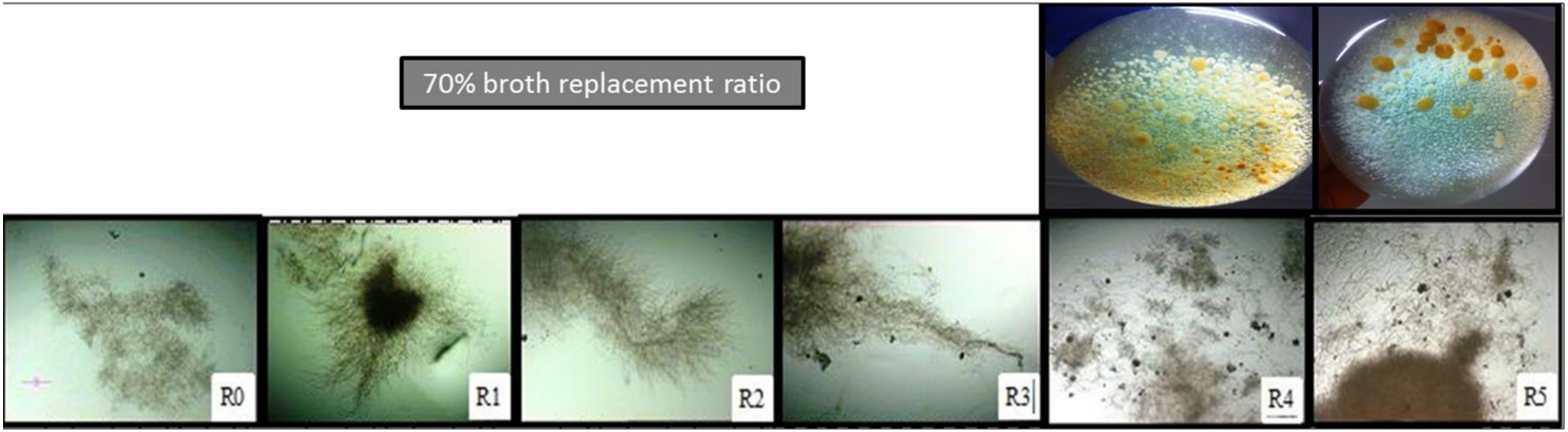


Figure 3.7 The comparison of morphological changes during shake flask RBF on *G. lucidum* BCCM 31549 (Leuven, Belgium) using 70% broth replacement ratio. Images were taken at 4-fold magnification. Bar = 150 µm. R0 – R5 means fermentation repetition in cycles. Macroscopic observations of the pellet colour changes were shown at R4 and R5, respectively.

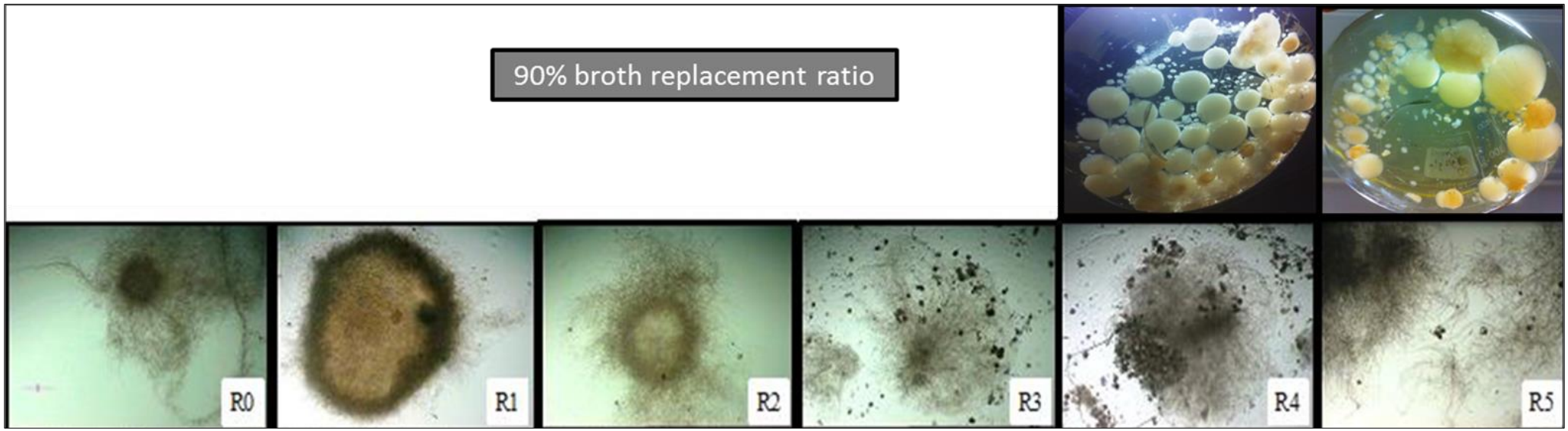


Figure 3.8 The comparison of morphological changes during shake flask RBF on *G. lucidum* BCCM 31549 (Leuven, Belgium) using 90% broth replacement ratio. Images were taken at 4-fold magnification. Bar = 150 µm. R0 – R5 means fermentation repetition in cycles. Macroscopic observations of the pellet colour changes were shown at R4 and R5, respectively.

Overall, from the third cycle (R3) to fifth cycle (R5), the highest autolysed pellet was observed at 50% broth replacement ratio (brown pellets) followed by 90% (yellowish pellets) and 70% (fewer yellowish-brown pellets).

According to the pattern of the RBF cycles, they were halted when the production of each cycle has reduced to half of the value of the batch (R0), making any additional cycles pointless. This is because, the rate of pellet colouration (autolysis) in the system has disrupted the physical activity of the mycelia in the shake flasks (McNeil and Harvey, 2008). Therefore, the repeated-batch system was discontinued at the fifth cycle (R5) for all of the three broth replacement ratios (50%, 70% and 90%).

This might be linked to the colour of pellets in the shake flasks (yellowish to brown) during RBF, which changed during the fifth cycle (R5), suggesting an unhealthy fermentation culture condition compared to the initial RBF stage (RO). This colour change may have led to an increase in viscosity which leads to the culture fluid displaying non-Newtonian behaviour, typical of those containing dispersed filamentous fungi (Wang and McNeil, 1995). These phenomena were believed to be caused by cell autolysis which is defined as a self-inflicted disruption of the cell membrane integrity, for example, following the death or starvation (McNeil and Harvey, 2008), starting at the third (R3) until the fifth (R5) cycle. Besides, White et al., (2002) explained that this natural autolysis was due to self-digestion of aged hyphal cultures, arising as a result of hydrolase activity, causing cell wall disruption and vacuolation.

3.4.4.2 Kinetics for broth replacement ratio

As for the kinetics, there was no significant difference (P value = 0.14) in biomass and EPS production between 70% and 90% of broth replacement ratios; as a result the technique was repeated with 80% broth replacement ratio to identify the optimum broth replacement ratio between them for future work. Therefore, all kinetic parameters for 50%, 70%, 80% and 90% were calculated and compared in Table 3.3.

The biomass concentrations of (g/L): 1.04, 1.17, 1.94 and 1.39 were obtained on 50%, 70%, 80% and 90% of broth replacement ratios, and the third cycle (R3) was the highest for all strategies using RBF. The corresponding biomass productivities for 50%, 70%, 80% and 90% of broth replacement ratios were (g/L day^{-1}): 0.173, 0.195, 0.324, and 0.231, respectively. To date, there have been no reports on the use of RBF on the filamentous fungi *G. lucidum*. The present work indicates that 80% broth replacement ratio was the finest biomass producer (1.94 g/L) from the viewpoint of cell growth and also has the highest biomass productivity (0.324 g/L day^{-1}) compared with others.

On the other hand, the EPS concentrations for 50%, 70%, 80% and 90% of broth replacement ratios were (g/L): 0.13, 0.19, 0.21, and 0.21. The corresponding EPS productivities were (g/L day^{-1}): 0.022, 0.031, 0.035, and 0.035, respectively. Observations have shown that 80% broth replacement ratio produces also the highest EPS concentration and EPS productivity compared to 50%, 70%, and 90% broth replacement ratios.

Table 3.3 Kinetic parameters on different broth replacement ratios by *G. lucidum* BCCM 31549 repeated-batch fermentation in the shake flask.

Broth replacement ratio [a]	Biomass concentration (max) x (g/L) (X_2 - X_1) [b]	Biomass productivity P_x (g/L day^{-1})	EPS concentration (max) , $_{EPS}$ (g/L) (EPS_2 - EPS_1) [c]	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, Q_{EPS}/x [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g_{EPS}/g_{GLU})	Yield $_{DCW}$ (DCW yield on sugar), (g_{DCW}/ g_{GLU})
50%	1.04 ± 0.04	0.173 ± 0.04	0.13 ± 0.03	0.022 ± 0.01	0.021 ± 0.006	0.037 ± 0.01	0.29 ± 0.1
70%	1.17 ± 0.07	0.195 ± 0.03	0.19 ± 0.08	0.031 ± 0.002	0.027 ± 0.007	0.022 ± 0.01	0.14 ± 0.02
80%	1.94 ± 0.10	0.324 ± 0.02	0.21 ± 0.01	0.035 ± 0.004	0.018 ± 0.006	0.042 ± 0.01	0.38 ± 0.05
90%	1.39 ± 0.09	0.231 ± 0.03	0.21 ± 0.01	0.035 ± 0.003	0.025 ± 0.004	0.015 ± 0.01	0.10 ± 0.02

[a] All broth replacement ratios were at the third cycle (R3) of RBF in the shake flask.
[b] (X_2-X_1) means the value of end biomass concentration minus initial biomass concentration for the each of the cycles.
[c] (EPS_2 - EPS_1) means the value of end EPS concentration minus initial EPS concentration for the each of the cycles.
[d] Fermentations were carried out in shake flasks with the conditions and medium compositions of [(g/L): Glucose 50, KH_2PO_4 0.5, K_2HP_{O4} 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl $_4$], 100 rpm, initial pH 4, 10% (v/v) initial inoculum and temperature at 30 ^oC.

1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair (s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration (max) was significantly different (P < 0.05) against Biomass productivity, EPS concentration (max), EPS productivity, Specific production of EPS, Yield $_{EPS}$ and Yield $_{DCW}$, respectively.

The yield of extracellular polysaccharides (EPS) and dry cell weight (DCW) that have been produced were calculated as: $\frac{\text{the compound produced}}{\text{glucose consumed}}$ at the end of the process. The EPS yields on sugar for 50%, 70%, 80% and 90% broth replacement ratios were (g_{EPS}/ g_{GLU}): 0.037, 0.022, 0.042 and 0.015 while DCW yields on sugar were (g_{DCW}/ g_{GLU}): 0.29, 0.14, 0.38 and 0.10, respectively. As expected, 80% broth replacement ratio gave both the highest $Yield_{EPS}$ (0.042 g_{EPS}/ g_{GLU}) and $Yield_{DCW}$ (0.38 g_{DCW}/ g_{GLU}) compared to other broth replacement ratios.

Overall, the results for different broth replacement ratios (50%, 70%, 80%, 90%) experiment in Table 3.3 indicate that 80% broth replacement ratio kinetic parameters showed the most sustainable and highest concentration, productivity, and yield for both EPS and biomass. By using 80% broth replacement ratio, 80% of the total fermented culture was harvested and the residue represents 20% (v/v) of an active inoculum for the subsequent cycles. Hence, the fermentation results have confirmed that 80% broth replacement ratio was the optimum strategy both for EPS and dry cell weight production in RBF cycles. This conclusion is in agreement with the previous work by Zhang et al., (2014b), which used 80% (v/v) of replaced volume during their reported RBF process of *Bacillus coagulans*.

3.4.5 Effects of broth replacement time point

Table 3.4 Experimental design for the effect of broth replacement time point during the *G. lucidum* BCCM 31549 RBF using shake flask.

Repeated-batch	Time point (day) *	Phases
A	11	at the end of logarithmic phase
B	12	transition phase
C	13	stationary phase

* These time points were obtained from the normal shake flask batch fermentation based on their EPS production curve from Fig. 3.4

These experiments were aimed at identifying the best process time point at which to carry out broth replacement. In Table 3.4, the experiments were conducted at the optimised 80% broth replacement ratio (v/v) and the replacement time points were set at A = end of logarithmic growth phase (day 11), B = transition phase (day 12), and C = stationary phase (day 13). Samples for RBF experiments were taken at six day intervals from each cycle. Based on Fig. 3.9, sustainable fungal production of EPS and DCW were observed in the three experiments (repeated batch A, B and C). Repeated-batch cells have shown their ability to undergo repeated use and seven batches could be fermented consecutively using a 500 mL flask, which mean the *G. lucidum* cells demonstrated robustness to repeated-fermentation cycles.

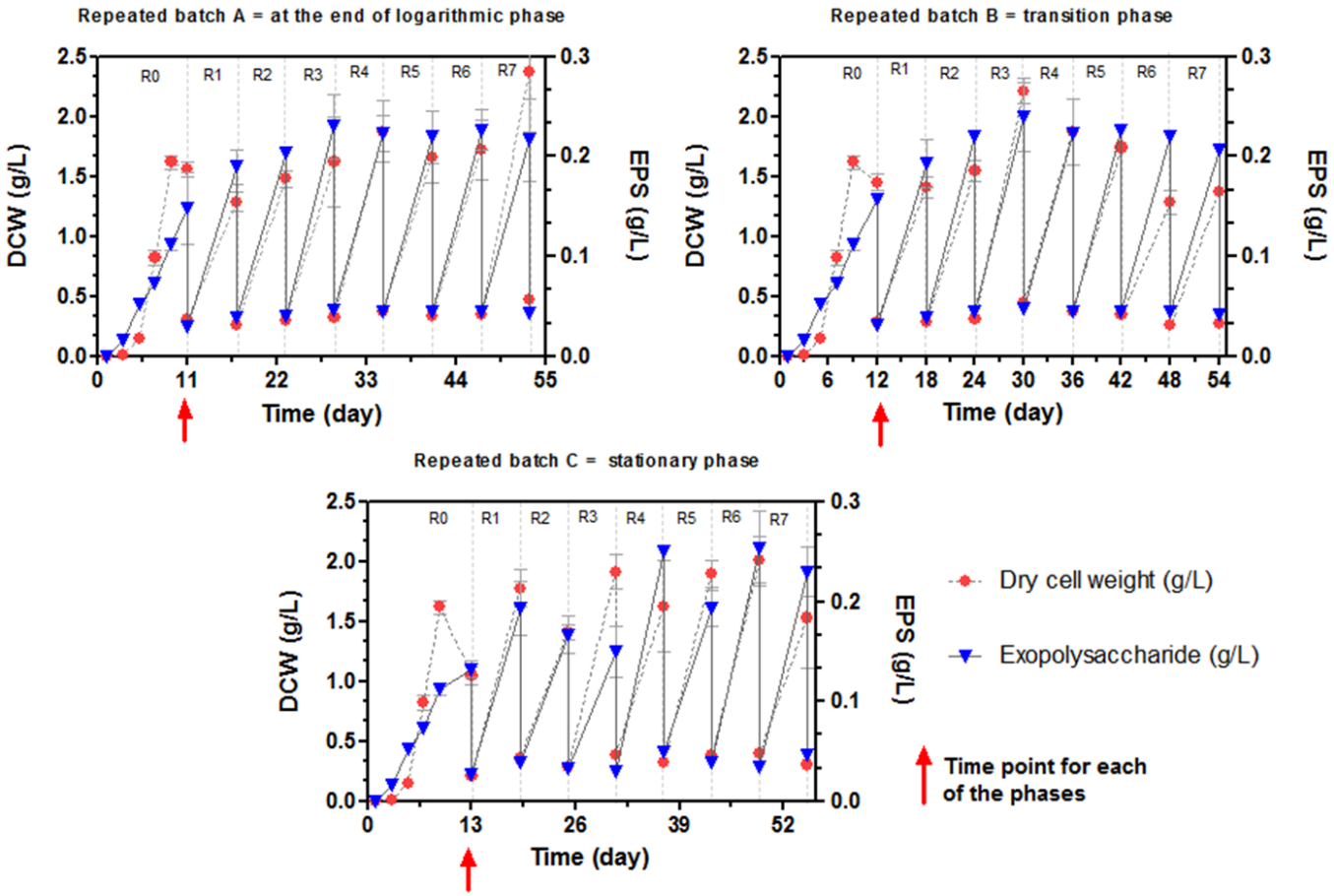


Figure 3.9 Effect of broth replacement time point on DCW and EPS production during RBF of *G. lucidum* BCCM 31549 (Leuven, Belgium) at A = at the end of logarithmic phase, B = transition phase, and C = stationary phase. All other fermentation conditions were all the same [(g/L): Glucose 50, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl 4], 10% inoculum, 100 rpm, pH 4, and temperature 30 ^oC. R1-R7 means fermentation repetition in cycles. Letters indicated R0 = Batch, R1= first cycle, R2 = second cycle, R3 = third cycle-, R4 = fourth ycle, R5 = fifth cycle, R6 = sixth cycle, R7 = seventh cycle. T-test showed the P value is <0.000 for all conditions. All means were significantly different ($P < 0.05$).

Between the first to seventh cycle (R1-R7), the lag phase reduced from each initial batches (R0) and all of the repeated-batch phases were able to produce EPS and DCW continuously until the seventh cycle. The approximate amount of EPS obtained was the same, or higher, than the batch fermentation (R0). These results were comparable to previous research by Naritomi et al., (2002), stating that RBF methods have often been used to obtain higher productivity than with normal batch culture. These were confirmed by their kinetic parameters (Section 3.4.5.1) to analyse the best broth replacement time point for further work.

3.4.5.1 Kinetics for broth replacement time point

Table 3.5 shows the growth and fermentation characteristics of three different broth replacement time point (A= at the end of logarithmic phase, B= transition phase and C= stationary phase) using 80% (v/v) repeated-batch process technique. It can be observed that the biomass productivity and EPS productivity of the seven RBF cycles (R1 - R7) were more or less similar to each other with one (time point B) of the phases (time point A and C) showing a better result. Overall, stable and consistent biomass productivity (P_x) of 0.163 – 0.318 g/L day^{-1} , EPS productivity (P_{EPS}) of 0.021 – 0.037 g/L day^{-1}, and specific production of EPS ($Q_{EPS/x}$) of 0.012 – 0.031 (g/g) day^{-1} were obtained for all time points (A, B and C). On average, these time points had produced a balanced value for P_x (A = 0.230, B = 0.217, C = 0.235 g/L day^{-1}), P_{EPS} (A = 0.029, B = 0.029, C = 0.028 g/L day^{-1}) and $Q_{EPS/x}$ [A = 0.022, B = 0.023, C = 0.021 (g/g) day^{-1}], respectively for the average of seven cycles.

Table 3.5 Effect of different broth replacement time point (growth phase) on DCW and EPS productivity by *G. lucidum* BCCM 31549 repeated-batch fermentation [c].

Time point [a]	Kinetic values [b]	RBF cycles [d]							**Average** (R1-R7)
		R1	R2	R3	R4	R5	R6	R7	
A	P_x (g/L day $^{-1}$)	0.163	0.204	0.221	0.258	0.215	0.232	0.318	1.611 ± 0.05
	P_{EPS} (g/L day $^{-1}$)	0.027	0.028	0.032	0.030	0.030	0.031	0.029	0.207 ± 0.002
	$Q_{EPS/x}$ [(g/g) day $^{-1}$]	0.028	0.023	0.024	0.019	0.023	0.022	0.014	0.153 ± 0.004
B	P_x (g/L day $^{-1}$)	0.187	0.211	0.318	0.238	0.228	0.156	0.187	1.525 ± 0.05
	P_{EPS} (g/L day $^{-1}$)	0.027	0.030	0.033	0.029	0.030	0.029	0.028	0.206 ± 0.002
	$Q_{EPS/x}$ [(g/g) day $^{-1}$]	0.024	0.024	0.017	0.021	0.022	0.031	0.025	0.164 ± 0.004
C	P_x (g/L day $^{-1}$)	0.262	0.176	0.272	0.208	0.263	0.274	0.188	1.643 ± 0.04
	P_{EPS} (g/L day $^{-1}$)	0.028	0.021	0.020	0.037	0.024	0.036	0.033	0.199 ± 0.07
	$Q_{EPS/x}$ [(g/g) day $^{-1}$]	0.018	0.020	0.012	0.030	0.015	0.022	0.030	0.147 ± 0.007

[a] A= at the end of logarithmic phase, B= transition phase and C=stationary phase.
[b] P_x (g/L day $^{-1}$) = biomass productivity, P_{EPS} (g/L day $^{-1}$) = EPS productivity and $Q_{EPS/x}$ [(g/g^{-}) day $^{-1}$] = specific production of EPS
[c] Fermentations were carried out in shake flasks with conditions and medium compositions of [(g/L): Glucose 50, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 10% (v/v) inoculum, 100 rpm, initial pH 4, temperature 30 ºC and there was 6 days interval between each cycle.
[d] R1-R7 means repetition in cycles

For the maximum biomass productivity (P_X), time point A reached the highest value of 0.318 g/L day^{-1} at seventh cycle (R7) and 0.318 g/L day^{-1} for time point B at third cycle (R3). Meanwhile, time point B and C generated the highest EPS productivity (P_{EPS}) of 0.033 g/L day^{-1} at third cycle (R3) and 0.036 g/L day^{-1} at fourth cycle (R4), respectively. It seems that there were no significant differences ($P > 0.05$) on EPS productivity between time point B and C. However, time point B was chosen as the ideal broth replacement time point for RBF as it showed the most stabilised growth curve (Section 3.4.5: Fig. 3.9) compared to time point A and C, and reached the highest total value of $Q_{EPS/x}$ [0.164 (g/g) day^{-1}]. The reasons for higher values are because the time points were different morphologically. In the last two cycles, the EPS production rate did not change despite the viscous broth of *G. lucidum* pellets which adhered to the inside wall of the flask (Section 3.4.5.2).

3.4.5.2 Morphological changes during broth replacement time points

Figure 3.10, 3.11 and 3.12 showed the comparison of morphological changes during RBF of *G. lucidum* with different broth replacement time points. Each phase started at a different initial time point which was at day 11 for A, day 12 for B and day 13 for C. These three different days have showed diverse initial morphology (R0) which was bulge-pellets (protuberance) for A, starburst-like pellets for B and detached-pellets (second generation pellets) for C. These can be explained since each of them had a different starting pellet type which was related to the number of days of pellet formation during the previous batch fermentation (Section 3.4.2: Fig. 3.4).

Subsequently, *G. lucidum* produces different pellet shapes throughout the first cycle (R1) to seventh cycle (R7). The pellet shape (branched filaments) came from

the event of pellet break-up which was observed in the work by Wagner et al., (2004) at day 13 of shake flask fermentation (this corresponds to time point C at day 13 showing detached-pellets in the present study).

For time point A (Fig. 3.10), at the first cycle (R1), the bulge-pellets from batch (R0) have detached from the pellet surfaces and formed scattered (small) starburst-like pellets. This starburst-like pellets may have coagulated with mycelial fragments at second cycle (R2), thus forms a small dense-core structure. Reaching third cycle (R3), this dense-core pellet structure has broken into several branched filaments and later these filaments were torn into a higher-branched network called feathers at fourth cycle (R4). By the fifth cycle (R5), torn filament-branches have tied with the outer mycelia thus creating dense-branched feathers. These dense-branched feathers were thickened into intertwined branched-feathers at the sixth cycle (R6) and later the thick branches deteriorated into hairy feathers at seventh cycle (R7) in Fig. 3.10.

For time point B (Fig. 3.11), the morphology initially showed starburst-like pellets (R0). At this time, the pellets were at the start of the pellet break-up or the onset of production of second progeny from the first pellet, thus suggesting an active growing environment for the fungus. Later, the broken second generation pellets were compacted and became hairy at R1. These hairy pellets were intertwined with each other and became irregular shaped at R2. They disintegrated during the following cycle and formed smoother ovoid pellets at third cycle (R3).

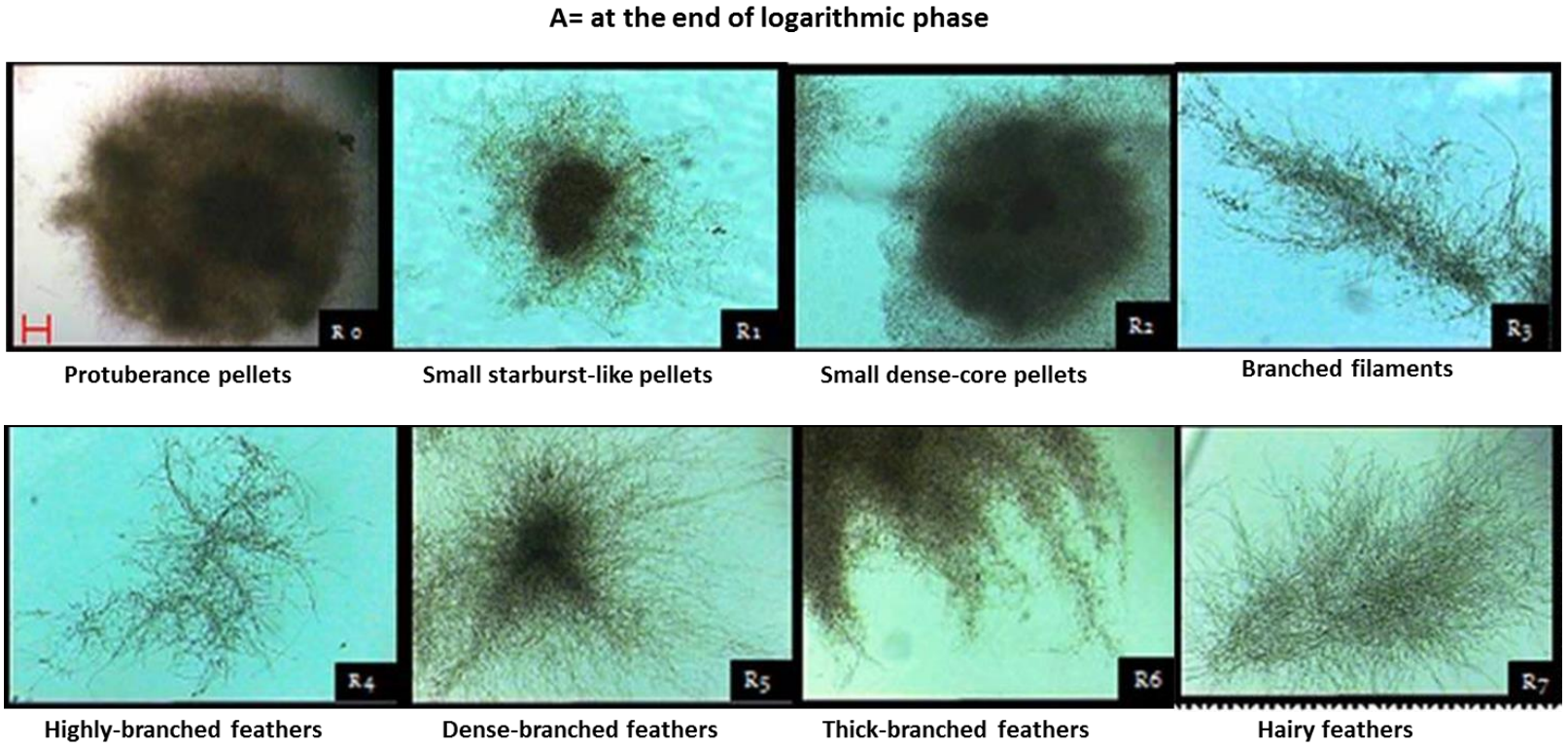


Figure 3.10 The comparison of morphological changes during shake flask RBF on *G. lucidum* BCCM 31549 (Leuven, Belgium) with a specific broth replacement time point (at the end of logarithmic phase). Images were taken at 4-fold magnification. Bar = 150 µm. R0 – R7 means fermentation repetition in cycles.

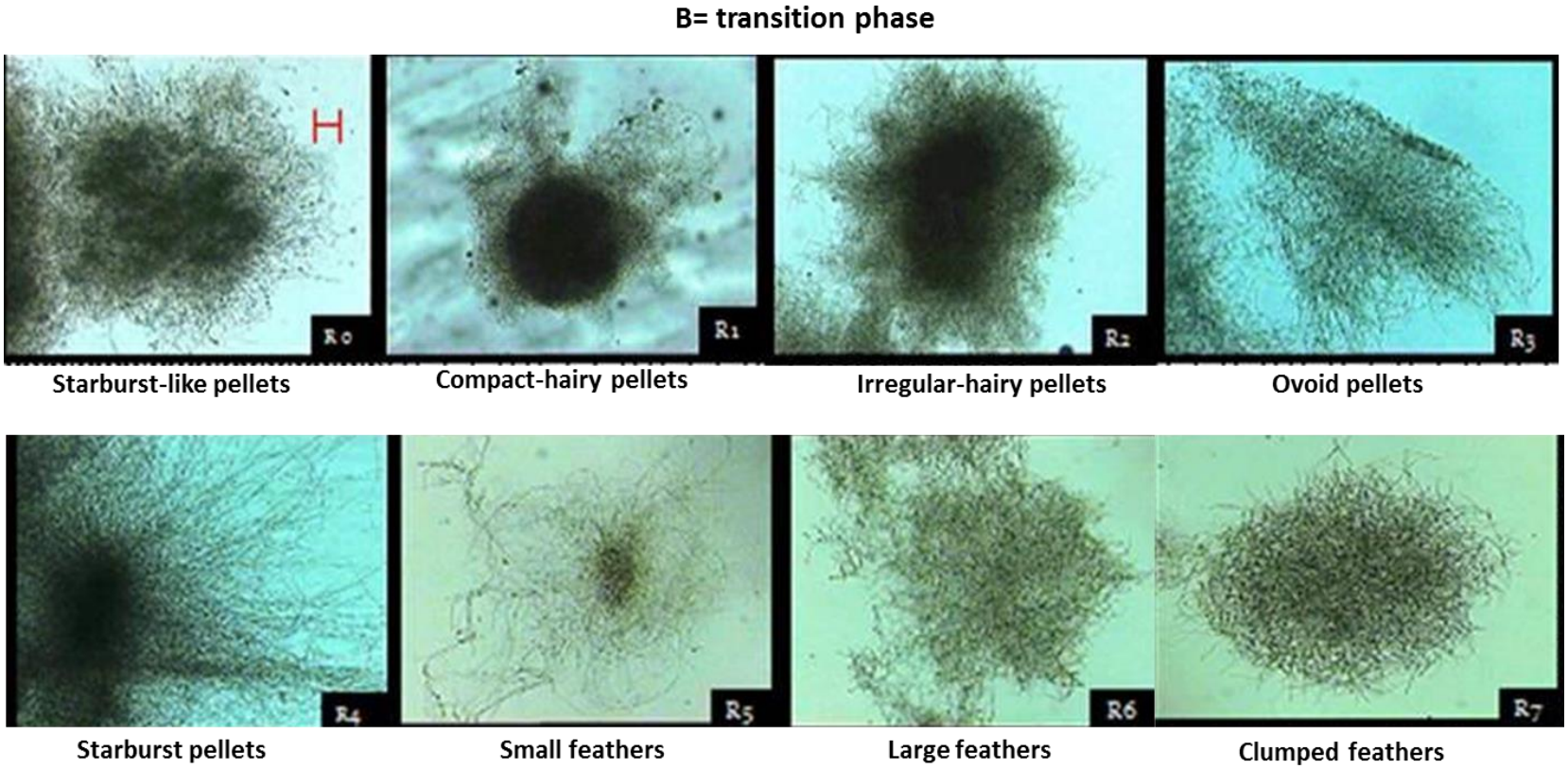


Figure 3.11 The comparison of morphological changes during shake flask RBF on *G. lucidum* BCCM 31549 (Leuven, Belgium) with a specific broth replacement time point (transition phase). Images were taken at 4-fold magnification. Bar = 150 µm. R0 – R7 means fermentation repetition in cycles.

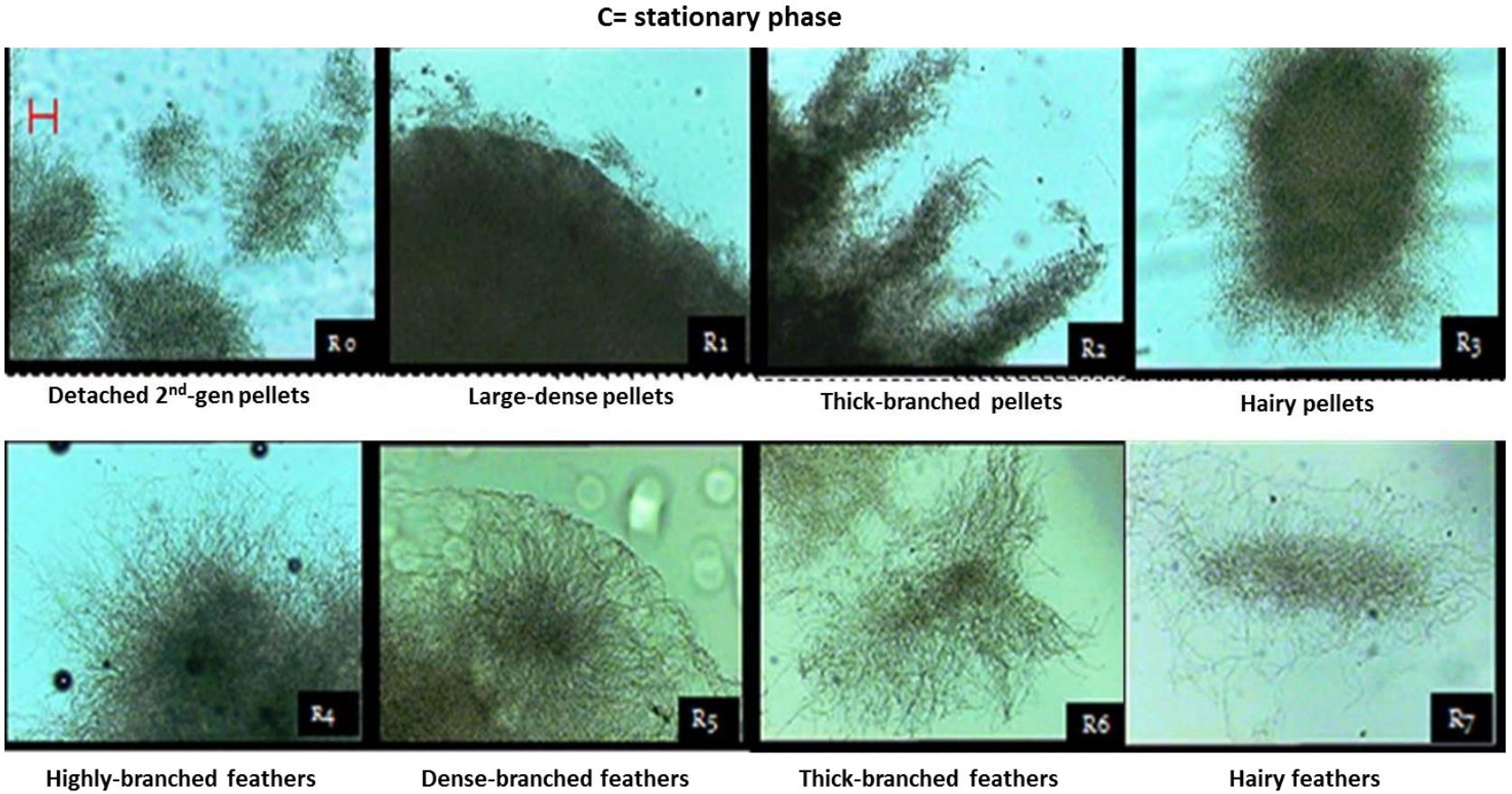


Figure 3.12 The comparison of morphological changes during shake flask RBF on *G. lucidum* BCCM 31549 (Leuven, Belgium) with a specific broth replacement time point (stationary phase). Images were taken at 4-fold magnification. Bar = 150 µm. R0 – R7 means fermentation repetition in cycles.

Later, these pellets engulfed the nearby mycelia and formed starburst pellets at fourth cycle (R4) which later broke into small feather-like dispersed clumps or aggregates by fifth cycle (R5). These little loose hyphal aggregates increased in size and became large at sixth cycle (R6), then clumped together at seventh cycle (R7).

For time point C (Fig. 3.12), the second generation pellets were completely dispersed during the initial stage (R0). These dispersed new pellets were intertwined with each other and then formed larger dense structures during the first cycle (R1). Later on, protuberances extended from the pellet surfaces, forming thick-branched pellets during the second cycle (R2). The hairy feathers surrounding the thick-branched pellet detached from the branch's tips and generated small fluffy pellets at third cycle (R3). The hair surrounds the pellets, which elongated and increased in number before becoming highly-branched hyphal trees. The hairy structure entangled again and clumped at fifth cycle (R5) and burst from the core at sixth cycle (R6). Ovoid pellets were shaped at seventh cycle (R7) once the hyphal elements growing outwards were sheared off.

These three-time points showed a variety of pellet morphology throughout the batches but only some of them resulted in EPS production. According to work by Wagner et al., (2004), EPS was the highest for *G. lucidum* once the ovoid pellets have formed. As observed in the present study, time point B has produced ovoid pellets at R3, while time point C managed to produce ovoid pellets at R7 and time point A was incapable of producing ovoid pellets throughout the cycles. The formation of ovoid pellets at R3 of time point B corresponds to its kinetic parameter showing the EPS productivity, thus time point B technique was chosen for future work.

With the implementation of RBF technique, there was evidence of fungal rejuvenation and renewed growth by the culture due to the influx of fresh nutrient supplies at each repeated-batch cycle, and possibly also to the reduction in levels of metabolic waste compounds in the culture medium due to the dilution with fresh medium. Our culture has produced a series of hairy pellets which consist of active tip regions and this may contribute to the success of RBF strategy.

The results of this study have confirmed that *G. lucidum* was stable for extended biomass and EPS production periods. To the best of our knowledge, no other filamentous fungus, especially from a *Ganoderma* species has been reported to produce bioactive metabolites over an extended fermentation period. However, because the same fungal cells in the culture were used in the RBF, it is critical to keep the biomass and EPS concentration below the restricting level by changing the media at an appropriate fermentation time as mentioned by Wenyan et al., (2014). From the results of the present study, RBF using shake flask has to be stopped at the seventh cycle due to pellet-colour changes (possible autolysis) and perhaps toxic metabolites build up. Therefore, experiments in the bioreactor should be done to determine the highest cycle number that RBF can be extended. These successful repeated-batch processes are now compared with the batch in terms of kinetic parameters.

3.4.6 Comparison of batch and RBF kinetic parameters in shake flasks

Table 3.6 shows the kinetic parameters of batch and RBF of *G. lucidum* in shake flasks. As expected, the biomass and EPS concentration of RBF (third cycle) were higher than the batch by 1.2 fold and 1.3 fold, respectively.

Table 3.6 Comparison of batch and RBF kinetic parameters in the shake flask using *G. lucidum* BCCM 31549.

Fermentation technique [a]	Biomass concentration, x (g/L)	Biomass productivity P_x (g/L day^{-1})	EPS concentration, EPS (g/L)	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPSs/x}$ [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g_{EPS}/g_{GLU})	Yield $_{DCW}$ (DCW yield on sugar), (g_{DCW}/g_{GLU})
Batch	1.62 ± 0.1	0.180 ± 0.03	0.16 ± 0.02	0.013 ± 0.001	0.009 ± 0.001	0.030 ± 0.01	0.19 ± 0.07
RBF [b]	1.91 ± 0.1	0.318 ± 0.2	0.20 ± 0.03	0.033 ± 0.001	0.018 ± 0.003	0.042 ± 0.01	0.38 ± 0.06
Increase [c]	1.2	1.8	1.3	2.5	2	1.4	2

[a] Fermentations were carried out in shake flasks with the conditions and medium compositions of [(g/L): Glucose 50, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, initial pH 4, 10% (v/v) inoculum and temperature at 30 ºC.
[b] RBF was done at 80% broth replacement ratio (v/v) during the transition phase. The repeated-batch values were taken at third cycle (R3) for repeated-batch strategy
[C] The increment values were calculated based on the difference of repeated-batch and batch

*1 way ANOVA has been carried out for each row with the P value of < 0.0001 and the pairing was significantly effective with the P value of 0.0485. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different (P < 0.05) vs Biomass concentration, EPS concentration, EPS productivity, Specific production of EPS, Yield $_{EPS}$, and Yield $_{DCW}$, respectively.

The biomass productivity of RBF was 1.8 times higher than batch while surprisingly the EPS productivity of RBF was 2.5 times higher than the batch itself. Specific production of EPS was doubled for RBF (third cycle), which shows the effectiveness of this strategy in EPS production.

By application of RBF, the productivity of EPS was increased. The likely cause could be the synergistic effect of cell reuse which was defined as the interaction of the reused mycelial cells with its adaptations to RBF. Consequently, the RBF yielded 1.4 times higher EPS than the batch and had doubled the biomass yield successfully (Table 3.6). Without using extra resources, RBF has proved to be a better fermentation technique than the batch which is in agreement with Yang et al., (2005). Hence, the repeated-batch process of *G. lucidum* with the same optimum broth replacement ratio and broth replacement time point was executed again in the fermentor to validate the achievability of these processes and compare the efficiency of RBF with a batch process using bioreactor.

Based on the optimised conditions, batch fermentation was carried out in a 2.5-L (total volume) jacketed bioreactor vessel (New Brunswick Bioflow 3000, Edison L.N, USA). A mycelial suspension was used to provide an inoculum to a 2.5 L fermentor containing 2-L medium (working volume) either with condition A or condition B, which have been described in Section 3.4.3, thus considered optimum for the current EPS production.

3.5 Upscaling

Fig. 3.13 below shows the time courses including morphological changes of two bioreactor batch fermentation of *G. lucidum* BCCM 31549 using condition A: Fig. 3.13 (1) and condition B: Fig 3.13 (3). Based on the time courses in Fig. 3.13 (1 and 3), the bioreactor fermentation process for both conditions have successfully followed the normal fungal growth curve pattern whereas the morphological changes in Fig. 3.13 (2 and 4) showed that *G. lucidum* grew with a pellet morphology as reported by Wagner et al., (2004).

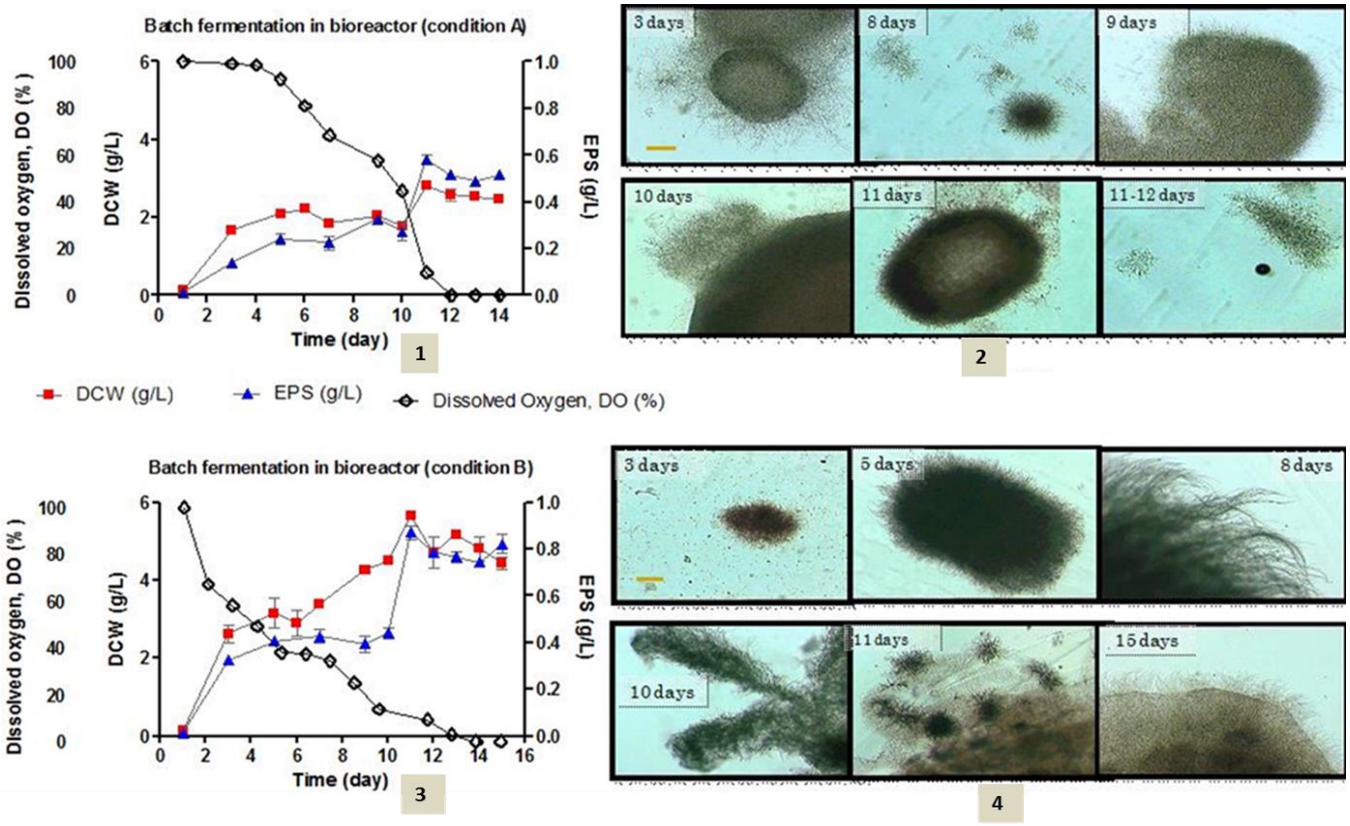


Figure 3.13 Time courses and morphological changes in bioreactor batch fermentation of *G. lucidum* BCCM 31549 (Leuven, Belgium) using Condition A: [50 g/L of Glucose, 10% (v/v) inoculum] and Condition B: [30g/L of Glucose, 20% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl 4], 2% vvm of air, 100 rpm, controlled pH 4, and temperature 30 ^oC.

*(1) T-test assesses whether the means of two groups are statistically different from each other. Therefore, paired T-test has been run for EPS and DCW for both conditions: Condition A, at 95% CI of discrepancy, the P value is <0.000 [two-tailed P value] and Condition B, at 95% CI of discrepancy, the P value is <0.000 [two-tailed P value]. Both means were significantly different ($P < 0.05$). (2) Images were taken at 4-fold magnification. Bar = 150 µm.

During the bioreactor fermentation using condition A [Fig. 3.13 (1)], biomass concentration started to increase at day 3 (1.69 g/L) and remained unchanged just before day 10. Afterwards, the biomass increased reaching its highest value at day 11 (2.82 g/L) and later it slowly reduced reaching a constant level. On the other hand, the EPS was low at an early stage but also increased at day 10 (0.27 g/L) reaching 0.58 g/L by day 11, and then slowly tapering off. Day 11 was the critical point for important events such as the onset of EPS acceleration and biomass growth. At this time, the dissolved oxygen (DO) percentage was at 10% [Fig. 3.13 (1) at day 11]. This corresponds to the criterion of a limiting environment condition, which is favourable for EPS production (Hsieh et al., 2006).

The time course in Fig. 3.13 (1) of condition A shows significant morphological changes in Fig. 3.13 (2). At day 3, the pellets were in solid sphere shape with some filaments surrounding the surface. As the day continues, the pellets broke into small feathers on day 8, and they clumped together to form large fluffy pellets at day 9. Here, the onset of EPS production occurred on day 10 which coincided with pellet break-up. The protuberances arising from the pellet surface detached from its parent (first generation pellet), forming new progeny, and this may have caused the increase in the EPS production.

On the other hand, as for the bioreactor fermentation using condition B [Fig. 3.13 (3)], both biomass and EPS started to increase/change at day 2. Later, they proceeded slowly until day 10; however the biomass growth began to be more active from day 8 onwards. The highest biomass was obtained at day 11 with the value of 5.66 g/L as well as EPS (0.87 g/L). At this time, the DO percentage was at 10%,

which is in agreement with the bioreactor process using condition A, thus confirming the limiting environment condition stimulates the EPS and biomass growth. Later, both of them slowly disintegrated into the early death phase (day 15).

In morphological terms, the changes occurring in the process under condition B are shown in Fig. 3.13 (4). At day 3, the fungus formed sphere-shaped pellets. Those pellets were then engulfing each other to create a larger pellet at day 5, producing short filaments at their surfaces. At this time around (from day 5 to day 9), the EPS values were constant as the shape of the pellets were not ready for metabolite secretion (Wagner et al., 2004). At day 10, the hairy pellets broke into starburst-shapes consistent with the onset of EPS production as mentioned by Wagner et al., (2004). Here, a dense fungal mycelium grew as a network of filaments which extended apically, branching away from each other across, to form a cluster-pellet structure.

These pellets degraded into small hyphal trees at day 11 and they clumped together to reshape the pellet structure. Here, the EPS level was the highest as the second generation pellets were being formed. By day 15, the reshaping of pellet structure was complete and the cycle of producing the third generation pellets were taking place, however due to possible nutrient exhaustion and waste build-up, the process slowed down.

Overall, the EPS and biomass were higher for bioreactor batch fermentation using condition B compared to condition A. Both processes produced two different sets of pellets. These two formation patterns were possibly formed due to different osmotic pressures of condition A (high glucose) and B (low Glucose), thus affecting

the stress level of the fungus in producing their second generation and third generation pellets, respectively. Furthermore, the kinetic parameters of these experiments were calculated and compared to understand their different productivities.

3.5.1 Kinetics of up-scaled bioreactor processes

Table 3.7 describes the kinetic parameters of bioreactor batch fermentation of *G. lucidum* using condition A and condition B. The biomass of condition B (5.66 g/L) was 2-fold than condition A (2.82 g/L) whereas the biomass productivity of condition B (0.515 g/L day^{-1}) was 2 times higher than condition A (0.256 g/L day^{-1}). In the case of EPS, condition A (0.58 g/L) was lower than condition B (0.87 g/L), while the EPS productivity of condition B (0.079 g/L day^{-1}) was 1.5 times higher than condition A (0.053 g/L day^{-1}). However, the specific production of EPS for condition A was slightly higher than condition B. When compared with the previous shake flask fermentation (Section 3.4.2.1), higher osmotic pressure of condition A may have affected the EPS production, while a higher inoculum percentage of condition B had possibly led to the improved biomass production compared to condition A, thus to a smaller pellet size and higher EPS production.

Observations on the yield for both conditions have shown that the yield of biomass on the sugar consumed for condition A was 0.19 g_{X}/g_{GLU}, while the value was 0.33 g_{X}/g_{GLU} for condition B. The yield of EPS on the sugar consumed for condition B (0.05 g_{EPS}/g_{GLU}) was 2.5 times higher than condition A (0.02 g_{EPS}/g_{GLU}). Therefore, condition B was the right candidate for the following investigation. Meanwhile, at the process end, residual N concentration for condition B was 3.6 g/L

of NH_4Cl compared to condition A (3.5 g/L NH_4Cl). Hence, approximately less than 1 g/L of nitrogen was consumed by the fungus for both conditions.

Table 3.7 Kinetic parameters of cultures during bioreactor batch fermentation of *G. lucidum* BCCM 31549 (Leuven, Belgium) using condition A: [50 g/L of Glucose, 10% (v/v) inoculum] and condition B: [30 g/L of Glucose, 20% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, 2% vvm of air, controlled pH 4, and temperature 30 ^oC.

Kinetic parameters	Condition A	Condition B
Biomass concentration, x (g/L)	2.82 ± 0.3	5.66 ± 0.9
Biomass productivity, P_x (g/L day $^{-1}$) *Increase*	0.256 ± 0.01 1	0.515 ± 0.02 2
EPS concentration, $_{EPS}$ (g/L)	0.583 ± 0.1	0.87 ± 0.1
EPS productivity, P_{EPS} (g/L day^{-1}) *Increase*	0.053 ± 0.01 1	0.079 ± 0.01 1.5
Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]	0.018 ± 0.01	0.014 ± 0.01
Yield $_{biomass}$, (g $_X$ /g $_{GLU}$)	0.19 ± 0.03	0.33 ± 0.03
Yield $_{EPS}$, (g $_{EPS}$/g $_{GLU}$)	0.02 ± 0.01	0.05 ± 0.01
End N (ppm)	914 ± 4.0	878 ± 2.0
End NH_4Cl concentration (g/L)	3.49 ± 0.09	3.36 ± 0.08

*1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different (P < 0.05) vs End N, Biomass productivity vs End N, Specific production of EPS vs End N, Yield DCW vs End N, Yield $_{EPS}$ vs End N, End N vs End NH_4Cl concentration, respectively.

Following these processes, RBF of *G. lucidum* under the optimal conditions was carried out in the same bioreactor to compare the efficiency of the two different fermentation techniques (batch and repeated-batch) which reportedly can enhance EPS and biomass productivity.

3.6 Repeated-batch fermentation in the bioreactor

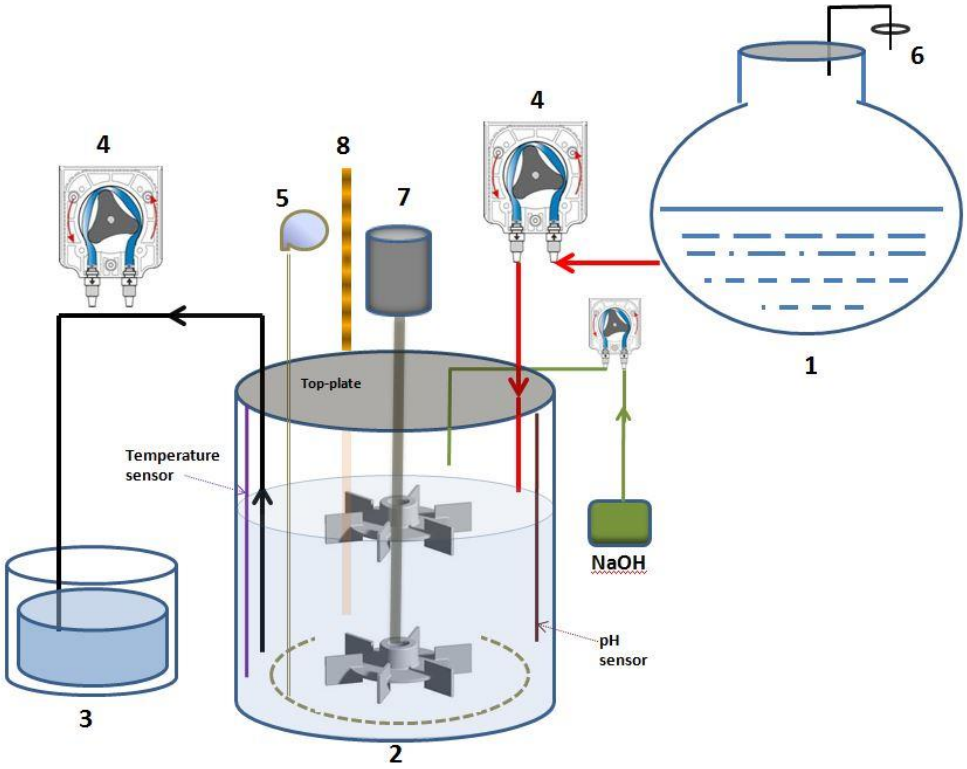


Figure 3.14 Diagram of experimental equipment:(1)medium reservoir; (2)fermentor; (3)broth receiver; (4)peristaltic pump; (5)air distributing implement; (6)filter; (7) impeller motor; (8) dissolved oxygen sensor. [Figures are by the author]

Based on Section 3.5, RBF was carried out with the bioreactor setup shown in Fig. 3.14. A mycelial suspension (10 days old: 400 mL in the shake flask) was used to provide an inoculum to a 2.5 L fermentor containing 1600 mL medium incorporated with condition B (total working culture is 2 L), which has been described in Section 3.15. At the transition phase of the fermentation process (Table 3.8 below), 80% of the broth (1.6 L) was withdrawn, and then fresh medium was pumped into the fermentor using a peristaltic pump as shown in Fig. 3.14. The pH sensor and temperature sensor at the bioreactor maintained the conditions in the system while a long sampling line made the 80% harvesting achievable. Whole broth samples from the bioreactor were analysed for EPS productivity and when maximum EPS productivity was detected the fermented broth was replaced.

3.6.1 Baffle effects on the RBF system

Table 3.8 Mode of operation for *G. lucidum* BCCM 3159 RBF in the bioreactor

Time point (day) *		Growth phase
Unbaffled [a]	Baffled [b]	
9	10	at the end of logarithmic
10	11	transition
11	12	stationary

* These time points were obtained from the batch fermentation growth curve using baffled and unbaffled conditions

[a] Unbaffled batch fermentation time course can be observed from Appendix 3.12C

[b] Baffled batch fermentation time course is shown in Fig. 3.13 (Section 3.5)

During RBF with a baffled-bioreactor, the fungal cells were unable to grow (Appendix 3.12D). Thus, the baffle had to be removed and a new set-up was

implemented. Following that, the fungus managed to adapt to the RBF system in an unbaffled bioreactor. As a result, the mode of operation for RBF in the bioreactor was improved and compared in Table 3.8. Based on the table, day 10 was chosen in the current work as the broth replacement time point. The fermentation results of the repeated cycle's experiments were taken at five day intervals from each cycle.

Table 3.9 below displays the kinetic parameters of these processes. Once the baffle was removed, the EPS production increased remarkably from 0.87 g/L (baffled) into 8.10 g/L (unbaffled) while EPS productivity experienced a 10-fold increase from 0.08 g/L day^{-1} (baffled) to 0.81 g/L day^{-1} (unbaffled). Also, both specific production of EPS and Yield $_{EPS}$ were also raised from the baffled condition [0.01 (g/g) day^{-1} and 0.05 g $_{EPS}$/g $_{GLU}$] compared with the unbaffled condition [0.81 (g/g) day^{-1} and 0.50 g $_{EPS}$/g $_{GLU}$]. This EPS enhancement under unbaffled condition was associated with higher N source consumption than the baffled condition. However, implementing unbaffled condition led to a significant reduction in biomass concentration (5.66 g/L), biomass productivity (0.52 g/L day^{-1}) and Yield $_{DCW}$ (0.33 g $_{DCW}$/ g $_{GLU}$) compared to the baffled condition [1.60 g/L, 0.16 g/L day^{-1} and 0.18 g $_{DCW}$/ g $_{GLU}$, respectively). Therefore, it can be concluded that, manipulation of the bioreactor environment by removing the baffle reduces the shear stress (reduced pellet size) of the working culture, hence by changing one condition (unbaffled) it resulted in the increased EPS with a reduced fungal growth (Seviour et al., 2011; Znidarsic and Pavko, 2001).

Table 3.9 Comparison of *G. lucidum* BCCM 31549 batch fermentation in the bioreactor.

Batch fermentation technique [a]	Biomass concentration, (g/L) (X_2 - X_1) [c]	Biomass productivity, $P\,x$ (g/L day^{-1})	EPS concentration, (g/L) (EPS_2 - EPS_1) [d]	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g $_{EPS}$/g $_{GLU}$)	Yield $_{DCW}$ (DCW yield on sugar), (g $_{DCW}$/ g $_{GLU}$)	End N (ppm)	End NH_4Cl (g/L)
Baffled [b] (at 11 days)	5.66 ± 0.9	0.52 ± 0.02	0.87 ± 0.1	0.08 ± 0.01	0.01 ± 0.01	0.05 ± 0.01	0.33 ± 0.03	878.0 ± 2	3.36 ± 0.08
Unbaffled [b] (at 10 days)	1.60 ± 0.1	0.16 ± 0.02	8.10 ± 0.3	0.81 ± 0.03	0.50 ± 0.03	0.89 ± 0.08	0.18 ± 0.02	662.4 ± 2	2.50 ± 0.08

[a] Fermentations were carried out in the bioreactor with the conditions and medium compositions of [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 2% vvm air ,100 rpm, controlled pH 4, 20% (v/v) inoculum and temperature at 30 ^oC.

[b] The values were taken at day 11 (baffled) and day 10 (unbaffled) as which the EPS was the highest for each condition

[c] (X_2 - X_1) means the value of end biomass concentration minus initial biomass concentration

[d] (EPS_2 - EPS_1) means the value of end EPS concentration minus initial EPS concentration

*1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different (P < 0.05) vs End N, Biomass productivity vs End N, EPS concentration vs End N, Specific production of EPS vs End N, Yield $_{EPS}$ vs End N, Yield $_{DCW}$ vs End N, End N vs End NH_4Cl concentration, respectively.

The successful RBF in the unbaffled bioreactor involved 5 cycles as displayed in Section 3.6.2 and this may due to some fixed objects (probes, sampling line, harvesting line) mounted in the headplate served a similar purpose as the baffle (Cherry and Papoutsakis, 1988). This achieved an improvement in EPS productivity compared to the baffled-batch fermentation.

3.6.2 RBF growth in unbaffled-bioreactor system

Initially (R0), the unbaffled-batch fermentation at day 10 (transition phase of the growth) produced biomass and EPS of 1.6 g/L and 8.10 g/L, respectively. When 80% (v/v) of culture was harvested and replaced with the same percentage of fresh medium, the first cycle (R1) showed a slight decreased in biomass growth, but the increase in EPS production (Fig. 3.15).

As reported by Naritomi et al., (2002), in the present study the second cycle (R2) and the third cycle (R3) during the current RBF in Fig. 3.15 produced nearly twice the EPS and biomass compared to batch culture. From here, it was observed that *G. lucidum* started to adapt to the RBF conditions with increasing EPS productivity until the fifth cycle (R5). The biomass and EPS concentrations were highest during the fifth cycle (R5) with values of 3.18 g/L and 0.92 g/L respectively. Following that, the RBF process was interrupted at the seventh cycle (R7) as the fungus was unable to continue growth. It could be that the culture might have aged or decayed (autolysis) and had lost its ability to produce the active hyphae during the extended culture process (R6 to R7) (White et al., 2002). Likewise, the kinetic parameters of these experiments were calculated and compared later to understand their different productivity.

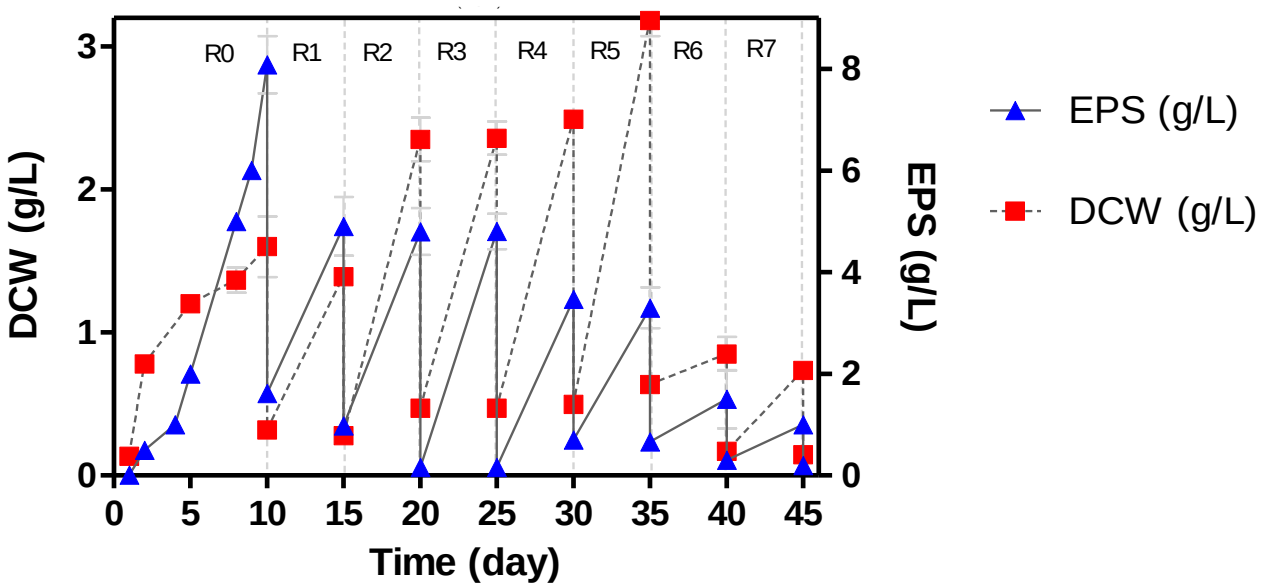


Figure 3.15 Time course of a bioreactor repeated-batch fermentation of *G. lucidum* BCCM 31549 (Leuven, Belgium) using condition B:[30 g/L of Glucose, 20% inoculum]. All other fermentation conditions were identical [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl 4], 100 rpm, 2% vvm of air, controlled pH 4, and temperature 30 ^oC. The bioreactor baffle was removed in this technique.

*The error bars were slightly obscured to improve the graph appearance. T-test assesses whether the means of two groups are statistically different from each other. Therefore, paired T-test was used for EPS and DCW: At 95% CI of discrepancy, the P value is 0.0092 [two-tailed P value]. The means were significantly different ($P < 0.05$).

3.6.3 Kinetics of RBF using unbaffled-bioreactor system

Table 3.10 summarises the kinetics of the RBF process using an unbaffled-bioreactor system. Biomass productivity of RBF from the first cycle (R1) to the fifth (R5) was somewhat higher than the batch (R0) whereas their average EPS productivities were higher. Meanwhile, biomass concentration and productivity surged during RBF and both reached the highest values (2.68 g/L and 0.54 g/L day^{-1}) at the fifth cycle (R5). On average, biomass concentrations of RBF (R2 to R5) were higher than the batch (R0). These were different for EPS concentration (4.64 g/L) and productivity (0.93 g/L day^{-1}) which attained the highest values at third cycle (R3). Largely, these values have successfully followed the concept of RBF as described in the literature (Birhanli and Yesilada, 2006; Birhanli and Yesilada, 2010; Yang et al., 2005).

In the meantime, the specific productivity of EPS from the first cycle (R1) to the seventh (R7) was in the range of 0.25 to 0.62 (g/g) day^{-1}, which is 50% higher than R0 [0.50 (g/g) day^{-1}], although R6 [0.79 (g/g) day^{-1}] shows the highest value and R5 [0.19 (g/g) day^{-1}] the lowest. The Yield $_{EPS}$ showed consistent values (0.53 – 0.62 g $_{EPS}$ /g $_{GLU}$) from the first cycle (R1) to the fourth (R4) while the Yield $_{DCW}$ (0.17 – 0.35 g $_{DCW}$/ g $_{GLU}$) doubled the values of the batch (R0). These values suggested that the applied RBF technique was economically effective compared to batch.

Table 3.10 Kinetic parameters of *G. lucidum* BCCM 31549 RBF using an unbaffled-bioreactor setup.

RBF cycle [C]	Biomass concentration, (x) (g/L) (X_2 - X_1)[a]	Biomass productivity P_x (g/L day^{-1})	EPS concentration, (EPS) (g/L) (EPS_2 - EPS_1)[b]	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g $_{EPS}$ /g $_{GLU}$)	Yield $_{DCW}$ (DCW yield on sugar), (g $_{DCW}$/ g $_{GLU}$)
R0 (batch)	1.60 ± 0.37	0.16 ± 0.06	8.08 ± 1.0	0.81 ± 0.02	0.51 ± 0.03	0.89 ± 0.07	0.18 ± 0.03
R1 (RBF starts)	1.07 ± 0.04	0.21 ± 0.03	3.28 ± 1.0	0.66 ± 0.02	0.62 ± 0.02	0.53 ± 0.02	0.17 ± 0.02
R2	2.07 ± 0.26	0.41 ± 0.03	3.82 ± 0.8	0.75 ± 0.05	0.36 ± 0.06	0.60 ± 0.05	0.32 ± 0.02
R3	1.89 ± 0.20	0.38 ± 0.08	4.64 ± 0.6	0.93 ± 0.02	0.49 ± 0.08	0.73 ± 0.02	0.30 ± 0.02
R4	2.02 ± 0.09	0.40 ± 0.02	3.34 ± 0.3	0.67 ± 0.06	0.33 ± 0.03	0.62 ± 0.02	0.37 ± 0.03
R5	2.68 ± 0.19	0.54 ± 0.02	2.60 ± 0.7	0.52 ± 0.02	0.19 ± 0.03	0.34 ± 0.05	0.35 ± 0.04
R6	0.21 ± 0.21	0.04 ± 0.01	0.84 ± 1.0	0.17 ± 0.04	0.79 ± 0.04	0.31 ± 0.01	0.08 ± 0.01
R7	0.56 ± 0.10	0.11 ± 0.01	0.70 ± 0.2	0.14 ± 0.03	0.25 ± 0.04	0.23 ± 0.03	0.18 ± 0.03

[a] (X_2 - X_1) means the value of end biomass concentration minus initial biomass concentration for the each of the cycles. [b] (EPS_2 - EPS_1) means the value of end EPS concentration minus initial EPS concentration for the each of the cycles.[c] Fermentations were carried out in bioreactor without a baffle. The conditions and medium compositions were at [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, 2% vvm of air**,** controlled pH 4, temperature at 30 ºC and 20% (v/v**)** inoculum. 80% fresh media replacement was used during the cycles.

*1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different (P < 0.05) vs EPS concentration, Biomass productivity vs EPS concentration, EPS concentration vs EPS productivity, vs Specific production of EPS, vs Yield $_{EPS}$, vs Yield $_{DCW}$, respectively.

3.6.4 Morphological changes during RBF

The morphological changes in the culture during the RBF process are shown in Fig. 3.16. Morphologically, this fungus forms pellets throughout the batch fermentation (R0) at day 10. At that culture phase, the fungus was largely in the form of spherical pellets. When 80% (v/v) of fresh medium was inserted in the system, the pellets had degraded or dispersed at first cycle (R1) to generate starburst-like pellets with mycelial strands protruding from the surface and the culture became cloudy in appearance. The fungus grew exponentially in the second cycle (R2) and the culture became cloudier (macroscopic observation) than the previous cycle. The vigorous mycelial growth was observed as the morphology shows a dense fluffy structure as the pellet shape disintegrated. This corresponds with the growth curve in Table 3.10 (Section 3.6.3) starting from the second cycle (R2) onwards, showing an increasing EPS production.

It is proposed that the fluffy-like pellets were linked to the liberation of the second generation of fungal progeny (R1 to R2) as mentioned in the shake flask work, and were associated with the rise in EPS production. This event continued, reaching the third cycle (R3) as the intertwined mycelia started to fuse together to form denser pellets. With the growth increase, the engulfed mycelia haltingly formed pellets at the fourth cycle (R4). Later, the irregular-pellet shape was generated during the fifth cycle (R5). The R5's morphology typically a starburst-like pellet was associated with EPS production during the present RBF cycles.

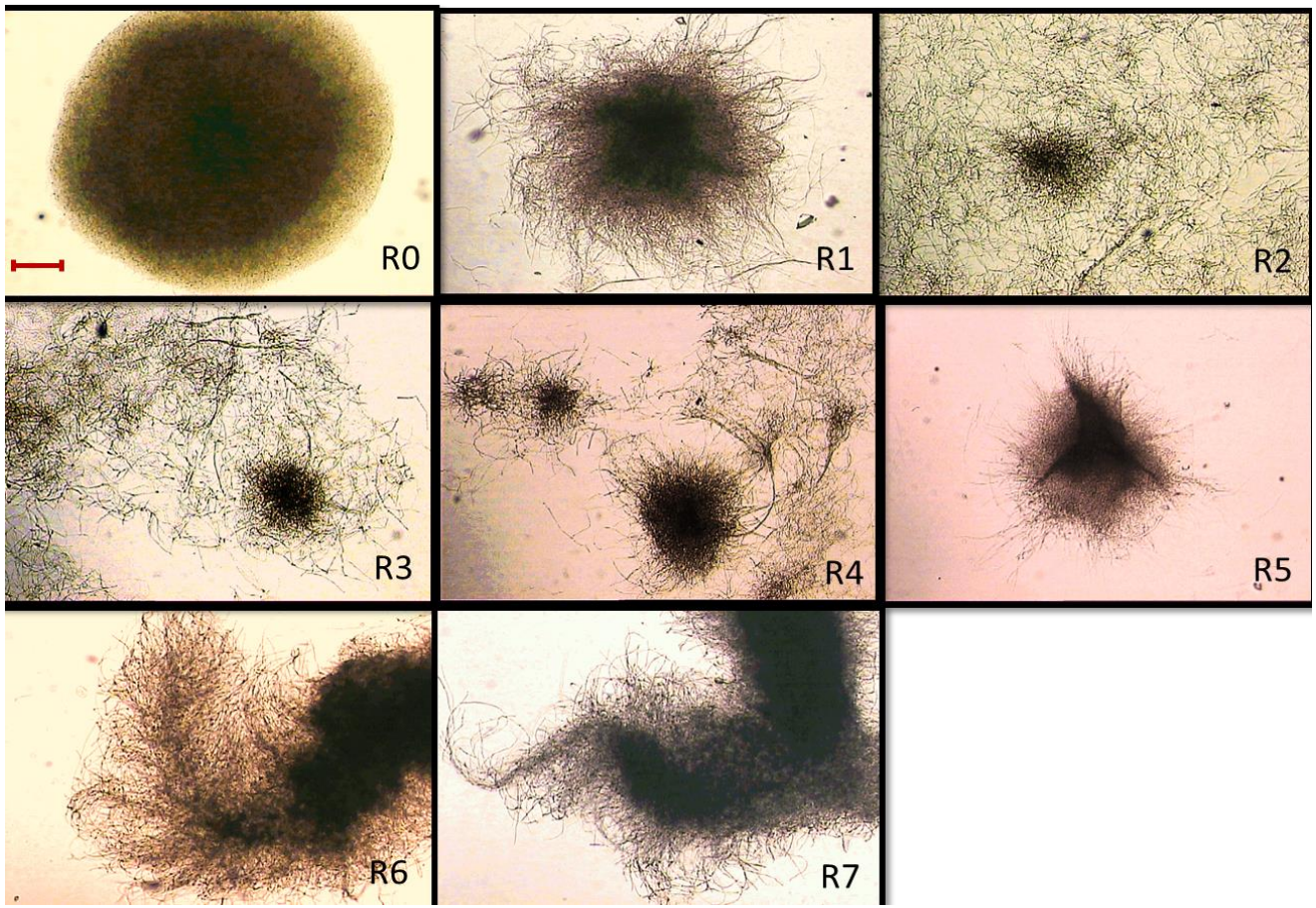


Figure 3.16 Morphological changes in a bioreactor repeated-batch fermentation of *G. lucidum* BCCM 31549 (Leuven, Belgium).The conditions and medium compositions were at [(g/L): Glucose 30, KH_2PO4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, 2% vvm of air, controlled pH 4, temperature at 30 ^oC and 20% (v/v) inoculum. Images were taken at 4-fold magnification. Bar = 150 µm. The baffle was removed in this RBF.

After R5, the fungus lost its ability to sustain the high growth rate reaching the sixth cycle (R6) possibly due to loss of vigour due to the successive subculturing event (RBF), as both biomass and EPS production were substantially reduced. At R6, the fungus showed an absence of small hyphal elements indicating the reduced growth conditions. The same morphology was observed during the last cycle (R7), as a thicker, branched, and denser structure was present. To conclude, the highest cycle that bioreactor RBF using *G. lucidum* BCCM 31459 can endure was the fifth (R5). This successful RBF process was compared with the normal bioreactor batch fermentation.

3.6.5 Comparison of batch and RBF in the unbaffled-bioreactor

In the present study, both unbaffled batch and RBF processes by *G. lucidum* were investigated and compared for effective fungal EPS productivity. The batch production time values were at 10 days while the RBF were at 5 days (R3). These results were carried out in the 2.5-L bioreactor and the results are listed in Table 3.11 to give the overall comparison for their EPS production. In the table, EPS productivity for RBF (0.93 g/L day^{-1}) was much higher than the batch (0.81 g/L day^{-1}).

The batch fermentation biomass (1.60 g/L), biomass productivity (0.16 g/L day^{-1}), and Yield $_{DCW}$ (0.18 g $_{DCW}$/ g $_{GLU}$) were lower than the RBF [(x) = 1.89 g/L, (P_x) = 0.38 g/L day^{-1} and Yield $_{DCW}$ = 0.30 g $_{DCW}$/ g $_{GLU}$, respectively], while the EPS counterparts for RBF [$(Q_{EPS/x})$ = 0.49 g/g day^{-1}, Yield $_{EPS}$ = 0.73 g $_{EPS}$/g $_{GLU}$] were approximately the same as the batch [($Q_{EPS/x}$) = 0.50 g/g day^{-1}, Yield $_{EPS}$ = 0.89 g $_{EPS}$/g $_{GLU}$].

Table 3.11 Comparison of batch and RBF kinetics of *G. lucidum* BCCM 31549 in the unbaffled bioreactor.

Fermentation technique/ Time [a]	Biomass concentration, (x) (g/L) ($X_2 - X_1$) [d]	Biomass productivity P_x (g/L day^{-1})	EPS concentration, ($_{EPS}$) (g/L) ($EPS_2 - EPS_1$) [e]	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g $_{EPS}$/g $_{GLU}$)	Yield $_{DCW}$ (DCW yield on sugar), (g $_{DCW}$/ g $_{GLU}$)	End N (ppm)	End NH_4Cl (g/L)
Batch [b] (10 days)	1.60 ± 0.1	0.16 ± 0.02	8.10 ± 0.3	0.81 ± 0.03	0.50 ± 0.03	0.89 ± 0.08	0.18 ± 0.02	662.4 ± 2	2.50 ± 0.08
RBF [c] (5 days)	1.89 ± 0.2	0.38 ± 0.08	4.64 ± 0.6	0.93 ± 0.02	0.49 ± 0.08	0.73 ± 0.02	0.30 ± 0.02	843.7 ± 2	3.09 ± 0.08

[a] Fermentations were carried out in the unbaffled bioreactor with the conditions and medium compositions of [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, 2% vvm of air, controlled pH 4, 20% inoculum and temperature at 30 ºC. RBF was done at 80% (v/v) broth replacement ratio and during the transition phase of broth replacement time point.

[b] The values were taken at day 10 as which the EPS was the highest.

[c] The values were taken at R3 as which the EPS was the highest.

[d] ($X_2 - X_1$) means the value of end biomass concentration minus initial biomass concentration for the each of the cycles.

[e] ($EPS_2 - EPS_1$) means the value of end EPS concentration minus initial EPS concentration for the each of the cycles.

*1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different ($P < 0.05$) vs End N, Biomass productivity vs End N, EPS concentration vs End N, Specific production of EPS vs End N, Yield $_{EPS}$ vs End N, Yield $_{DCW}$ vs End N, End N vs End NH_4Cl, respectively

These kinetics indicate that the RBF was a more effective cultivation strategy than the batch process for fungal EPS production by *G. lucidum* BCCM 31549. In the future, the differences in their N consumptions are worth examining especially in terms of impact on EPS concentration and productivity.

3.7 RBF Interactions on the life cycle of *G. lucidum*

As observed, the fungus *G. lucidum* showed different morphological characteristics throughout the various cycles of the RBF processes both in the shake flask and bioreactor. During their early stage, this fungus formed small loose mycelial aggregates called clumps which later develop into spherical aggregates (pellets), consisting of highly entangled networks of hyphae as mentioned by Znidarsic and Pavko (2001). Similar to the previous work by Fazenda et al., (2010), the pelleted morphology in the shake flask was different from the dispersed hyphal elements in the 10-L STR bioreactor fed-batch cultivation. In the present RBF study, there were morphological differences in the shake flask (low shear) possibly due to a higher hydromechanical stress present inside the bioreactor (high shear). Moreover, the shear rate (velocity of the moving plate/ distance between two parallel plates) in the bioreactor was up to a factor of 10 greater than the lowest average limit usually seen in the shake flask cultures (Reyes et al., 2003). As a result, the life cycle of *G. lucidum* might be altered, possibly also changing the formation of clamp connections.

The clamp connections are designed to function in ensuring that each Basidiomycetes ‘’cell’’ or compartment has a compatible pair of nuclei, and so the dikaryotic state is maintained (Fazenda et al., 2008). In the present work, the

increment of the shear rate in the bioreactor effects the morphology of *G. lucidum*, breaking the pellets into more fragmented hyphae, and henceforth encouraging the development of the filamentous form (Fazenda et al., 2010).

Based on these events, the means of transferring nuclei from one hyphal fragment to another or also called clamp connections were found in Fig. 3.17, which illustrate the details of *G. lucidum* hyphae during the RBF that are both in the bioreactor (image A and B) and shake flask (image C and D). The red arrow indicates clamp connections, yellow arrow indicates septum and blue arrow indicate hyphal tip. Overall, the finding of clamp connections in RBF cultures were both astonishing and inspiring, since in liquid cultures submerged hyphae may fail to form clamp connections (Carlisle et al., 2001), which attributed to the use of severe agitation in the current stirred-tank bioreactor (Fazenda et al., 2010). Without the clamp connections, the primary fungal mycelium would not divide synchronously (Deacon, 2013) and the dikaryotic stage of the cells will get disrupted, thus halting the growth.

Therefore, maintaining the dikaryotic stage during the RBF process even with the presence of shear stress is crucial for fungal rejuvenation and EPS production. As part of the comparative analysis, liquid culture fermentation of ectomycorrhizal fungi (*Laccaria proxima*) by Carlisle et al., (2001) revealed that clamp connections were always lost and hence reduced the ability to infect the plant symbiont, ultimately resulted in halting the potential commercialisation opportunity. The presence of key elements in the present RBF work proved that the investigation herein will widen the applicability of Basidiomycetes in extending batch cultures by using high-scale bioreactor.

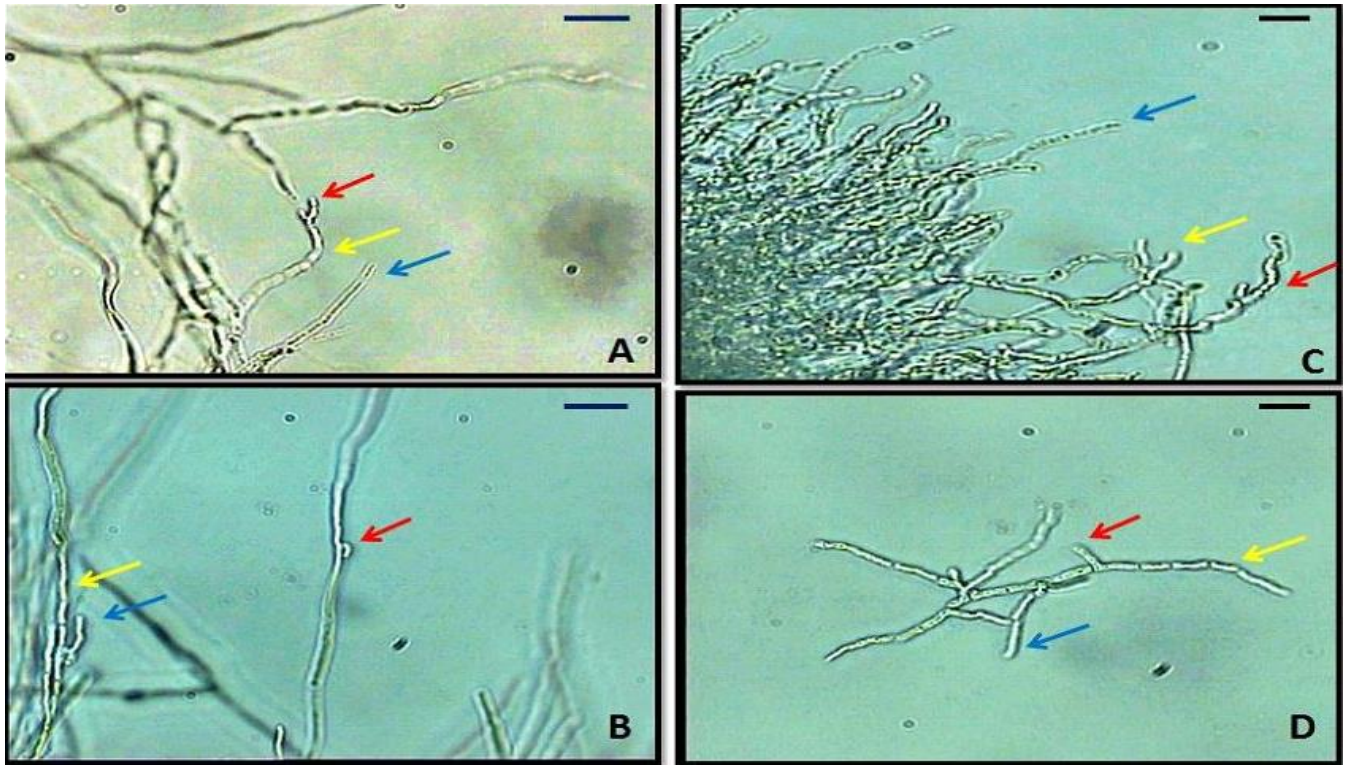


Figure 3.17 Details of RBF of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) with hyphae showing the presence of clamp connections/crozier (red arrow), septum (yellow arrow) and hyphal tip (blue arrow). Image A and B showed the condition in the unbaffled bioreactor at the fifth cycle (R5) and the second cycle (R2), respectively. Image A and B were taken at 25-fold magnification and bar = 150 μm. Image C and D depicted the condition in the shake flask at day 10 and day 48, respectively. Image and D were taken at 20-fold magnification and bar 150 = μm. All other fermentation conditions were all the same [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, 2% vvm of air, controlled pH 4, 20% (v/v) inoculum and temperature 30 ^oC.

3.8 Nitrogen strategy during RBF using unbaffled bioreactor

The nitrogen (N) limitation is an efficient strategy for increasing EPS production for some *Ganoderma* species (Zhao et al., 2011b). Ammonium from ammonium chloride utilised in this work is the primary N source for *G. lucidum* (Zhao et al., 2011b), which are also the key compounds involved in N regulation of EPS metabolism (Marzluf, 1997). By using either N-limiting or N-free medium on RBF process, it could enhance the EPS growth and productivity. Therefore, the accumulations of EPS in response to N-limiting and N-free media were measured in the RBF system.

In this method, the N-limiting and N-free media were used during the first cycle (R1) and its subsequent RBF cycles. The N-limiting medium contained 0.4 g/L of NH_4Cl (source of N) which is 10% (w/v) of the original 4 g/L of NH_4Cl (N-normal) in the process. Meanwhile, the N-free medium contained no NH_4Cl during RBF. Both of these media were prepared and implemented during the broth replacement events for each RBF cycle. The aim of this procedure was to determine the effect of N limitation on *G. lucidum* EPS production during RBF process.

3.8.1 The impact of N-free medium on RBF

Based on the results in Fig. 3.18 below, it can be seen that the N-free RBF in the unbaffled bioreactor failed to undergo any further RBF cycles. In the early process stage (R0), by day 10 (transition phase of the batch fermentation growth), biomass and EPS concentration were 1.56 g/L and 8.08 g/L, respectively.

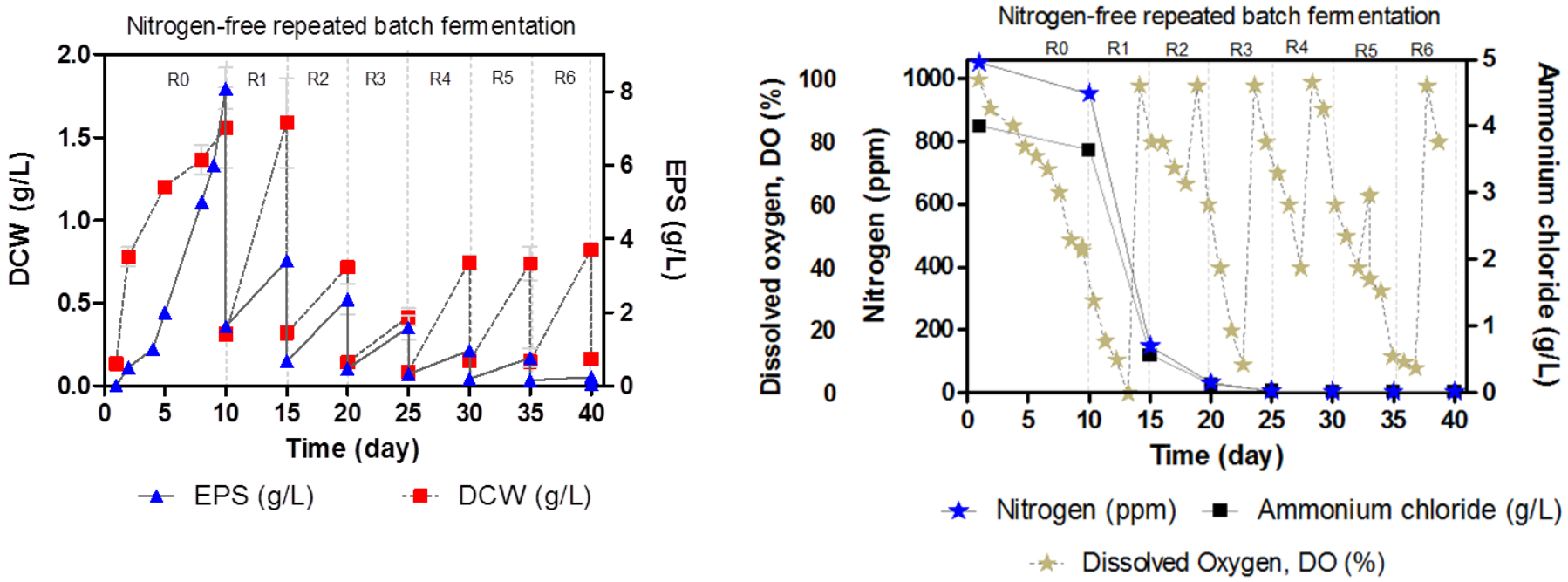


Figure 3.18 Time course of an unbaffled-bioreactor RBF of *G. lucidum* BCCM 31549 (Leuven, Belgium) using condition B: [30 g/L of Glucose, 20% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl (4 at initial stage and zero for consecutive cycles)], 100 rpm, 2% vvm of air, controlled pH 4, and temperature 30 ^oC. The error bars were slightly obscured to improve the graph appearance. T-test assesses whether the means of two groups are statistically different from each other. Therefore, paired T-test was used for EPS and DCW: At 95% CI of discrepancy, the P value is 0.0295 [two-tailed P value]. The means were significantly different ($P < 0.05$). Nitrogen levels during the RBF were shown on the right graph.

However, when 80% (v/v) of culture was harvested during the first cycle (R1), the biomass (1.28 g/L) was stable but with an important substantial decrease in its EPS (1.78 g/L). During the subsequent cycles, they were unable to reproduce further due to the absence of N starting from the first cycle (R1).

Consequently, the EPS production was greatly reduced from the first cycle (R1) to the sixth (R6) without any increment while it struggled to produce the biomass (DCW) throughout the subsequent cycles (R2 to R6). It can be observed that a N supply was crucial in EPS production both in batch and RBF process and this is in agreement with the findings of Fang and Zhong (2002a) which discussed the influences of N source on the accumulation of polysaccharide during submerged fermentation of *G. lucidum* in the shake flasks.

As N ran out during the third cycle (R3), the biomass started to increase at the expense of EPS production. This was a surprising result, but suggests that the culture was prioritising growth, perhaps by means of cryptic growth on stored N sources (Harvey et al., 1998). RBF using N-free medium kinetics are reported in Section 3.8.1.1.

3.8.1.1 Kinetic parameters of N-free RBF

Based on the Table 3.12, both biomass concentration and biomass productivity were greatly reduced starting from the batch (R0) and continued to the RBF (R1, R2, R3, R4, R5 and R6, respectively). The EPS concentration and productivity experienced the same drawbacks, as both of them were halted until the end of the cycle (R6).

Table 3.12 Kinetic parameters of RBF of *G. lucidum* BCCM 31549 using an unbaffled bioreactor setup (N-free).

Cycle [C]	Biomass concentration, (x) (g/L) (X_2 - X_1)[a]	Biomass productivity, $P\,x$ (g/L day^{-1})	EPS concentration, ($_{EPS}$) (g/L) (EPS_2 -EPS_1)[b]	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g $_{EPS}$ /g $_{GLU}$)	Yield $_{DCW}$ (DCW yield on sugar), (g $_{DCW}$/ g $_{GLU}$)
R0 [batch]	1.56 ± 0.35	0.16 ± 0.04	8.08 ± 1.2	0.81 ± 0.04	0.52 ± 0.04	0.89 ± 0.09	0.17 ± 0.04
R1[RBF starts]	1.28 ± 0.05	0.26 ± 0.02	1.78 ± 0.5	0.36 ± 0.04	0.28 ± 0.03	0.74 ± 0.04	0.53 ± 0.07
R2	0.40 ± 0.05	0.08 ± 0.05	1.67 ± 0.9	0.33 ± 0.06	0.84 ± 0.07	0.55 ± 0.07	0.13 ± 0.04
R3	0.28 ± 0.09	0.05 ± 0.09	1.12 ± 0.5	0.19 ± 0.04	0.68 ± 0.09	0.38 ± 0.03	0.10 ± 0.08
R4	0.67 ± 0.05	0.11 ± 0.05	0.65 ± 0.1	0.11 ± 0.07	0.16 ± 0.04	0.20 ± 0.03	0.20 ± 0.04
R5	0.59 ± 0.18	0.10 ± 0.04	0.57 ± 0.04	0.10 ± 0.03	0.16 ± 0.02	0.20 ± 0.08	0.20 ± 0.06
R6	0.67 ± 0.20	0.11 ± 0.03	0.07 ± 0.03	0.01 ± 0.05	0.02 ± 0.01	0.04 ± 0.01	0.33 ± 0.02

[a] (X_2 - X_1) means the value of end biomass concentration minus initial biomass concentration for the each of the cycles.
[b] (EPS_2 - EPS_1) means the value of end EPS concentration minus initial EPS concentration for the each of the cycles.
[c] Fermentations were carried out in bioreactor without a baffle. The conditions and medium compositions were at [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl (4 at initial stage and zero for consecutive cycles)], 100 rpm, 2% vvm of air, controlled pH 4, temperature at 30 ^oC and 20% inoculum (v/v). 80% (v/v) fresh media replacement was used during the repeated-batch cycles.
*1 way ANOVA has been carried out for each row with the P value of 0.0207. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass productivity was significantly different ($P < 0.05$) vs EPS concentration.

When the fungus was forced into the exhaustion mode (N-free medium addition), EPS yield was reduced which correspond with the glucose consumed as there were not enough growing hyphae in the system to reach the secondary stage of the fungal growth (onset of EPS production). Values of the first cycle (R1) and onwards were not 50% more than the R0, which means the RBF was clearly unsuccessful.

Consequently, the N-free condition might initiate fungal autolysis as the culture differentiates and ages. In the present study, in the absence of a N supply, we have observed that it has a surge in product (EPS) degradation, similar to the response described by White et al., (2002). In order to accomplish the RBF technique, it is essential to avoid decreased availability of nutrients which might cause cellular death (Hibbett et al., 2007; White et al., 2002). The future RBF needed to have a sufficient optimum nutrients to ensure both fungal survivability and avoid any possible hyphal fragmentation or degradation due to autolysis and associated cryptic growth described in Section 3.8.1.2.

3.8.1.2 Image analysis of N-free RBF

N-free RBF significantly affects the mycelial morphology in Fig. 3.19. Round fluffy-pellets were observed prior to RBF (R0). When the first cycle (R1) took place in Fig. 3.19, the pellet burst into branched filaments proving that the nutrients were still sufficient. By the second cycle (R2), the depletion of N was noticeable as distorted-dense pellets were formed. Here, it suggests that the culture struggled to produce active hyphae.

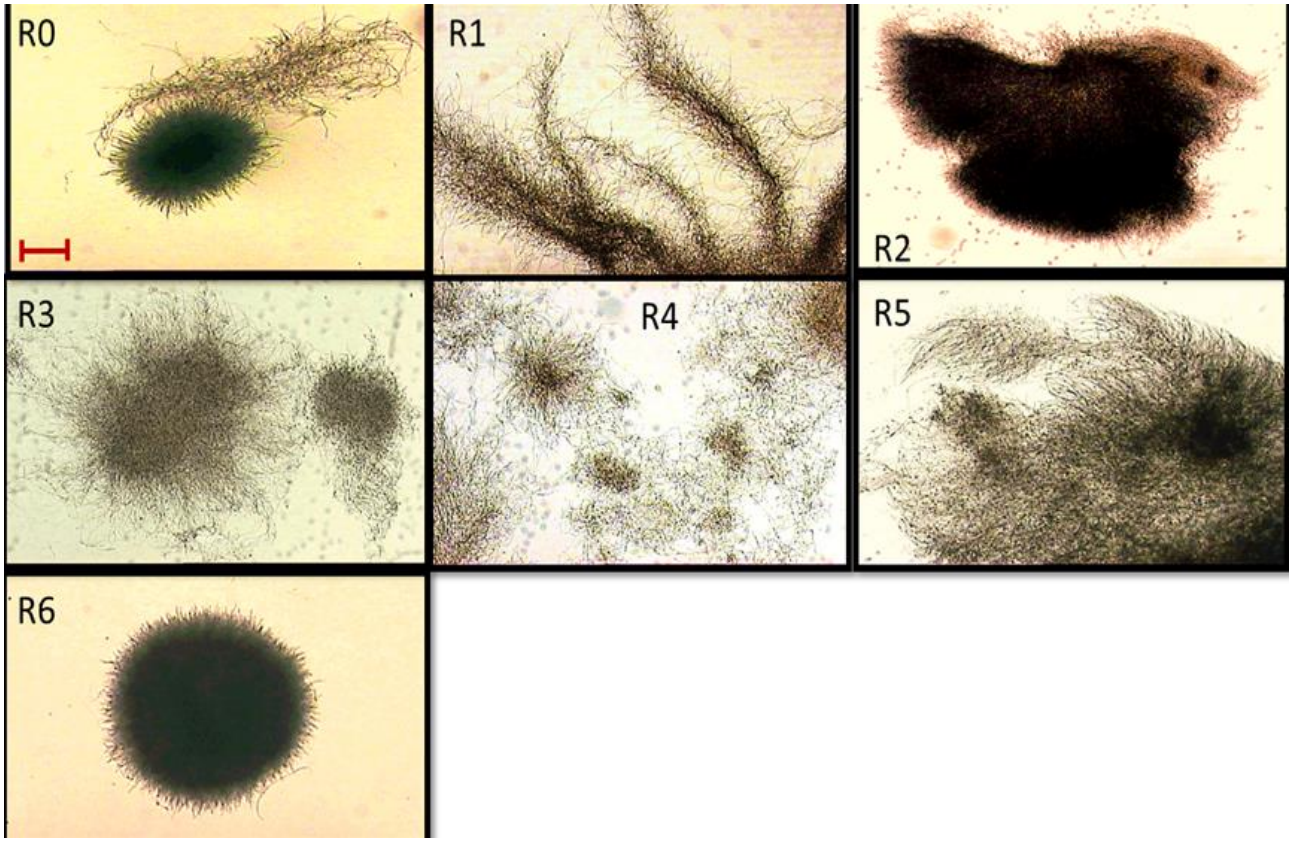


Figure 3.19 Morphological changes of an unbaffled bioreactor RBF using *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium). The conditions and medium compositions were at [(g/L): Glucose 30, KH_2PO4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, Yeast Extract 1, NH_4Cl (4 at initial stage and zero for consecutive cycles)], 100 rpm, 2% vvm of air, controlled pH 4, temperature at 30 ^oC and 20% (v/v) inoculum. Images were taken at 4-fold magnification. Bar = 150 µm

At the third cycle (R3), N supply ran out and smooth-pellets were scattered in the culture. Culture condition became worse by the fourth cycle (R4) with broken fluffy pellets. During the fifth cycle (R5), most likely the fungus has undergone some cryptic growth, as a result of the nutrient starvation (White et al., 2002) due to the low N. Biomass was reduced by the sixth cycle (R6). The cryptic growth of *G. lucidum* is comparable with the results of Nitsche et al., (2013) in the ageing submerged cultures of *Aspergillus niger*. The possibility of cryptic growth is supported by the biomass concentrations (R3 = 0.28 g/L, R4 = 0.67 g/L, R5 = 0.59 g/L and R6 = 0.67 g/L, respectively), whereby cryptic growth has maintained the growth of active hyphae during N-free RBF (R3 to R6).

Accordingly, since N-free condition had shown such a clear negative effect (Fig. 3.19 and Table 3.12) on RBF, N limitation effects were investigated as discussed in Section 3.8.2.

3.8.2 The impact of N-limiting medium on RBF

Based on the results shown in Fig. 3.20, it can be seen that the N-limiting medium (N levels were kept to minimum) in an unbaffled 2.5-L bioreactor has supported EPS formation for seven consecutive cycles of RBF. During the early stage (R0), the fermentation reached day 10 (transition phase), producing biomass and EPS at 1.56 g/L and 8.08 g/L, respectively. When 80% (v/v) of culture was harvested during the first cycle (R1), the biomass (0.20 g/L) was vastly decreased but with a substantial increase in its EPS production (4.86 g/L).

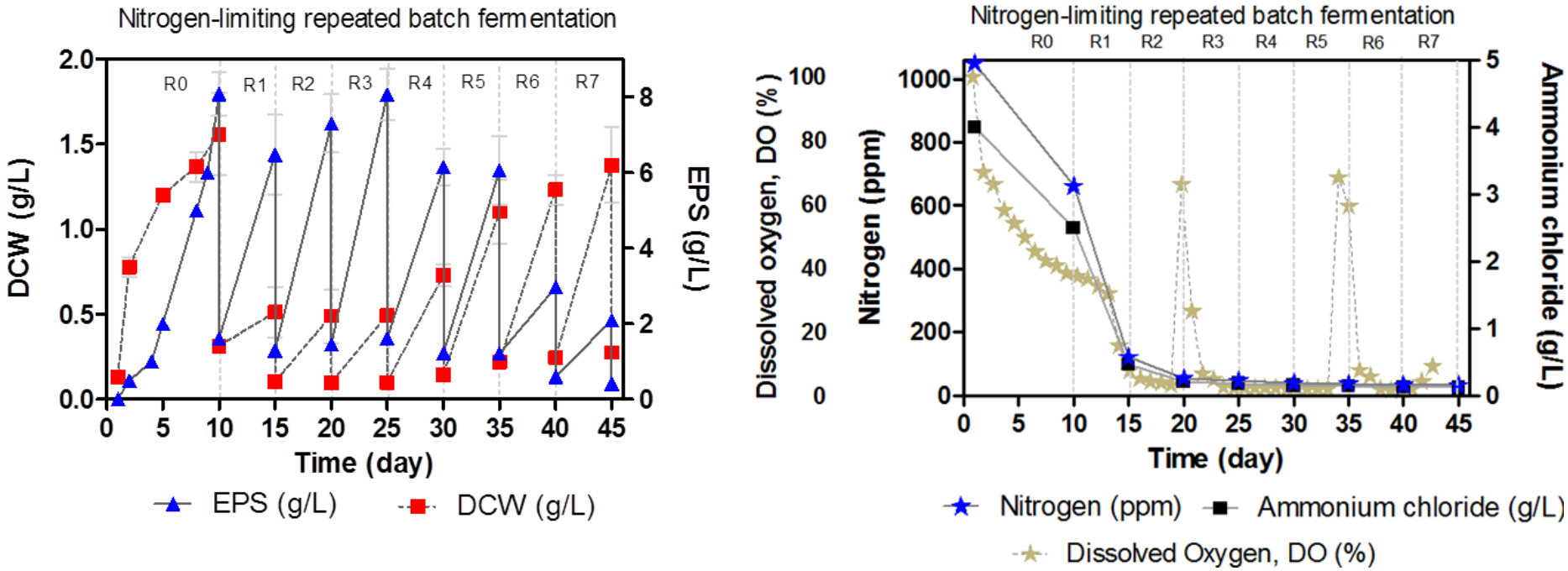


Figure 3.20 Time course of an unbaffled-bioreactor RBF of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) using condition B:[30 g/L of Glucose, 20% inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl (4 at initial stage and 0.4 for consecutive cycles)], 100 rpm, 20% (v/v) inoculum, 2% vvm of air, controlled pH 4, temperature 30 ^oC, and 80% (v/v) broth replacement ratio. The error bars were slightly obscured to improve the graph appearance.

*T-test assesses whether the means of two groups are statistically different from each other. Therefore, paired T-test has been run for EPS and DCW: At 95% CI of discrepancy, the P value is 0.0003 [two-tailed P value]. The means were significantly different ($P < 0.05$).

During the N-limiting RBF (Fig. 3.20), EPS was produced substantially until the fifth cycle (R5), and then it dropped at the sixth cycle (R6) and onwards. RBF stops at the seventh cycle (R7) because the EPS concentration fell drastically 50% below the batch value (R0) as the ageing mechanism may have occurred. When compared with the literature, in the current RBF strategy, N source limitation promotes the fermented culture (Hsieh et al., 2006) and the secondary metabolites production (Zhou and Zhong, 2009).

When the N supply was limited during RBF, the DO percentage was around 8-10% which has been shown to favour EPS production by Fazenda et al., (2010). The low level of N in the fermentation culture has stimulated EPS productivity which may increase internal broth viscosity and leads to a reduction of DO in the system. Kinetics and morphological changes in these RBF cultures are discussed in the following sections.

3.8.2.1 Kinetic parameters of N-limiting RBF

Table 3.13 describes the kinetics of N-limiting RBF. Overall, EPS productivities of five RBF cycles (R1-R5) were higher than the batch (R0). These productivities (R1-R5) showed a mean value of 1.07 g/L day^{-1} which was higher than R0 (0.81 g/L day^{-1}). Specific production of EPS showed the same trend with a mean value of 2.76 (g/g) day^{-1} from R1 to R5 compared to R0 [0.52 (g/g) day^{-1}]. Meanwhile, the yield of EPS was in the same region as the batch for the first four RBF cycles.

Table 3.13 Kinetic parameters of RBF of *G. lucidum* BCCM 31549 using an unbaffled bioreactor setup (N-limiting)

Cycle [C]	Biomass concentration, (x) (g/L) (X_2 - X_1)[a]	Biomass productivity $P\,x$ (g/L day^{-1})	EPS concentration, (EPS) (g/L) (EPS_2 -EPS_1)[b]	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g $_{EPS}$ /g $_{GLU}$)	Yield $_{DCW}$ (DCW yield on sugar), (g $_{DCW}$/ g $_{GLU}$)
R0 [batch]	1.56 ± 0.30	0.16 ± 0.04	8.08 ± 1.2	0.81 ± 0.03	0.52 ± 0.04	0.89 ± 0.05	0.17 ± 0.04
R1 [RBF starts]	0.20 ± 0.08	0.04 ± 0.06	4.86 ± 1.1	0.97 ± 0.04	4.83 ± 0.06	0.78 ± 0.04	0.03 ± 0.06
R2	0.39 ± 0.20	0.08 ± 0.04	6.01 ± 0.6	1.20 ± 0.07	3.10 ± 0.08	0.94 ± 0.06	0.06 ± 0.09
R3	0.40 ± 0.25	0.08 ± 0.05	6.61 ± 0.5	1.32 ± 0.04	3.34 ± 0.09	1.03 ± 0.08	0.06 ± 0.05
R4	0.63 ± 0.05	0.13 ± 0.04	4.53 ± 0.4	0.91 ± 0.08	1.44 ± 0.05	0.84 ± 0.04	0.12 ± 0.06
R5	0.96 ± 0.20	0.19 ± 0.03	4.83 ± 0.9	0.97 ± 0.03	1.01 ± 0.06	0.64 ± 0.08	0.12 ± 0.03
R6	1.01 ± 0.23	0.20 ± 0.05	1.76 ± 1.2	0.35 ± 0.08	0.35 ± 0.03	0.65 ± 0.05	0.37 ± 0.02
R7	1.13 ± 0.11	0.23 ± 0.03	1.51 ± 0.4	0.30 ± 0.05	0.27 ± 0.02	0.49 ± 0.04	0.36 ± 0.07

[a] (X_2 - X_1) means the value of end biomass concentration minus initial biomass concentration for the each of the cycles. [b] (EPS_2 - EPS_1) means the value of end EPS concentration minus initial EPS concentration for the each of the cycles.[c] The conditions and medium compositions were at [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl (4 at initial stage and 0.4 for consecutive cycles], 20% (v/v) inoculum, 2% vvm of air, 100 rpm, controlled pH 4, and temperature at 30 ºC. 80% (v/v) fresh media replacement was used during the repeated-batch cycles. 1 way ANOVA has been carried out for each row with the P value of <0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair (s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different ($P < 0.05$) vs EPS concentration, Biomass productivity vs EPS concentration and vs Specific production of EPS, EPS concentration vs EPS productivity and vs Specific production of EPS and vs Yield $_{EPS}$ and vs Yield $_{DCW}$, respectively.

On the other hand, when the N was limited, biomass performances were stunted from the first cycle (R1) to the fifth (R5). Biomass was 50% less than the batch while their productivity fell in the same manner. The yields of biomass for the first five RBF cycles were lower compared to batch (R0). These kinetic values affected the morphology as discussed in the following sections.

3.8.2.2 Morphological analysis of RBF cultures subject to N-limitation

Morphological changes in the bioreactor with N-limiting RBF are described in Fig. 3.21. During the initial stage (R0), the mycelia engulfed and formed a pellet. When the first RBF started (R1), the N-limiting environment ruptured the pellet structure and formed feather-like morphology, stimulating the surge in EPS and descent in biomass production. Reaching the second cycle (R2), the feather-like structure became denser. This structure detached into an individual feather-like pellet at the third cycle (R3) as EPS formation was the highest. At the fourth cycle (R4), it grows heavily with protruding filaments. When the biomass level started to increase at the fifth cycle (R5), the structure fuses with neighbouring pellets.

This structure was observed as the dark zone colour in the Figure at R5. The fused pellet disintegrates at the sixth cycle (R6), showing two fluffy-pellets. At the last cycle (R7), these pellets deformed, indicating EPS reduction and increment in biomass. Based on N-limiting RBF, the third cycle (R3) morphology favoured EPS but it was uncharacteristic for biomass production. This feather-like pellet at R3 agrees with Fazenda et al., (2010) as the ideal morphology for EPS production in a stirred-tank bioreactor.

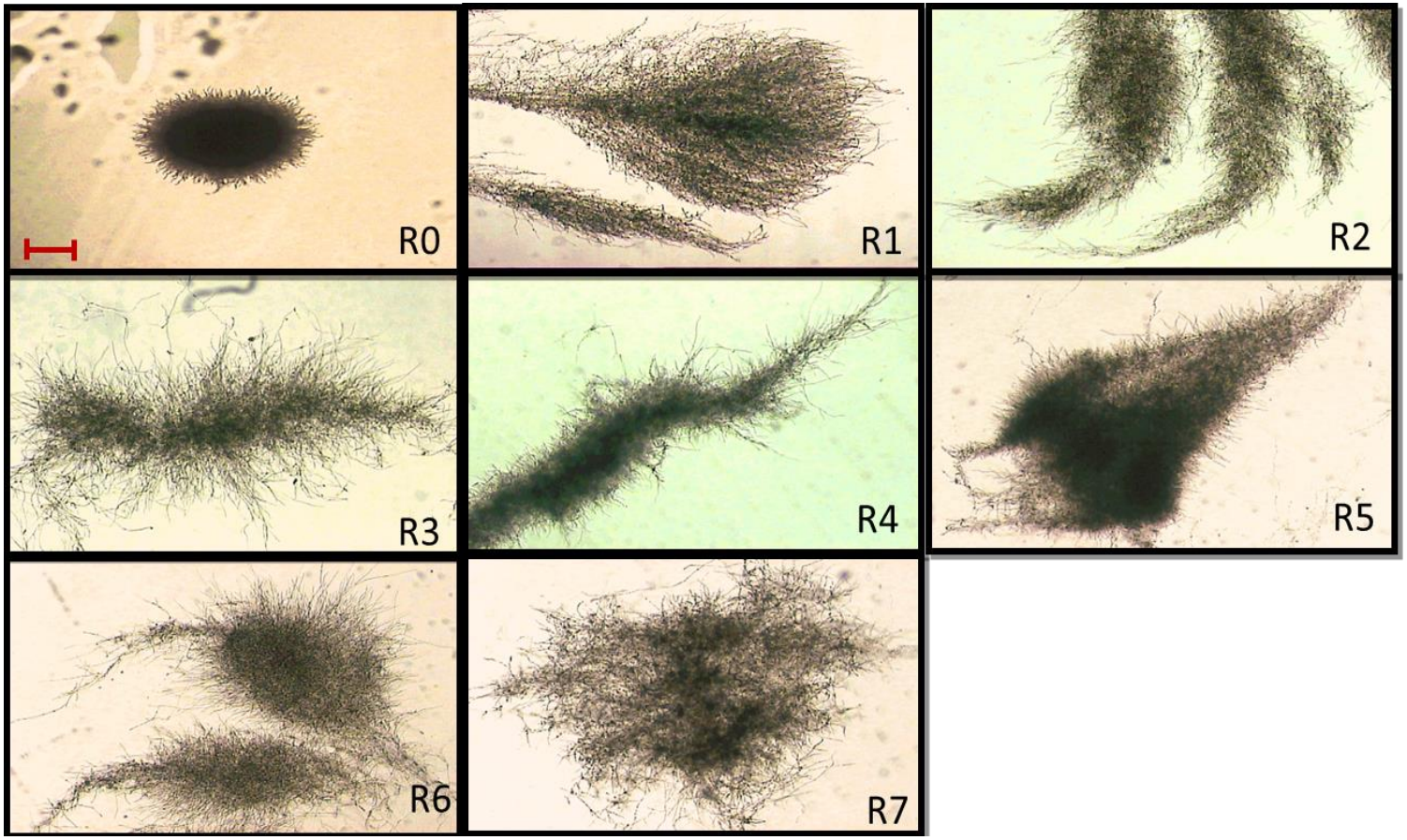


Figure 3.21 Morphological changes of an unbaffled bioreactor RBF using *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium).The conditions and medium compositions were at [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_4 7H_2O$ 0.5, YE 1, NH_4Cl (4 at initial stage and 0.4 for consecutive cycles)], 20% (v/v) inoculum, 2% vvm of air, 100 rpm, controlled pH 4, and temperature at 30 ^oC. Images were taken at 4-fold magnification. Bar = 150 µm for all figures.

3.9 Comparison of RBF and batch cultures in the unbaffled bioreactor

RBF has successfully generated five days of cycles for every five successful batches, resulting in a total of 55 days fermentation (25 + 30 days of seed culture). If the conventional batch strategy were applied, 10 days with five successful batches would generate 80 days of total fermentation time (50 + 30 days of seed culture). These reductions have supported the practicality (reducing fermentation time) of RBF strategy for improving the extended batch cultures. As shown in Table 3.14 below, compared to the batch, the EPS productivity, specific production of EPS, and Yield $_{EPS}$ of the N-limiting RBF strategy were higher. Therefore, the RBF could successfully enhance the productivity of fungal EPS fermentation. When compared with the previous RBF work on *Mortierella alpina* (Zygomycota) by Ji et al., (2014), the extended batch strategy has proven to shorten the fermentation time similarly to the current work on *G. lucidum* (Basidiomycota).

It is known that N supply plays a significant role in fungal metabolite overproduction, and likewise may affect fungal morphology (Fazenda et al., 2008), especially when RBF technique was applied with different N conditions. Based on Table 3.14, the N-limiting medium (0.4 g/L NH_4Cl) used in this study clearly promotes EPS production and was shown to be higher than N-normal conditions (4 g/L NH_4Cl), while N-free medium (0 g/L NH_4Cl) was the lowest. These phenomena were similar to the work of Zhao *et al.*, (2011b), where the secondary metabolite production was improved from *G. lucidum* under N-limiting environment. Overall, the biomass and biomass productivity were descending in the order of N-normal > N-free > N-limiting, while the EPS production, specific production of EPS and EPS productivity were ascending in the order of N-free < N-normal < N-limiting.

Table 3.14 Comparison of batch and repeated-batch strategy in the unbaffled bioreactor using *G. lucidum* BCCM 31549.

Culture strategy [a]	Time (day)	Biomass concentration, x (g/L) (X_2 - X_1) [b]	Biomass productivity, P_x (g/L day^{-1})	EPS concentration, $_{EPS}$ (g/L) (EPS_2 - EPS_1) [c]	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]	Yield $_{EPS}$ (EPS yield on sugar), (g_{EPS}/g_{GLU})	Yield $_{DCW}$ (DCW yield on sugar), (g_{DCW}/ g_{GLU})	End N (ppm)
Batch (Table 3.9)	10	1.56 ± 0.35	0.16 ± 0.04	8.08 ± 1.2	0.81 ± 0.04	0.52 ± 0.04	0.89 ± 0.09	0.17 ± 0.04	878 ± 2.00
RBF									
N-normal (3rd cycle)	5	1.89 ± 0.20	0.38 ± 0.08	4.64 ± 0.6	0.93 ± 0.02	0.49 ± 0.08	0.73 ± 0.02	0.30 ± 0.02	991 ± 2.00
N-free (1st cycle)	5	1.28 ± 0.05	0.26 ± 0.02	1.78 ± 0.5	0.36 ± 0.04	0.28 ± 0.03	0.74 ± 0.04	0.53 ± 0.07	149 ± 2.00
N-limiting (3rd cycle)	5	0.40 ± 0.25	0.08 ± 0.05	6.61 ± 0.5	1.32 ± 0.04	3.34 ± 0.09	1.03 ± 0.08	0.06 ± 0.05	46.3 ± 3.00

*The values were taken at the day as which the EPS was the highest for each RBF strategy [N-normal at third cycle, N-free at first cycle and N-limiting at third cycle]

[a] Fermentations were carried out in the bioreactor with the conditions and medium compositions of [(g/L): Glucose 30, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, Yeast Extract 1], 100 rpm, pH 4, 20% (v/v) inoculum, 2% vvm of air and temperature at 30 ^oC. Repeated-batch fermentation was done at 80% (v/v) broth replacement ratio and during the transition phase of broth replacement time point.

[b] (X_2 - X_1) means the value of end biomass concentration minus initial biomass concentration

[c] (EPS_2 - EPS_1) means the value of end EPS concentration minus initial EPS concentration

In Table 3.14, Yield $_{EPS}$ work well with N-limiting compared to N-normal and N-free. If compared with *G. lucidum* that is grown in the wild, they live on substrates that are low in most nutrients except carbon, which is due to the low N content in wood. It is found that the *G. lucidum* behaviour in the current RBF strategy was the same with *G. lucidum* in the wild, as most of these cultures are naturally N-limited (Fazenda et al., 2008).

With respect to biomass growth in a high concentrated N medium, it was observed that biomass concentrations were higher as the N increased, which is the case with the RBF system (Table 3.14) and this is also notified by Hsieh et al., (2006). Under these conditions, EPS synthesis demonstrated an opposite pattern to that of observed for growth. This is because EPS production was higher at lower N concentration. Thus, maximum EPS levels were reached in an N-limiting medium compared to N-free medium in RBF strategy.

In the RBF process, the time for seed culture and inoculation between each fermentation cycle was saved. Thus, both production and downstream cost may be reduced. RBF strategy was previously applied for producing different biochemical products, such as lactic acid (Zhao et al., 2010), hyaluronic acid (Abouelenien et al., 2009), citric acid (Moeller et al., 2011), ethanol (Watanabe et al., 2012), and laccase (Yup Jang et al., 2002), yet the work on EPS production are scarce.

3.10 Comparison of the current work with the literature

Table 3.15 shows the comparison of the current *G. lucidum* fermentation applications with previously reported research either using batch, fed-batch or repeated-batch fermentation (RBF) for the purpose of improving EPS production with the utilisation of a bioreactor. There were only six previous studies involving *G. lucidum* in bioreactors which produced EPS, including four fed-batches and two batches (Berovic et al., 2003; Fazenda et al., 2010; Tang and Zhong, 2002; Tang et al., 2011; Wei et al., 2014; Yang and Liau, 1998c). To the best of our knowledge, the RBF study reported here is the most recent application of *G. lucidum* EPS production.

The current work was most effective in producing EPS (6.61 g/L for 5 days of RBF) compared to the closest counterpart (4.55 g/L for 5 days of fed-batch) (Fazenda et al., 2010) in Table 3.15. Also, even though the current RBF applied 2.5-L bioreactor as the growing vessel, they had managed to produce EPS consistently for at least three consecutive cycles compared to other higher vessel volumes. Besides, the removal of the baffle for the current RBF work might favour EPS production as compared to the other work as homogeneity in the bioreactor improves significantly.

Table 3.15 Comparison of the current work on *G. lucidum* EPS fermentation using bioreactor

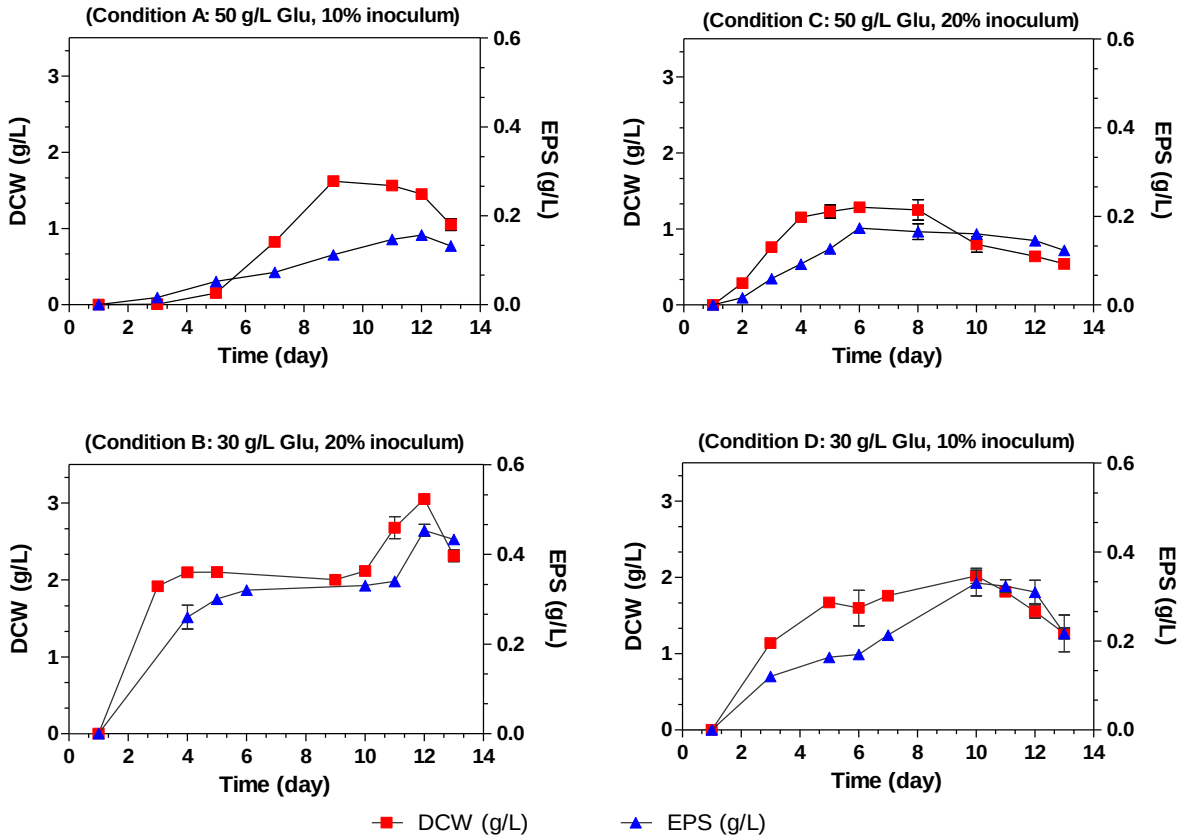
Technique	Bioreactor	EPS production, ($_{EPS}$) (g/L)	EPS productivity, P_{EPS} (g/L day^{-1})	Fermentation period (days)	Yield $_{EPS}$ (g_{EPS}/g_{GLU})	Reference
RBF	2.5-L jacketed bioreactor vessel (New Brunswick Bio-flow 3000, Edison L.N, USA) with Rushton turbine impeller (without baffle)	6.61	1.32	5	1.03	*Current work*
Batch	10-L stirred-tank bioreactor (7-L working volume, Baoxing Company) 300-L stirred-tank bioreactor (Baoxing Co.)	1	0.08	12	NA	(Wei et al., 2014)
		1.2	0.24	5	NA	(Wei et al., 2014)
Fed-batch	7.5-L stirred-tank bioreactor Biotech JS bioreactor system by Baoxing Bioengineering (Shanghai, China)	2.59	0.14	17	NA	(Tang et al., 2011)
Fed-batch	15-L stainless steel bioreactor (Bio-Stat C. DCU; B. Braun Biotech International, Switzerland)	4.55	0.65	5	NA	(Fazenda et al., 2010)
Fed-batch	10-L stirred tank bioreactor (Bioengineering AG, Switzerland)	9.6	NA	NA	1.37	(Berovic et al., 2003)
Fed-batch	2-L agitated bioreactor	0.53	0.05	10	NA	(Tang and Zhong, 2002)
Batch	2-L bioreactor (Bioflow Model C32, New Brunswick Scientific)	1.5	0.5	3	NA	(Yang and Liau, 1998c)

* NA means data not available or not reported

3.11 Conclusions

Comparative experimental results of EPS production by *G. lucidum* BCCM 31549 under different culture strategies is presented in this chapter. It has demonstrated that RBF was effective in reducing fermentation time by eliminating the lag phase, reducing the time for seed culture, reducing process operator time input, eliminating the inoculation time between each fermentation cycle, and enhancing the EPS productivity. Furthermore, the robustness of *G. lucidum* pellet to survive in long-term fermentation cycles was effective in generating a constant EPS production with the desired morphology. The presence of clamp connections in RBF strategy ensures the reproducibility of the fungus growth in the liquid environment for both in the shake flask and bioreactor. N-limiting medium aids RBF strategy significantly. This technique was optimised and the results have shown that this process had a high potential for the industrial extended-batch production of EPS from Basidiomycetes.

3.12 Appendix



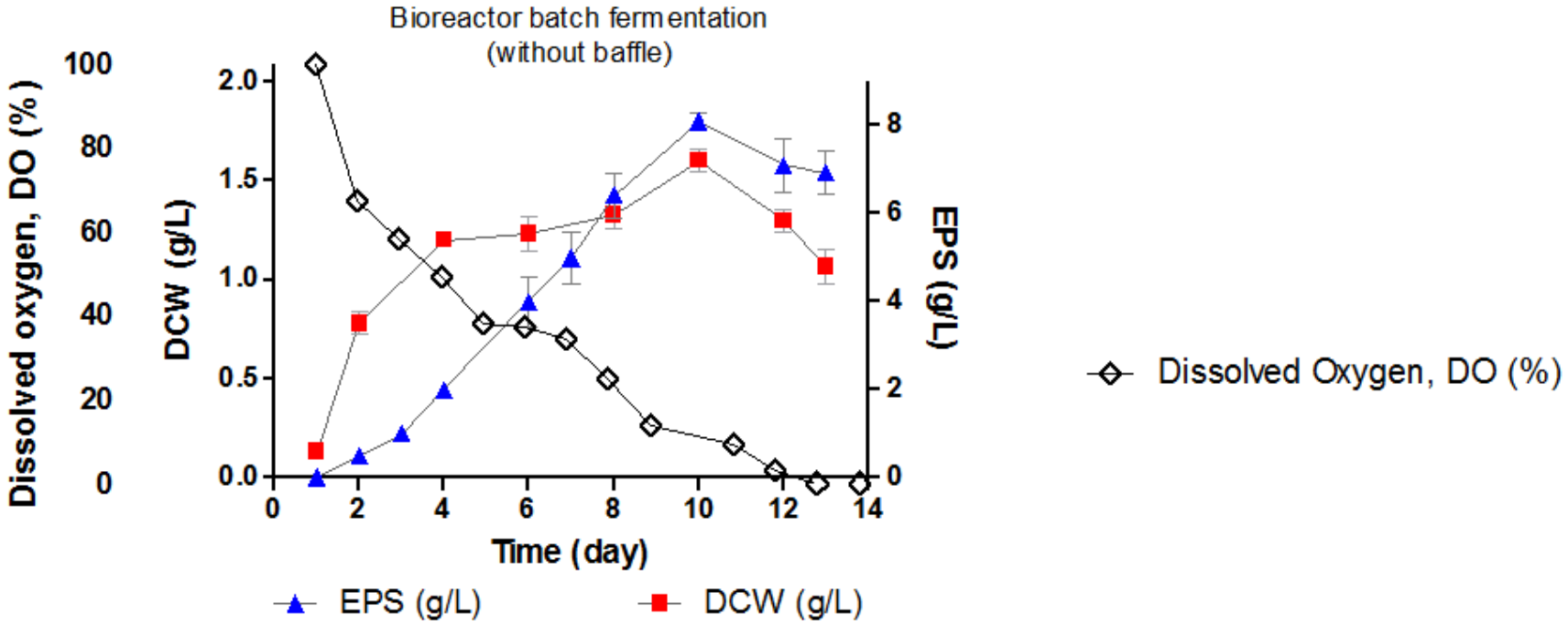
Appendix 3.12A Time courses during shake-flask batch fermentation of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) using condition A, B, C and D. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl 4], 100 rpm, initial pH 4, and temperature 30 ^oC.

*T-test assesses whether the means of all groups are statistically different from each other. Therefore, paired T-test has been run for EPS and DCW for all conditions: Condition A, at 95% CI of discrepancy, the P value is 0.0135, Condition B the P value is 0.0014, Condition C the P value is 0.0004 and Condition D the P value is < 0.0001. All means were significantly different ($P < 0.05$)

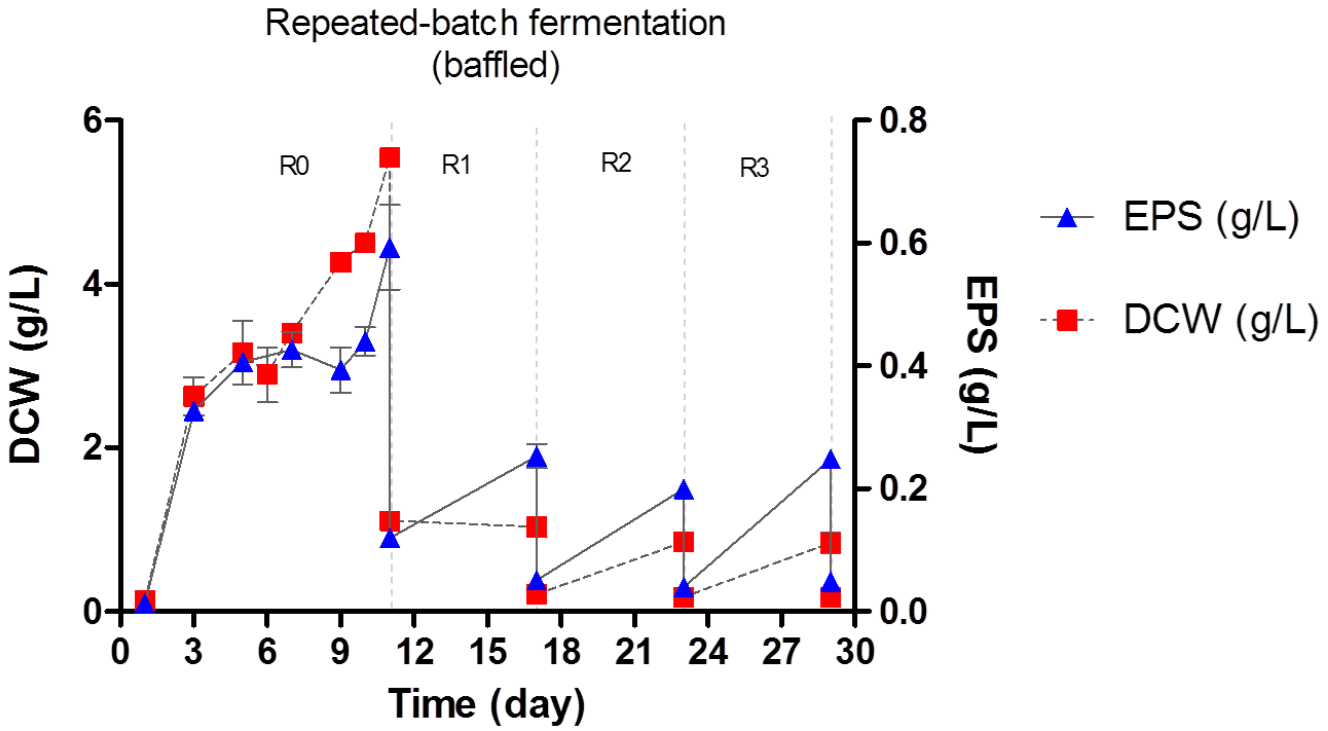
Appendix 3.12B Kinetic parameters during shake-flask batch fermentation of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) using condition A [50 g/L of Glucose, 10% (v/v) inoculum], B [30 g/L of Glucose, 20% (v/v) inoculum], C [50 g/L of Glucose, 20% (v/v) inoculum] and D [30 g/L of Glucose, 10% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl 4], 100 rpm, pH 4, and temperature 30 ^oC.

Condition	Biomass concentration x (g/L)	Biomass productivity, P_X (g/L day^{-1}	EPS concentration, P_{EPS} (g/L)	EPS productivity, P_{EPS} (g/L day^{-1})	Specific production of EPS, $Q_{EPS/x}$ [(g/g) day^{-1}]
A	1.62 ± 0.10	0.180 ± 0.008	0.157 ± 0.1	0.013 ± 0.002	0.009 ± 0.0008
B	3.05 ± 0.20	0.235 ± 0.010	0.453 ± 0.09	0.040 ± 0.030	0.011 ± 0.0008
C	1.28 ± 0.01	0.213 ± 0.002	0.170 ± 0.05	0.028 ± 0.006	0.022 ± 0.002
D	2.02 ± 0.22	0.202 ± 0.006	0.330 ± 0.15	0.033 ± 0.003	0.016 ± 0.0008

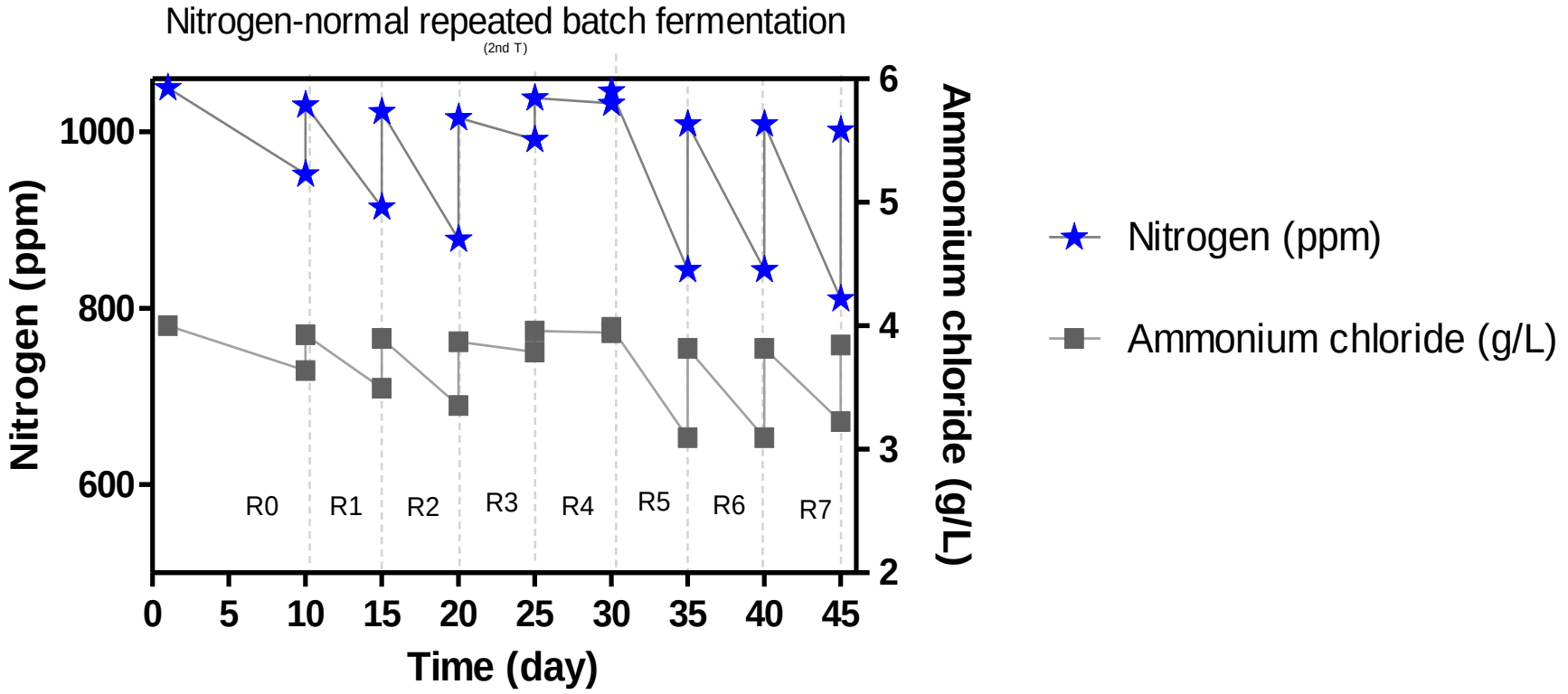
*1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a post-hoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the Biomass concentration was significantly different (P < 0.05) vs Biomass productivity and vs EPS concentration and vs EPS productivity and Specific production of EPS, respectively.



Appendix 3.12C Time course in an unbaffled bioreactor batch fermentation of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) using condition B: [30 g/L of Glucose, 20% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl 4], 2% vvm of air, 100 rpm, controlled pH 4, and temperature 30 ^oC. *T-test assesses whether the means of two groups are statistically different from each other. Therefore, paired T-test has been run for EPS and DCW: At 95% CI of discrepancy, the P value is 0.0138 [two-tailed P value]. The means were significantly different ($P < 0.05$).



Appendix 3.12D Time course in a bioreactor repeated-batch fermentation of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) using condition B: [30 g/L of Glucose, 20% inoculum]. All other fermentation conditions were all the same [(g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5], YE 1, NH_4Cl 4], 2% vvm of air, 100 rpm, controlled pH 4, and temperature 30 ^oC. *T-test assesses whether the means of two groups are statistically different from each other. Therefore, paired T-test has been run for EPS and DCW: At 95% CI of discrepancy, the P value is 0.0020 [two-tailed P value]. The means were significantly different ($P < 0.05$).



Appendix 3.12E Nitrogen changes in an unbaffled bioreactor repeated-batch fermentation of *Ganoderma lucidum* BCCM 31549 (Leuven, Belgium) using condition B: [30 g/L of Glucose, 20% (v/v) inoculum]. All other fermentation conditions were all the same [(g/L): KH2PO4 0.5, K2HPO4 0.5, MgSO47H20 0.5, YE 1, NH4Cl 4], 2% vvm of air, 100 rpm, controlled pH 4, and temperature 30 ^{o}C**.**

CHAPTER 4

PREPARATION AND IDENTIFICATION OF GLUCAN SULPHATE EXTRACTED FROM THE EXTENDED BATCH CULTURE OF *Ganoderma lucidum* BCCM 31549

4.0 Introduction

The extracted mycelial *G. lucidum* β-glucan is known to act as biological response modifier. Therefore, much research has focused on this fungal polysaccharide as a functional foodstuff and source for the development of biomedical drugs (Heleno et al., 2013). The clinical utilisation of β-glucans has one main difficulty in addition to the limited availability referred to above, that is, their comparative absence of solubility in aqueous solution, which leads to difficulties in product analysis, formulation and delivery. This is usually ascribed to the high number of –OH groups in the β-glucan leading to the native polymer adopting a compact triple stranded helix conformation, which determines their poor solubility in aqueous condition (Wang et al., 2005b). These demonstrate the failure of existing glucan products and the proposed glucan sulphate would not.

Upon preliminary isolation from *G. lucidum*, the β-glucan mainly, (1-3)-β-D-glucan exists as an insoluble microparticulate. Thus, a technique such as sulphation is needed to alter the molecule's hydrophobicity thus making it water-soluble and potentially more bioactive in aqueous systems. The proposed sulfation technique has been used as an effective approach to improve the antibacterial, antiproliferative, anti-inflammatory, antitumor, and immunomodulatory activity of a range other polysaccharides (Chen et al., 2010; Karnjanapratum et al., 2012; Xu et al., 2011; Zhang et al., 2012). A previous effort by Williams et al.,(1992) demonstrated that insoluble (1-3)-β-glucan was able to dissolve in water by a sulphation process, while increasing the positive biological functions (Chen et al., 2010).

Although there has been a number of investigations on optimal culture conditions and the medicinal properties of *G. lucidum*, the preparation of soluble β-glucan (from this mushroom or from mycelial cultures) and its role as a bioactive sulphated glucan have not been studied in detail (Han et al., 2008).The goals of the present study are to develop a water-soluble polysaccharide based on the introduction of a sulphated group in enhancing the bioactive activity of mushroom (mycelia) polysaccharide. This work will offer useful information on the features of glucan sulphate from the mycelia of *G. lucidum*, and will be helpful for further studying its activity.

4.1 Materials and methods

4.1.1 Fungal material

G. lucidum BCCM 31549 was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL), [Agro] Industrial Fungi and Yeast Collection (Leuven, Belgium) as a well-preserved culture slant tube and considered as a master culture of the Basidiomycete. The fungus was subcultured onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK) and maintained according to Chapter 3. The repeated batch fermentation strategy was implemented, and the mycelial pellets were extracted.

4.1.2 Extraction and isolation

Distilled water (D_2O) was functioned to rinse the pellets (Fig. 4.1) off the sieves from the fermented culture broth. Through Whatman filter paper; they were filtered and vaporised to 50 mL at 60-80 ^{o}C (under reduced pressure). This volume was added to 150

mL of ethanol, for macromolecules precipitation, containing the desired polysaccharide-derived β-glucan. A glass rod was used to obtained the product by twirling. Based on the macromolecules precipitation, the precipitate was attached or adsorbed onto the glass rod and harvested from the solution. Usually, the mixture of three volumes of ethanol per volume of water would not precipitate the glucose. However, numerous soluble glucose may be confined within the extracted precipitate, which was then splashed using 96% (v/v) ethanol. Subsequently, the solution was dialysed against distilled water for three days (MW cut-off = 10,000 Da) using a dialysis tube (Fisher Scientific, Loughborough, UK). The residual glucan was aerated and pre-chill in -20 ^{o}C freezer.

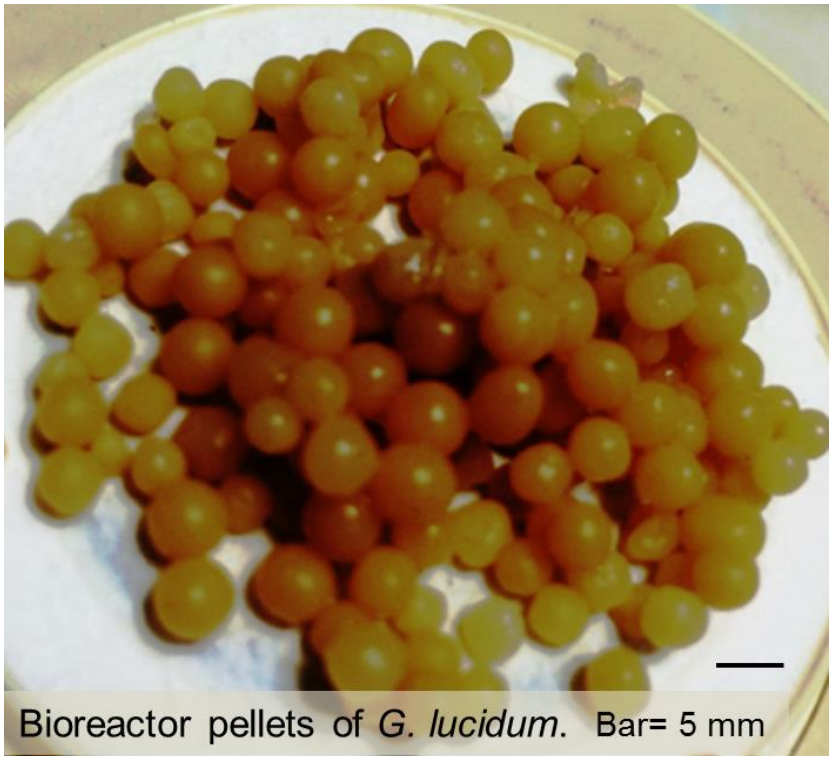


Figure 4.1 Bioreactor pellets of *G. lucidum* BCCM 31549 obtained from Chapter 3

After a couple of hours (h), the samples were transferred to -80 ^{o}C freezer for 24 h and then freeze-dried for 48 h. Later on, the build-up moisture surrounding the precipitated glucan were completely evapourated. It was then re-suspended in distilled water, freeze-dried in -80 ^{o}C freezer and evaluated to yield a (1,3)-β-D-glucan (G).

The G produced from the repeated batch fermentation processes was water-insoluble; therefore an inevitable process needs to be implemented to increase its solubility in water. Suzuki et al., (1991) and Williams et al., (1992) did sulphation of active (1,3)-β-D-glucans to increase their solubility or increase their bioavailability. Hence, sulphation of the current water-insoluble G was executed in this experiment. The improvised method of G sulphation of Williams et al., (1992) was followed. Soluble (1,3)-β-D-glucan sulphate (GS) was produced as outlined in Fig. 4.2. Firstly, 1 g of microparticulate G was liquefied in 50 mL of dimethyl sulfoxide (DMSO) containing 6 M urea. Eight millilitre of concentrated sulphuric acid was added drop-wise directly erstwhile to heating. In a water bath, the solution was heated at 100 ^{o}C, and the reaction process continued for 3 to 6 hours. By 90 minutes, a crystalline precipitate (ammonium sulphate) was formed. The mixture solution was then vented at room temperature, and 1 L of ultrapure, pyrogen-free, D_2O (Millipore, Bedford, MA) was added. The GS solution was then pre-filtered to remove unreacted polymer in G. The GS solution was dialysed using a Vivaflow 200, using a 10,000 MW cut-out filter (Sartorius Stedim Lab Ltd, Binbrook, UK). The final volume was reduced to 500 mL and lyopholised to dryness.

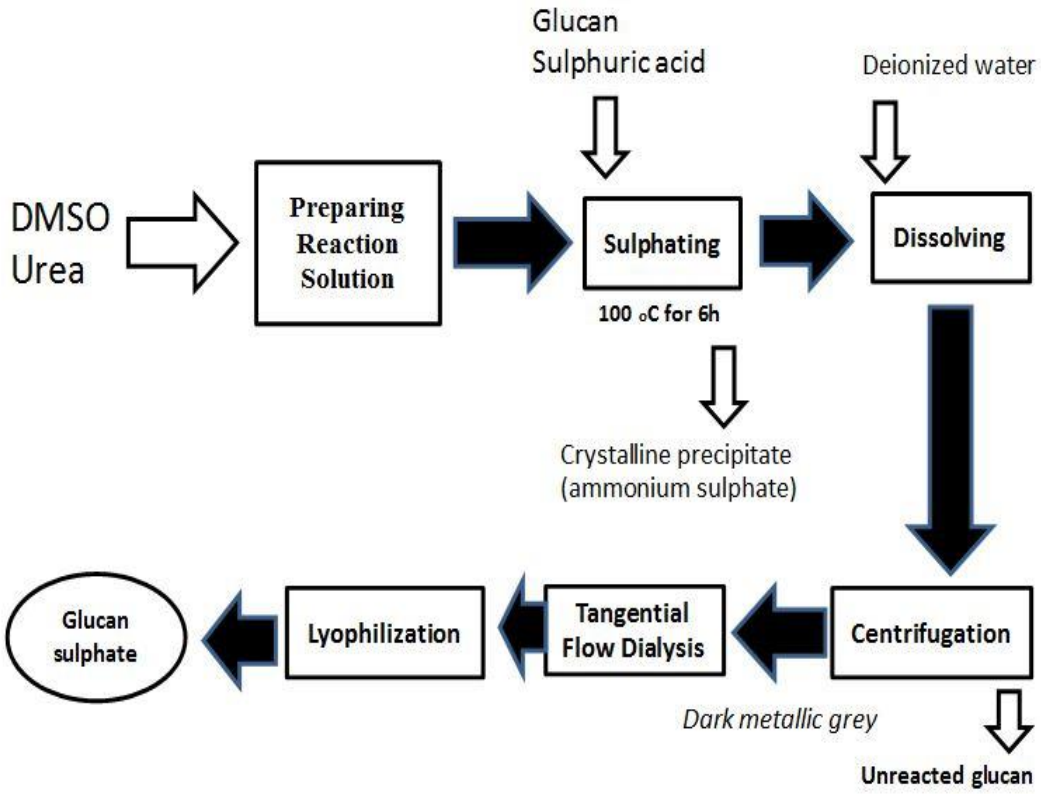


Figure 4.2 Homogenous reactions for sulphated (1-3)-β-D-glucan (GS) preparation: process scheme. Improvised from Wang et al., (2005b).

4.2 Compositional analysis of glucan and glucan sulphate

4.2.1 Elemental analysis

The content of C, H, O, N, and S were estimated using a Perkin Palmer 2400 Series II CHNS/O Elemental Analysis (Waltham, MA, USA) device. According to the recorded results of the elemental analysis, the degree of sulphation (DS) is defined by the following equation (A) according to Wang et al., (2005b).

$$DS = \frac{72s}{32c} \qquad (A)$$

Where *s* is the mass ratio of S element in the product glucan sulphate (GS). From now on, DS signifies the number of sulphate groups per glucose residue.

4.2.2 Infrared spectroscopy

FT-IR spectra of the G and GS samples were taken using a FT/IR 3000 spectrophotometer, (Jusco, Japan) following the method of Shi et al., (2013). The samples were ground with spectroscopic grade potassium bromide (KBr) powder and then pressed in a 1 mm pellet for FT-IR measurement in the frequency range of 4000-400 cm^{-1}. For jelly-like samples (GS), FT-IR Attenuated Total Reflectance (ATR) [Perkin Elmer, USA] was used to acquire the spectrum.

4.2.3 ^{1}H NMR Spectroscopy

In order to improve the solubility of G prior NMR analysis, ultrasonic treatment was performed using a JY-99 II ultrasonic reactor (Ningbo Xin Zhi Biotechnology Co., Ltd., China) of 20 kHz frequency and 1200 W maximum output power. Next, the NMR spectra of the both G and GS were obtained using a DXM 500 FT-NMR spectrometer (Bruker, Switzerland) equipped with a 5-mm multinuclear inverse probe at 500.13 and 125.78 MHz. Both compounds were liquefied in deuterium oxide $-d_6$ at the concentration of about 10 mg/mL to 30 mg/mL. All spectra were carried out at 80 ^{o}C, respectively. Scan number was 16 and the chemical shifts (δ) indicated in parts per million (ppm). Laminarin from *Laminaria digitata* (Sigma-Aldrich, UK) was used as the

comparison standard for G while Fucoidan originated from *Fucus vesiculosus* (Sigma-Aldrich, UK) was used as the comparison standard for GS.

4.3 Statistical Analysis

All analysis were carried out in triplicate and the respective mean ± S.D determined using the software, GraphPad Prism 5 (Version 5.01) and shown as in Chapter 3, 5, and 6.

4.4 Results and discussions

4.4.1 Sulphation of β-glucan and its solubility

In this study, the method for solubilisation of G employs DMSO to dissolve water-insoluble G preceding sulphation (Ferreira et al., 2015b). The DMSO and other reaction products were removed from G by extensive dialysis to ensure the purity of the GS produced. The solubility of the GS in water was measured post-sulphation to assess the effects of sulphation on G. In ultrapure distilled water, the final solubility of G was below 5% (w/v), but that of its GS was above 95% (w/v). Table 4.1 recaps the solubility and yields of the native G and soluble GS. Furthermore, the GS was readily dissolved without heating while the G needs 0.1 M of NaOH at 80 ^{o}C to assist dilution in water. The improved tractability of GS about G represents a significant aid in developing and implementing assays.

Table 4.1 Solubility properties of glucan sulphate (GS) prepared from glucan (G) derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

Characteristics	G	GS
Appearance	White powder	Reddish brown slime
Dry weight (g)	1.6	1.4
Yield (%)	-	87.5
Water solubility (%)	Below 5	Above 95

*Values are means of four batches

The introduction of sulphate group has several purposes. Based on the present study's findings, the aqueous solubility of the extracted G from the fermenter was as poor as that of G prepared from other procedures. Astonishingly, this was mentioned in the literature that G was less suitable for medicinal applications (Han et al., 2008). In terms of the commercial importance of bioactive glucan, the water insoluble G show slight bioactivity, although G by-products such as pullulan sulphate, lentinan sulphate, and dextran sulphate have been suggested to display high anti-HIV activities and small anticoagulant activities (Wang et al., 2005b). Wang and Zhang (2014) also revealed that the sulphation process on the fruiting bodies of*G. lucidum* producing G have led to enhanced antitumour and antiviral activities (Liu et al., 2012b; Peng et al., 2005). However, comparable bioactivities on the antimicrobial, antiproliferative, antifungal, NO stimulation, and cytotoxicity of mycelial-sourced GS are limited.

4.4.2 Compositional analysis

4.4.2.1 Elemental analysis

Elemental analysis was accomplished to attain the composition of the (1,3)-β-D-GS and, therefore, its degree of sulphation (DS). Basic examination of lyophilised GS gives a composition (w/w) of 24.5% C, 5.72% H, 49.92% O, 9.85% S, and 10.01% N (Table 4.2). Based on this, the DS of GS is thus 0.90 indicating 90 sulphate groups are present on every 100 glucose subunits within the polysaccharide on average. When compared with the previous DS value (0.94) of sulphated polysaccharide (S-GL) reported by Wang and Zhang (2009), the current DS value of GS (0.90) was broadly similar to each other.

Table 4.2 Elemental analysis of glucan (G) and glucan sulphate (GS) derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

	(w/w)%					
Sample	C	H	N	O	S	Degree of sulphation (DS)
G	28.60	3.28	1.79	65.08	1.25	-
Laminarin	39.36	6.47	0.32	52.84	1.01	-
GS	24.50	5.72	10.01	49.92	9.85	0.90
Fucoidan	24.10	4.24	0.3	64.49	6.87	0.64

* Laminarin is a standard for (1,3)-β-D-glucan from *Laminaria digitata*
* Fucoidan is a standard for sulphated-(1,3)-β-D-glucan from *Fucos vesiculosus*

4.4.2.2 IR spectroscopy

Table 4.3 and Fig. 4.3, summarise the results of using FTIR spectroscopy to assess the structural characteristics of the G and GS. Both molecules showed the typical IR absorptions of polysaccharides at 1,250 and 1,650 cm^{-1}: 1,170 and 1,651 cm^{-1}, respectively. These IR absorptions as well as those in the 'anomeric region' at 950 – 700 cm^{-1} allow us to differentiate β from α glucans spectroscopically (Wang et al., 2009). Overall, the D-glucosidic linkage arrangement is β-type both prior to and the following sulphation.

In the functional group region of the G-spectra, there were significant absorptions at 3,400, 1,077, 2,925, 1,374, 1,647 , 1,246, 1,540, 1,077, and 892 cm^{-1} (Table 4.3), which resembles the elongating absorption bands of poly -OH, C=O=C, -CH_2, -CH_3, C=0, amide, pyranose ring and β-configuration of D-glucose units. As compared with the previous work by Wang et al., (2005b) and Kiho et al., (2014), the specific absorption of G at 892.9 cm^{-1} demonstrates that the compound is a β-glucan. The characteristic peak of the β-configuration at 892.9 cm^{-1} was also noted in the spectra of GS with two new absorption peaks at 1,170 and 867 cm^{-1} also present (Fig. 4.3), which match to the S=0 asymmetrical stretching and C-S-C symmetrical vibration (Wang et al., 2009). These confirmed that the sulphated derivative had been efficiently synthesised from G.

Table 4.3 Infrared regions and assignments for glucan (G) and glucan sulphate (GS) derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

Wavenumber (cm^{-1})		Detail	Group
G	GS		
3,400	3,350	O-H stretching vibration	O-H
1,339, 2,925	2,922	C-H stretching vibration	$-CH_2-$
1,374	1,318	C-H stretching vibration	-CH3-
1,647, 1,246	1,651	Symmetric and asymmetric stretching vibration	C=O
1,077	1,000	C-O stretching vibration	C-O
-	1,170	S=O stretching vibration	$-O-SO_3$
-	867	C-O-S stretching vibration	$-O-SO_3$
1,077	949	Saccharide / Pyranose ring	C-O-C
892	867	β – configuration of sugar unit	C-O-C
1,540	-	Amide group	$R_nE(O)_xNR'_2$

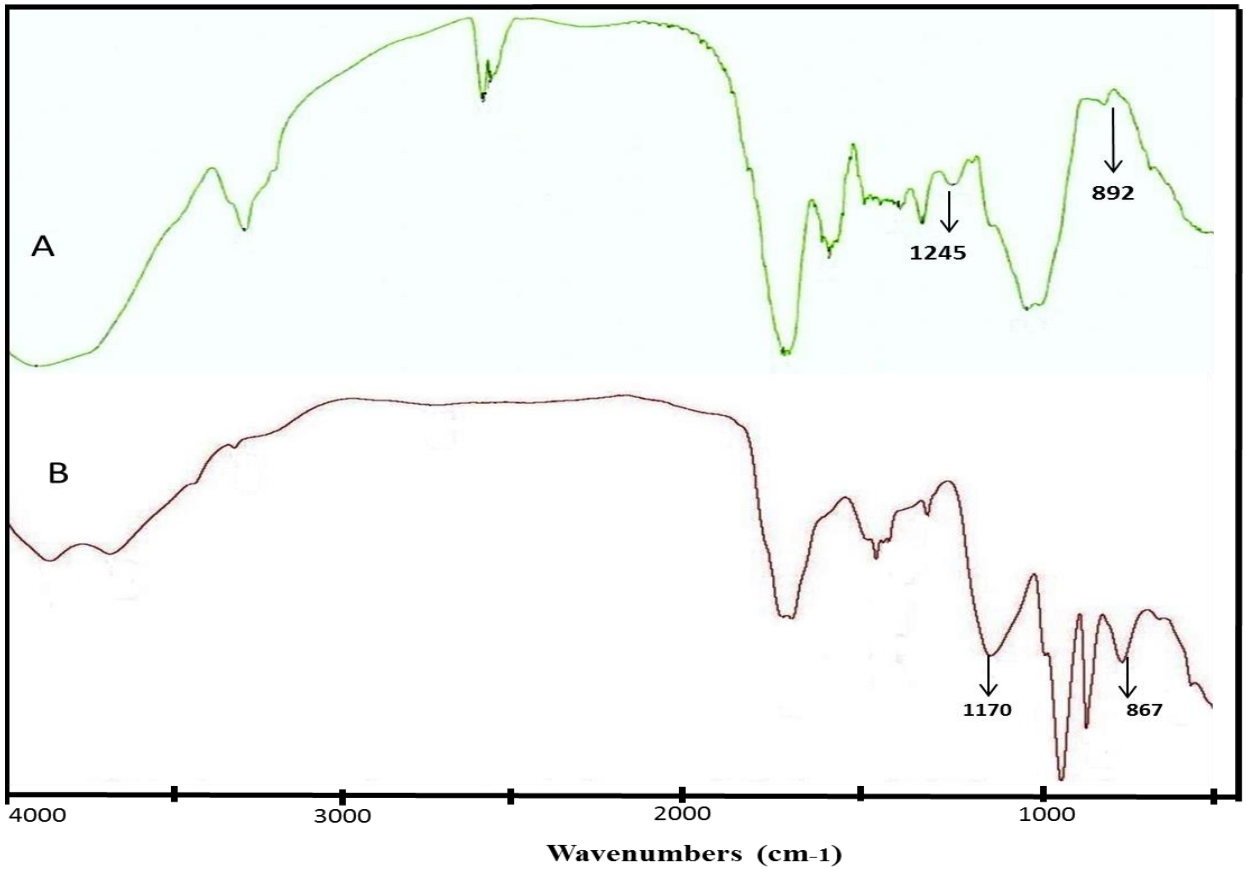


Figure 4.3 Comparison of β-glucan IR spectra. A: glucan (G) and B: glucan sulphate (GS) were derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

4.4.2.3 NMR studies of the β-glucan

As can be seen in Fig. 4.4 and Fig. 4.5, ^{1}H NMR spectroscopic analysis of the original G and GS from *G. lucidum* was conducted at 80 ^{o}C using D_2O-d_6 as a solvent. Using ppm as the standardised unit for NMR studies, ^{1}H NMR spectra of the G were compared with the standard laminarin (β-1,3-D-glucan) from *L. digitata* while the GS spectra were compared with the standard fucoidan (sulphated-β-1,3-D-glucan) from *F. vesiculosus*. The spectrum chemical shifts of δ 3.9 to 5.4 ppm and δ 2.6 to 5.5 ppm exhibited indicate that both compounds were glucans, as can be observed in both Fig. 4.4 and Fig. 4.5, respectively. The current work is comparable with previous research by Ji et al., (2012), which analysed laminarin and sulphated laminarin in the area of ^{1}H-NMR spectrum of δ 4.49-5.5 ppm. Thus, these spectra indicate that the glycosidic bonds in both G (Fig. 4.4) and GS (Fig. 4.5) were β-type.

Evaluation of the 'anomeric region' of ^{1}H NMR spectra in this study with those described previously specifies that they are of similar pattern (Liu et al., 2014; Wagner et al., 2003; Wang et al., 2005b). For G (Fig. 4.4) ^{1}H NMR spectra, the signals at δ 5.08, 4.50 and 4.40 were assigned to OH-2, OH-6, and OH-4 when compared with the reported work by Wagner et al., (2003). The GS (Fig. 4.5) ^{1}H NMR also exhibits similarity to the G with the signals at δ 5.21, 4.52 and 4.40. When compared, the anomeric signals for both compounds in the present study (G and GS) were at δ 4.5 ppm and δ 4.2 ppm, respectively indicating β-configuration for glucopyranosyl units as reported by Liu et al., (2014).

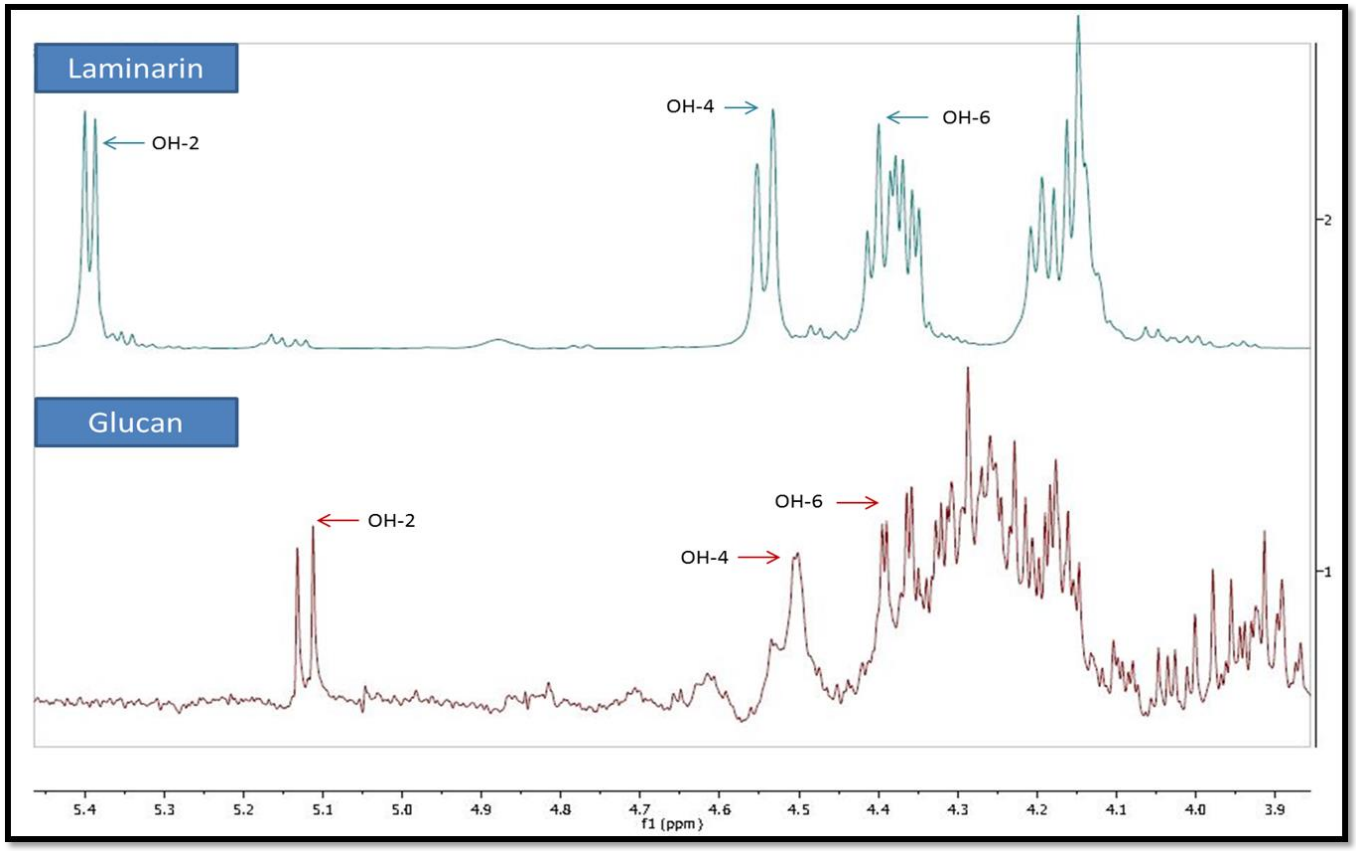


Figure 4.4 ^{1}H NMR spectra of (1-3)-β-D-glucan (G) which derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium and Laminarin (*Laminaria digitata*) standard in D_2O-d_6 at 80 ^{o}C.

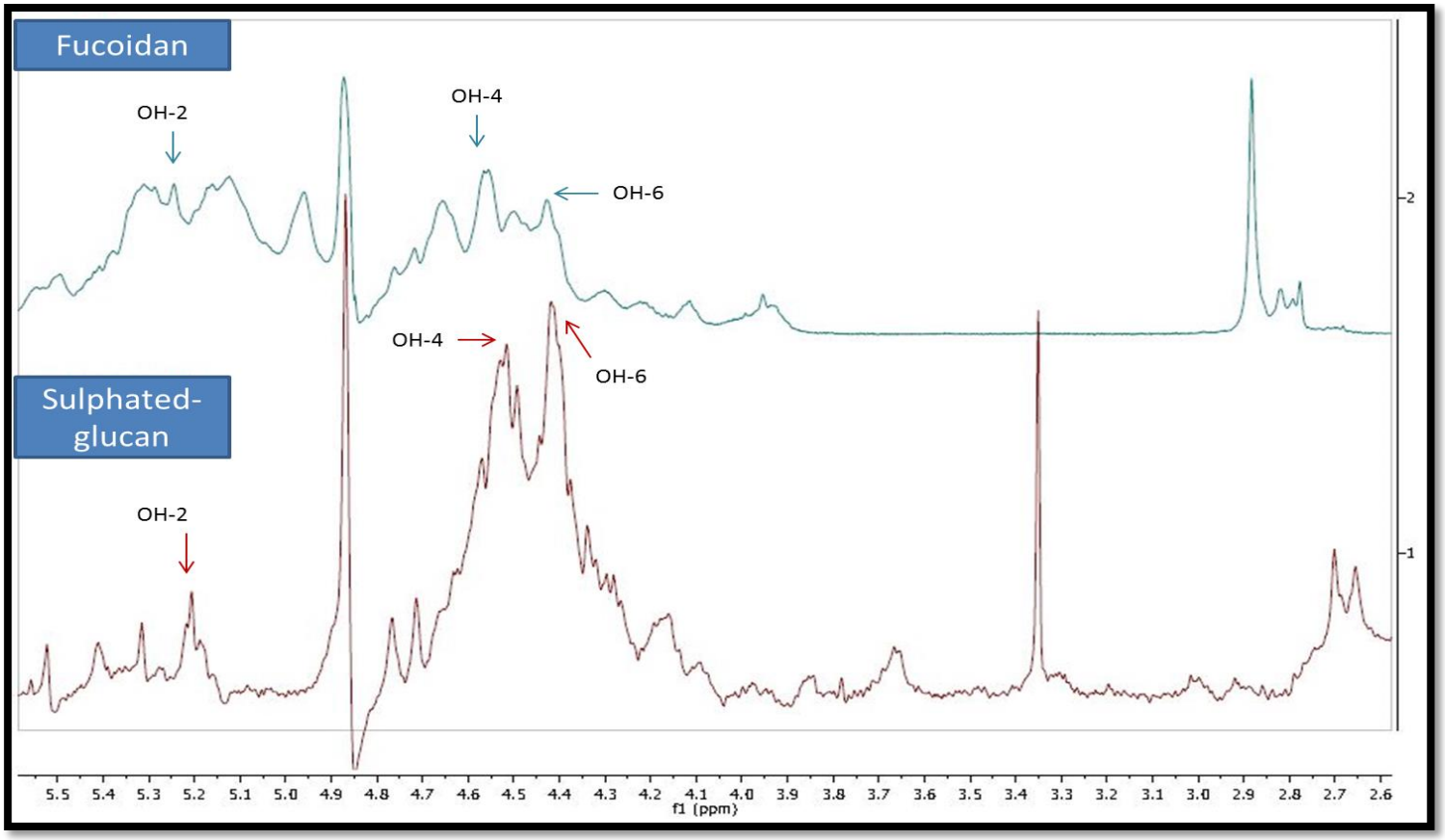


Figure 4.5 ^{1}H NMR spectra of sulphated (1-3)-β-D-glucan (GS) which derived from extended batch cultures of *G. lucidum* BCCM 31549 pellets and Fucoidan (*Fucus vesiculosus*) standard in D_2O-d_6 at 80 ^{o}C.

Moreover, the ^{1}H-NMR spectrum of the GS displayed that the chemical shift of hydrogen usually stimulated downfield relative to G, which showed that most of the hydroxyl groups in the G had been sulphated and similarly specified that GS had β-glycosidic bonds (Table 4.4). From the IR and ^{1}H NMR analysed, it is possible to conclude that the G compound is composed of (1-3)-β-D-linkages which gave the polymer structure apparently as a 1,3-β-D-glucan.

Table 4.4 Change of chemical shifts of G to GS from the extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

Sugar proton	Chemical shift (ppm)			
	G	Laminarin	GS	Fucoidan
OH-2	5.08	5.42	5.21	5.25
OH-4	4.57	4.56	4.52	4.56
OH-6	4.49	4.43	4.45	4.45

*G = glucan, GS = glucan sulphate. Laminarin (*Laminaria digitata*) was used as a standard for 1,3-β-D-glucan while Fucoidan was used as a standard for sulphated-1,3-β-D-glucan

4.5 Conclusions

In summary, it has been shown that the compounds extracted from these mycelial cultures were polysaccharides with a proposed structure of β-1,3-D-glucan, when compared with both standards, laminarin and fucoidan. The glucan sulphate (GS) was successfully produced from the glucan (G) by chemical conversion (sulphation), and will be tested for any possible therapeutic bioactivities in Chapter 5.

CHAPTER 5

ANTIMICROBIAL, ANTIFUNGAL, CYTOTOXIC AND IMMUNOMODULATORY ACTIVITITIES OF SULPHATED-(1,3)-β-D-GLUCAN DERIVED FROM THE EXTENDED BATCH CULTURE OF *Ganoderma lucidum* BCCM 31549

5.0 Introduction

Bacterial infection is one of the most significant causes of food degradation, and there is little attention on the role of food producers to prevent this phenomenon. Foodstuffs represent a rich source of nutrients often stored under conditions of permissible temperature and humidity. In addition to food degradation by microorganisms, high levels of multiplying microorganisms present in the food may initiate food poisoning which can contribute to public health problems (Alvarez-Suarez et al., 2010) and disrupting supply chain issues worldwide. Ideally, improving the safety and spoilage characteristics of foodstuffs by including other naturally occurring products which may possess both antimicrobial, and other desirable biological activities (e.g. cytotoxicity on cancer cells, health-giving), potentially offers a route to safer foods with enhanced health imparting charateristics. This approach make use of the potential for ‘’bifunctional’’ effects (Llaurado et al., 2015) of glucan materials derived from traditional food sources, including some species of mushrooms (Liao et al., 2013). These natural foods have been shown to be a relatively unexplored source for improvements in food safety, preservation while providing extra health benefits (Zhang et al., 2012).

Mushrooms of the genus *Ganoderma*, have been eaten for many centuries in Asia to encourage well being, durability and endurance (Ferreira et al., 2015b; Liu et al., 2014). *G. lucidum* has been lately shown to possesses varied properties, such as bactericidal (Heleno et al., 2013) and cytotoxicity effects on cancer cells (Kimura et al., 2002). A great deal of interest has also focused on *G. lucidum* immunomodulatory ability and its potential anti-inflammatory effect which relates to the white blood cells

(macrophages). Activated macrophages release an inflammatory mediator called nitric oxide (NO) (Tung et al., 2013), which provide defence mechanisms against pathogens by modulating inflammation.

However, most of the reported positive bioactivities were obtained from the fruiting bodies and not from their mycelial cultures in bioreactors. Such cultures represent a much faster way to produce bioactive compounds from *G. lucidum* than extraction from mushrooms as discussed in Chapter 3. If *G. lucidum* derived materials having multifunctional effects are to be used to improve foodstuffs as described above, they will need to be produced in bulk, quickly, cheaply and to a consistent quality. Bioreactor submerged liquid fermentation has been shown to be capable to do this (Chapter 3), and these bioreactor derived materials are safe. In this study, mycelial β-glucan with potential bioactivities identified in Chapter 4 was tested.

To date, the cytotoxicity, antifungal, NO-stimulation, and antimicrobial activity of extracts from *G. lucidum* mycelia particularly the glucan sulphate (GS) have not been completely characterised. In Chapter 4, the glucan (G) from *G. lucidum* mycelia was sulphated. Both G and GS structures were screened and the results showed that GS exhibited significant bioactivities and hence could be utilised as a potential additive in food systems. It's presence would inhibit both spoilage and pathogenic bacteria, and impart significant health benefits noted in this study.

5.1 Materials and methods

5.2.1 Materials

Gentamicin susceptibility test discs (30 µg of concentration) were supplied by Thermo Scientific Oxoid (Fisher Scientific, Loughborough, UK) and antifungal standard, Bifonazole (10 mg) was obtained from Sigma-Aldrich, Dorset, UK. In this experiment, RAW264.7 macrophage cell line, (ZR-75-1) Human-Caucasian-Breast-Carcinoma, (U937) Human-Caucasian-Histiocytic-Lymphoma and (PNT2A) Human-Prostate-Normal cell were obtained from ECAAC, European Collection of Cell cultures, supplied by (Sigma-Aldrich, Dorset, UK). DMEM and TryLE™ Express were supplied by Gibco (Life technologies, Paisley, UK). RPMI - Bio Whittaker® without L-glutamine was supplied by Lonza, Vergiers, Belgium. HBBS –Hank's balanced salt solution was supplied by Sigma-Aldrich, St. Louis, USA, MA. The 96-wells plate, TPP 92096 was supplied by TPP, Trasadingen, Switzerland. Cell culture spectroscopy analysis was done using Wallac, Victor2 ™ $_{H20}$ Multi-label Counter with IR, high density TR-Fluorometry, stacker and robot loading (PerkinElmer, Waltham-MA, USA). All other chemicals and solvents were analytical grade.

5.2.2 Microorganism and fermentation

Ganoderma lucidum BCCM 31549 used was based on the previous chapters. The extended RBF has been previously described in Chapter 3.

5.2.3 Recovery of glucan (G) and glucan sulphate (GS)

The G and GS were recovered as previously described in Chapter 4.

5.3 Antimicrobial activity

5.3.1 Microbial strains and conditions

The test bacteria used for antimicrobial sensitivity testing comprise the bacteria *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli* that were obtained from the General Microbiology Lab Collections SIPBS, Glasgow, UK. In addition, *Escherichia coli* EPIC S17, *Salmonella* BA54 SL1344 pSsaG, *Listeria monocytogenes*, *Shigella sonnei* 20071599, and Methicillin-Susceptible-*Staphylococcus aureus* (MSSA) ATCC 292123 were kindly supplied by Dr. Jun Yu, SIPBS, Glasgow, UK. At 20 ^{o}C, the strains were kept in the suitable freshly-prepared medium and rejuvenated two times before being applied in the proposed assays. Bacteria were cultured with the oxygen supplied environment at 37 ^{o}C (Incubator- Bruker 200, Thermo, UK) in nutrient agar (NA) medium for bacteria.

5.3.2 Kirby-Bauer disk diffusion assay

Determination of antimicrobial activity was carried out using the Kirby-Bauer disk diffusion assay method. First, 20 mL of NA medium were decanted into each Petri dish. All test microorganisms were adjusted to 0.5 McFarland standards using sterile broth medium. Once hardened, about roughly 200 µL of suspension of the test bacteria was smeared on the prepared agar. The standardised sterile 11 mm discs (blank) (Sigma-

Aldrich, UK) with an indentical absorbed GS volume were soaked with a known amount of extract. It was positioned moderately onto the agar overlay. The plates were carefully incubated overnight at 37 ºC or 48 h or 35 ºC for two days depending on the growth requirement of the bacterium. Gentamicin was applied as the positive control while ethanol was the negative control. After the incubation, the diameters (mm) of the inhibition zone were measured. Inhibition zones that were higher than 11 mm were considered positive for antimicrobial reactions.

5.3.3 Evaluation of minimum inhibitory concentration (MIC)

The MIC was evaluated by microdilution using 96-well microtitre plates according to Li et al., (2012) with slight modifications. Sterile broth medium in conjunction with 0.5 McFarland standards was used as bacterial suspensions adjustment. GS compounds were dissolved in sterile ultrapure water and serially diluted into (mg/mL) 200, 100, 20, 10, 8, 5, 3, 2 and 1. The final mixture was 25 µL of compounds with 75 µL of a suspension of each bacterium (working volume of 100 µL). Each test culture was pipetted on the plates and incubated for 24 h at 30 ºC. Once the incubation time ended, the turbidity or cloudiness was taken as the signal or indication for bacterial growth. The lowest diluted concentration at which the incubated mixture persisted clear after microscopic assessment (at the binocular microscope) was thus selected as the MICs.

5.3.4 Evaluation of minimum bactericidal concentration (MBC)

Based on the MIC observation, the level at which the incubated mixture stayed clear after the microscopic estimation was selected as the MIC. The microscopic growth range were then pipetted (100 µL) to the nutrient agar. Sterile L-spreaders were used to make the spreading even. Following that, the concentration indicating the MIC and at least two of the more concentrated dilutions were plated and enumerated to determine viable colonies specifically for MBC determination. The media were cultured at 30 ^{o}C for 24 h to observe for any microorganism growth. For the MBC, the minimum or lowest concentration in the medium that had less than 5 colonies was used.

5.3.5 Anti-microbial screening assay *in vitro*

The method by SIDR (Strathclyde Institute of Drug Research) was used for the antimicrobial test on the bacteria include MRSA 16, MRSA 106, *Klebsiella pneumoniae* ATCC 13883, *Klebsiella pneumoniae* ATCC BAA-2146 NDM-1 positive and *Mycobacterium marinum* ATCC BAA 535 using the 96-well plates *in vitro* (Cechinel-Filho, 2012; Khalaf et al., 2012) . These tests were in triplicate, and the GS was supplied at 10 mg/mL. Gentamicin was used as the positive control for the bacteria while DMSO as the negative one.

5.4 Antifungal and demelanising activity on *Aspergillus niger* A60

Aspergillus niger A60 was obtained from the Fermentation Group, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, UK and used for screening study. This fungus was maintained on PDA agar and stored at 4 ^{o}C. Only

sources from the standardised stock culture in -80 ^{o}C freezer were used. In order to investigate the antifungal activity of GS, a modified micro-dilution technique was used (Heleno et al., 2013). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 x 10^5 in a final volume of 100 µL per well. Dilutions of the inocula were cultured on solid malt agar to rule out the absence of contamination and to check the validity of the inoculum. MIC evaluations were performed by a serial dilution technique using 96-well microtitre plates. GS was dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) at (mg/mL) 200, 125, 100, 60, 30, 20, 15, 10, 2, and 1 and added in potato-dextrose medium with inoculum. The lowest GS concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) was evaluated by serial sub-cultivation of a 2 µL of GS dissolved in medium 72 h cultivation, and later transferred into microtitre plates containing 100 µL of broth per well. Further incubation for 72 h at 28 ^{o}C was done. The lowest GS concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, and commercial fungicide, Bifonazole (Sigma-Aldrich, UK) was used as positive controls (1-3000 µg/mL).

5.4.1 Demelanising activity

For testing the demelanising activity of GS on *A. niger* A60, 96-well microtitre plates were used. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a suitable level of 1.0 x 10^5 in a final volume of 100 µL / well. Dilutions of the inocula were cultured on potato-dextrose-agar (PDA) to attest the absence of contamination and to verify the validity of the inoculum. Evaluation of minimum demelanising concentrations (MDCs) was accomplished by serial dilution. The GS was dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) and added in potato-dextrose broth with inoculum. The microplate was incubated for 72 h at 28 ^{o}C. A sample of mycelium was obtained from the edge of a colony grown on the medium with different concentrations of GS. GS were later dried and fixed with lacto-phenol and observed under a light microscope (Nikon Instruments Europe B. V., Surrey, UK) to inspect structural irregularities (Heleno et al., 2013). The lowest concentration that triggered demelanisation of fungal hyphae and conidia was determined as MDC. Samples from the control plate without added extracts were also stained and observed. DMSO (5%) was used as negative control.

5.5 Cell culture assays

All cell work was carried out in aseptic condition (Class II- Microbiological Hood, Thermo Scientific, Model SAFE 2020, Glasgow, UK). When confluent, the adherent cancer cells (PN2TA, and ZR-75-1) were passaged by aspirating medium and adding Hank's Balanced Salt Solution (HBBS) (Lonza, Belgium) without Ca^{2+} and Mg^{+}

ions, for 1-2 minutes, to help remove adhesion proteins. The HBBS was then discarded and the cells were then incubated in 5 to 10 mL of TrypLE™ Express (Life Technologies Ltd., Paisley, UK) for 10 minutes until the cells had dislodged from the flask surface. TrypLE™ Express was inactivated by adding an equal volume of HBBS containing Ca^{2+} and Mg^{+} (Lonza, Belgium). The cells were centrifuged at 1,200 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in complete medium. Cells were counted using a haemocytometer (Bright-Line™ Z359629-1EA, Gillingham, UK) and the cell density was calculated. Equations (1 and 2) were used to calculate the cells (survival rate).

$\frac{\text{Target density}}{\text{Calculated cells}}$ x Flask size (eg. 150 cm^2) for adherent cells – **Equation 1**

$\frac{\text{Target density}}{\text{Calculated cells}}$ x Target volume (eg. 20 mL) for non-adherent cells – **Equation 2**

All cells were grown in 75 cm^2 flasks, unless otherwise stated. The cells were maintained in the respected complete medium in a humidified incubator with an atmosphere of 95 % air, 5 % CO_2 at 37 ^oC.

5.5.1 Cytotoxicity on normal human prostate cell (PN2TA)

Cell lines were grown in appropriate freshly-prepared complete medium in a cell culture incubator (gaseous composition: 95% air, 5% CO_2) at 37 ^oC. The PN2TA normal human prostate cell line sustained in a complete medium comprising RPMI, penicillin-streptomycin (5 mL), 50 mL foetal bovine serum (FBS), L-glutamine (5 mL), and pH at 7.4. The cytotoxic effect of both G and GS were determined using the AlamarBlue®

assay. Initially, 96-well microtitre plates were seeded with the PN2TA cells 2 x 10^4 cells / mL for each well. Cells were permitted (to ensure healthy cell counts) to cultivate one day afore being introduced to GS: 500, 300, 50, 30, 5, and 3 µg/mL. For the negative control group, 4% (v/v) of Triton-X was added to the medium. After the incubation for the indicated hours, 10% (working volume per well) of alamarBlue® reagent was decanted to each well and incubated for an extra 6 h in a humidified incubator. Once 6 h of incubation completed, the resazurin in the alamarBlue® undergoes oxidation-reduction change in response to cellular metabolic change. The reduced form resazurin is pink and extremely fluorescent, and the strength of fluorescence produced is proportional to some living cells undergone respiration. Over-identifying the level of oxidation during respiration, the alamarBlue® reagent acts as a shortest indicator to quantitatively determine cytotoxicity and cell viability. For analysis, cytotoxic activity was calculated based on cell survival ratio (%). The wavelength of 570 nm was used for absorbance reading. For analysis, percentage (%) of the control was calculated based on cell survival ratio with Equation 3.

$$\text{Percentage of control (\%)} = \frac{\text{Each tested well values} - \text{Background}}{\text{Average of control}} \text{ X } 100 \text{ - } \mathbf{Equation\ 3}$$

5.5.2 Cytotoxicity on cancer cells (U937 and ZR-75-1)

The cytotoxicity effect of both G and GS were also tested on the cancerous cell U937 (Human Caucasian Histiocytic Lymphoma) and ZR-75-1 (Human Caucasian Breast Carcinoma) by 96-well plate alamarBlue® assay. These cancer cells at a density of 3 x 10^5 cells/well were being exposed to 60, 50, 30, and 10 µg/mL of both G and GS

at day one prior incubation except for ZR-75-1 (after cell attachment at 500-20 µg/mL). As for the control group, an identical volume of complete sterile medium was applied (positive control) with Triton X (4%) as the negative control. After incubation for the designated period, 10% of alamarBlue® reagent was pipetted to each well of the working microtitre plate and incubated for an extra 6 h in a humidified incubator. Following a 6 h incubation, the alamarBlue® reagent initiated resazurin to undergo oxidation-reduction change in response to the cellular metabolic modification. The principles were the same as explained in Section 5.5.1. The wavelength of 570 nm was used for absorbance reading. For analysis, cytotoxic activity was calculated based on cell survival ratio (%) using Equation 3.

5.6 Stimulation of macrophage cells

As described in the literature, RAW264.7 macrophages were cultured in phenol red-free DMEM with high glucose (4500 mg/L) and L-Glutamine (4 mM/L) supplemented with 10% heat-inactivated foetal bovine serum, penicillin (10,000 U/mL), and streptomycin (10,000 µg/mL) (Chiong et al., 2013). The cells were maintained in a humidified incubator containing 5% CO_2 at 37 ^{o}C. For all experiments, cells were grown to 80% - 90% confluence, and subjected to no more than 20 cell passages. Cells were scraped out from the plastic culture flasks using Sterile-Scrappers (Corning, USA), and then centrifuged at 110 rpm at 4 ^{o}C for 10 minutes. The medium was then removed and the cells were suspended with fresh DMEM containing the same supplements. The density was attuned to 2-4 x 10^5 (cells / mL) and cell viability was always more than 80%, as determined using a standard trypan blue cell-counting technique. Cells were

dispensed (75 µL) into wells of tissue culture-grade 96-well plates (i.e., 0.5 x 10^5 cells / well) and incubated for 6 h at 37 ^oC in 5% CO_2 atmosphere to attach the cells. Unattached cells were discarded gently after 2 h. The attached cells were then stimulated with (µg/mL) 500, 200, and 50 of glucan (G), glucan sulphate (GS), and 0.1M NaOH (as solvent or negative control) in the presence or absence of the treatment sample at a final volume of 200 µL/well. Lipolysaccharide (LPS) from *Escherichia coli* 0111: B4 (Sigma-Aldrich, UK) was used as positive control. Cells were then incubated for 24 h at 37 ^oC in a humidified 5% CO_2 atmosphere.

5.6.1 Measurement of NO production

The level of nitrite oxide (NO) in cell-free culture supernatants, which reflects intracellular NO synthase activity, was evaluated by the Griess reaction using a 23479 Nitrate/nitrite Assay Kit Colorimetric (Sigma-Aldrich, USA). To prepare the kit-samples, the reacted medium was centrifuged at 1000 rpm for 15 minutes and the supernatant was used as a sample solution. For determination of nitrite, using 96-wells plate, 80 µL of a sample solution was added to one well and later 20 µL of buffer solution was added to each well. Next, 50 µL of Griess Reagent A was added to each well and mixed. After 5 minutes, another 50 µL of Griess Reagent B was added to each well and mixed. The plate was incubated at room temperature, and the absorbance was measured at 570 nm with a microplate reader. For the calculations, the absorbance of the blank solution (well A) was subtracted from the absorbance of each well. The concentration of nitrite in the sample solution was determined from the calibration curve (Appendix 5A).

5.7 Statistical analysis

All analyses were carried out as described in Chapter 3 and 4.

5.8 Results and discussions

G. lucidum possess a range of biologically active substances, which have scarcely been investigated. To broaden their use in food and pharmaceutical industries, a comprehensive study of the effects exerted as biological response modifiers is still needed, thus increasing the impact of the compound. In order to expand the pharmacological property of glucan sulphate (GS) from extended batch cultures of *G. lucidum* mycelial extract, screening for several activities was carried out in this study, and found that it showed: (i) antimicrobial activity towards both Gram positive and Gram negative bacteria; (ii) cytotoxic activity on U937 cancer cells; (iii) Demelanising activity on *A. niger* A60 and (iv) macrophage-stimulating activity for NO production. All of these activities suggested it to be a good candidate as a ‘’quad-functional’’ drug substance.

5.9 Antimicrobial activity

5.9.1 Disk-diffusion method

The antimicrobial effect of the GS from *G. lucidum* was tested against 10 species of bacteria as G was not evident. Their strength was measured quantitatively and qualitatively by the absence or presence of inhibition zones, zone diameters, MBC and MIC values. The findings of these tests are summarised in Table 5.1 (zone diameters) and Fig. 5.1 (graphical analysis), respectively. Among the bacterial strains tested in

Table 5.1 (zone diameters), when the GS reached 500 mg/mL, the diameters (mm) of the inhibition zone were 34 ± 3.2, 24 ± 2.6, 32 ± 1.0, 25 ± 2.6, 23 ± 2.8, 27 ± 1.5, 28 ± 0.5, 26 ± 1.0, 30 ± 1.0, and 30 ± 3.1, for *E. coli* EPIC S17, *E. coli*, *L. monocytogenes*, *Shigella sonnei* 20071599, *P. aeruginosa*, *S. enteritidis*, *Salmonella* BA54 SL 1344 (pSsaG), *Staph. aureus*, *Staph. epidermis*, and Methicillin-Susceptible *Staph. aureus* (MSSA) ATCC 292123, individually. The inhibition zone diameters increased with increasing GS prepared concentrations (Table 5.1). These reactions displayed that the anti-bacterial effect of GS was dose-dependent and that the gentamicin positive control was clearly effective against all the test bacteria.

Furthermore, Table 5.2 shows the MIC concentrations for bacterial strains were in the range of 1- 5 mg/mL and the MBC concentrations range was 5-10 mg/mL except the resilient *Shigella sonnei* 20071599 (Table 5.2, no.3). Among four species of Gram-positive bacteria verified, the greatest antimicrobial activity of GS was shown against *Staph. aureus* (Table 5.2, no.8), and its MIC was 2 mg/mL. Meanwhile, the antimicrobial activity of GS was verified against six species of Gram-negative microbial strains. GS exhibited fairly strongest antimicrobial activity against *E. coli* (Table 5.2, no.2) (MIC = 1 mg/mL), and seven species of microbial strains were shown to have MIC concentrations at respective 3 mg/mL while the most resistant bacterium was *Staph. epidermis* (Table 5.2, no.9) (MIC = 5 mg/mL).

Table 5.1 Antimicrobial activities of GS derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

No. /Gram	Bacteria	Glucan sulphate (GS) [a] Diameter of inhibition zone [b] (mm) 200 mg/mL	300 mg/mL	400 mg/mL	500 mg/mL	GENT [c] 30 µg	Ethanol [c] 100%
1 (G-)	*Escherichia coli* EPIC S17	22 ± 2.6	26 ± 1.2	29 ± 1.0	34 ± 3.2	19 ± 2.5	11.4 ± 0.1
2 (G-)	*Escherichia coli*	20 ± 1.5	22 ± 1.5	20 ± 1.1	24 ± 2.6	23 ± 3.1	11.3 ± 0.1
3 (G-)	*Shigella sonnei* 20071599	16 ± 1.0	21 ± 0.5	23 ± 2.1	25 ± 2.6	22 ± 1.5	11.4 ± 0.1
4 (G-)	*Pseudomonas aeruginosa*	16 ± 1.0	20 ± 1.0	20 ± 0.5	23 ± 2.8	23 ± 1.1	11.2 ± 0.1
5 (G-)	*Salmonella enteritidis*	17 ± 1.5	20 ± 1.0	23 ± 1.0	27 ± 1.5	24 ± 2.5	11.1 ± 0.1
6 (G-)	*Salmonella* BA54SL1344 (pSsaG)	20 ± 2.1	24 ± 1.5	26 ± 1.0	28 ± 0.5	28 ± 2.0	11.2 ± 0.1
7 (G+)	*Listeria monocytogenes*	26 ± 2.1	28 ± 1.0	30 ± 1.5	32 ± 1.0	34 ± 1.0	11.1 ± 0.1
8 (G+)	*Staphylococcus aureus*	18 ± 1.0	20 ± 0.5	21 ± 2.3	26 ± 1.0	21 ± 1.0	11.1 ± 0.1
9 (G+)	*Staphylococcus epidermis*	22 ± 1.5	23 ± 4.0	28 ± 1.5	30 ± 1.0	27 ± 1.5	11.2 ± 0.1
10 (G+)	MSSA ATCC 292123	22 ± 2.1	24 ± 2.0	27 ± 1.2	30 ± 3.1	25 ± 2.5	11.2 ± 0.1

[a] Values represent averages ± standard deviations ($P < 0.05$) for triplicate experiments. All organisms were obtained from Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, UK. G indicates Gram positive (G+) or Gram negative (G-) bacteria

[b] Sterile disc size was 11 mm indicating a negative inhibition and positive inhibitions were more than 11 mm.

[c] Ethanol was used as the negative control while Gentamicin (GENT) was used as the positive control

When compared to the studies where derivatised fungal polymers have been examined as food preservatives and their antimicrobial activity has been assessed (Dutta et al., 2009), it showed that SC2 sulphated-polysaccharide (chitosan) has MIC values higher than 2 mg/mL (Shahidi et al., 1999) for *Staph aureus*, *L. monocytogenes*, *Vibrio parahaemolyticus*, *P. aeruginosa*, *Shigella dysenteriae*, *V. cholera*, *Aeromonas hydrophila* and *S. typhimurium*. SC2 shows a much higher MIC's against Gram-positive than Gram-negative bacteria. Devlieghere (2004), Muzzarelli (1990) and Hernandez-Lauzardo (2008) also tested the antimicrobial activity of chitosan as food preservatives and gave results for MIC's at or above 2.5 mg/mL. The closest comparison to the present study involved an assessment of MIC values of polysaccharide extracts of *G. atrum* (Li et al., 2012) and *Ganoderma* sp. (Kozarski et al., 2014) sourced from powdered fruiting bodies varying from 1.6 to 6.25 mg/mL for the common bacterial food contaminants which also reported by Ferreira et al., (2015b). Thus, the MIC's recorded (Table 5.2) for the GS in the present study are broadly similar to those reported in other studies for fungal-derived polymers.

Table 5. 2 MIC and MBC of GS against the tested organisms derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

No. / Gram	Bacteria	Glucan sulphate (GS) Visibility / Colony interpretation							
		200 mg/mL	100 mg/mL	20 mg/mL	10 mg/mL	5 mg/mL	3 mg/mL	2 mg/mL	1 mg/mL
1 (G -)	*Escherichia coli* EPIC S17	Clear	Clear	Clear	Clear / 1 colony (MBC)	Clear / 8 colonies	Clear / 30 colonies (MIC)	Turbid / TNTC	Turbid / TNC
2 (G -)	*Escherichia coli*	Clear	Clear	Clear	Clear	Clear	Clear	Clear / 3 colonies (MBC)	Clear / 6 colonies (MIC)
3 (G -)	*Shigella sonnei* 20071599	Clear	Clear	Clear / 2 colonies (MBC)	Clear / 8 colonies	Clear / 12 colonies	Clear / 17 colonies (MIC)	Turbid / TNTC	Turbid / TNC
4 (G -)	*Pseudomonas aeruginosa*	Clear	Clear	Clear	Clear	Clear / 2 colonies (MBC)	Clear / 10 colonies (MIC)	Turbid / TNTC	Turbid / TNTC
5 (G -)	*Salmonella enteritidis*	Clear	Clear	Clear / 2 colonies (MBC)	Clear / 15 colonies	Clear / 20 colonies	Clear / 25 colonies (MIC)	Turbid / 100 colonies	Turbid / 200 colonies

6 (G -)	*Salmonella* BA54 SL1344 (pSsaG)	Clear	Clear	Clear	Clear	Clear / 4 colonies (MBC)	Clear / 10 colonies (MIC)	Turbid / TNTC	Turbid / TNC
7 (G +)	*Listeria monocytogenes*	Clear	Clear	Clear	Clear	Clear / 3colonies (MBC)	Clear / 30 colonies (MIC)	Turbid / 300 colonies	Turbid / TNTC
8 (G +)	*Staphylococcus aureus*	Clear	Clear	Clear	Clear	Clear	Clear / 4 colonies (MBC)	Clear / 16 colonies (MIC)	Turbid / TNTC
9 (G +)	*Staphylococcus epidermis*	Clear	Clear	Clear	Clear 3 colonies (MBC)	Clear / 11 colonies (MIC)	Turbid / TNTC	Turbid / TNTC	Turbid / TNTC
10 (G +)	MSSA ATCC 292123	Clear	Clear	Clear	Clear / 4 colonies (MBC)	Clear / 12 colonies	Clear / 27 colonies (MIC)	Turbid / TNTC	Turbid / TNC

* Clear = no growth. Turbid = growth (Visibility assessment)
* The lowest concentration at which the incubated mixture remained clear after microscopic evaluation was taken as the MIC
* The lowest concentration which had fewer than five colonies was taken as the MBC
* TNTC = Too numerous to count

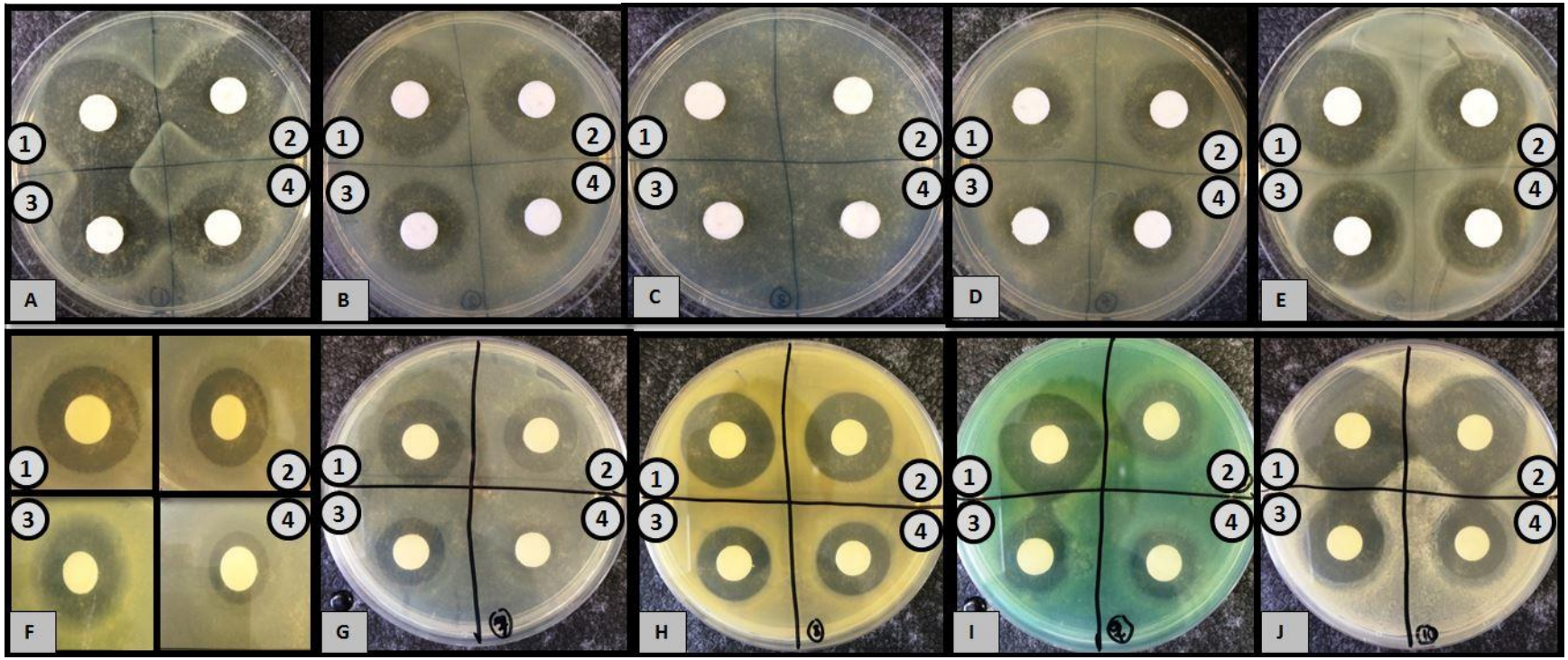


Figure 5.1 Antimicrobial activities of GS derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium shown on plate cultures.

***** Glucan sulphate (GS) concentrations were labelled as number 1 = 500 mg/mL, 2 = 400 mg/mL, 3 = 300 mg/mL and 4 = 200 mg/mL, respectively. Various bacteria were labelled as A = *Escherichia coli* EPIC S17, B = *Salmonella* BA54 SL1344 (pSsaG), C = *Listeria monocytogenes*, D = *Shigella sonnei* 20071599, E = MSSA ATCC 292123, F = *Salmonella enteritidis*, G = *Escherichia coli*, H = *Staphylococcus aureus*, I = *Pseudomonas aeruginosa*, J = *Staphylococcus epidermis*. The experiment was repeated 3 times to ensure reproducibility of the data.

5.9.2 Antimicrobial screening using 96-well plates

The antimicrobial activity of chosen bacteria including *K. pneumoniae* ATCC 13883 and *M. marinum* ATCC BAA 535 were tested via 96-well microtitre plates (*in vitro*) to assess the antimicrobial effects of GS. Overall, the results showed some clear inhibition of growth of both these test species (Table 5.3). The Gram-negative *K. pneumoniae* ATCC 13883 exhibited a survival of 52.8 ± 5.66 % (at 500 µg/mL and 24 h incubation) while the acid-fast bacteria *M. marinum* ATCC BAA 535 gave a survival value of 65 ± 3.39 % (at 100 µg/mL and 24 h incubation) compared to positive growth controls.

Table 5.3 Activity of GS derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium using 96-well microtitre plates.

Bacteria	*Klebsiella pneumoniae* ATCC 13883	*Mycobacterium marinum* ATCC. BAA. 535	Gentamicin	DMSO
Gram	(-)	Acid-fast bacteria		
GS (µg/mL)	500	100	100	100
Survival ratio [a] (% of control)	52.8 ± 5.66	65 ± 3.39	1 ± 1.20	99 ± 1.05
Inhibition status	Positive	Positive	(+) control	(-) control

[*] Values represent averages ± standard deviations ($P < 0.05$) for triplicate experiments
[a] Lower percentage of control value means greater antibacterial effect

Due to the significant and increasing occurrence of nosocomial infections and destructive changes to human lungs as mentioned by Daligault et al., (2014) antibiotic-

resistant *K. pneumoniae* ATCC 13883, the possibility of using novel antimicrobials from natural sources such as GS extracted from *G. lucidum* merits further investigation and refinement. Meanwhile, GS might have some potential in controlling the occurrence of common granulomatous diseases arising from *M. marinum* ATCC BAA 535 that affect individuals who work with fish or keep aquaria as described by Slany (2012).

Overall, at present it is not entirely clear what the mechanism(s) of the antimicrobial activity of a sulphated polysaccharide such as GS is likely to be, as there are few studies in this area, meanwhile the G was negative in terms of antibacterial impact (results not shown). The steric and repulsive electrostatic properties of sulphate groups and how these might alter the spatial construction of the glucan were proposed by Ji et al., (2012) as a possible contributor to the observed properties of GS. Others suggested that changes in the flexibility of the polysaccharide backbone, and the altered water solubility could lead to variations in biotic response (Bao et al., 2002; Ellington et al., 2010; Skalicka-Wozniak et al., 2012), which may also include the antimicrobial effects. However, no single clear mechanism for this is proposed.

Slany et al., (2012) discuss the impact of sulphation on structure and biological activity. In general, the sugar chain confirmation becomes modified by the process of sulphation such that non-covalent bonds form more readily when the –OH groups in a β-glucan element are replaced with sulphate groups. Similarly, repulsions between the anionic groups lead to elongation of the sugar chain. They propose that these events result in the polymer developing an active conformation, thus initiating the bioactivity surge. Hence, this might be the proposed mechanism for these antimicrobial reactions.

In the last 20 years, there have been insufficient reports on antimicrobial activities of biopolymers from *Ganoderma* species (Daligault et al., 2014; Heleno et al., 2013; Li et al., 2012). This genus has been commonly considered for its therapeutic properties, but less widely explored as a source of novel antibacterial agents (Gao et al., 2003; Heleno et al., 2013). However, certain polysaccharides from *Ganoderma* species employ antibacterial activity by hindering the growth of bacteria and, in some events, by eliminating pathogenic bacteria (Skalicka-Wozniak et al., 2012). Nearly all antibacterial investigations on *Ganoderma* species have been accomplished on the fruiting body and not on extracts from the liquid cultivated mycelium, a point which is made strongly in the recent review by Ferreira et al., (2015b). Meanwhile, most of the positive antibacterial compounds were from alcoholic extracts, hot-water extracts and triterpenoids of fruiting bodies. The current work is the first to show positive results using GS extracted from *G. lucidum* mycelium (pellets) produced in the bioreactor.

In the present study, GS has shown antimicrobial activity against a varied range of bacteria in test systems. The mechanisms of sulphation on glucan structure were proposed for these positive reactions. Consequently, it is essential to further studies in order investigate polysaccharide structure-activity relationships, which might deliver a detailed foundation for polysaccharide development and improvement.

5.9.3 Comparison of GS with the previous work

From the year 1994 to 2015, there were only a few reports on antimicrobial activities of polysaccharides from *Ganoderma* species (Table 5.4). This table depicts the comparison on the positive antimicrobial affects from the different source of countries, origins, extractions, bacteria strains tested, and the species type. Most of the reported studies were from Asian countries and there were only four previous works from European's *Ganoderma* (Poland, Portugal, and German) including the current work (France). Meanwhile, active antibacterial compounds were mostly from alcoholic extracts, hot-water extracts and triterpenoids. In the light of other people's work, the current work was the first to show significant results using GS which was extracted from *G. lucidum* mycelia produced in the RBF cultures (Chapter 3).

As can be observed in the Table, all polysaccharides from the mycelia and basidiocarp of *Ganoderma* species were found to possess activity against the common Gram positive and Gram negative bacteria include *E. coli*, *Bacillus cereus*, *Staph. aureus*, *P. aeruginosa*, *Staph. epidermis*, *S. typhimurium* and *K. pneumoniae*. The current GS has managed to inhibit the growth of acid-fast bacteria *M. marinum* ATCC BAA 535 and Gram negative *Shigella sonnei* 20071599, which added to the range of species reported to be inhibited by *G. lucidum* derived materials.

Table 5.4 Reports on the current positive antimicrobial effects from *Ganoderma* species worldwide.

Species type / Compound used/ Origin	Source	Common pathogenic microorganism	Compound/ Reference
G. lucidum 31549 (France, Europe)	Mycelia (RBF in the bioreactor)	*Staph epidermis, P. aeruginosa, Staph aureus, E. coli, S. enteritidis, E. coli* EPIC S17*, Salmonella* BA54 SL1344 pSsaG*, L. monocytogenes, S. s* 20071599, MSSA ATCC 292123, *K. pneumonia* ATCC 13883, *M. marinum* ATCCBAA.535	Glucan sulphate (Wan Mohtar *et al.*, 2015) Current work
G. lucidum (India, Asia)	Fruiting bodies	*B. cereus* 430*, Enterobacter aerogenes* 111, *E. coli* 1687, *P. aeruginosa* 2453	MetOH extract (Jonathan and Awotona, 2013)
G. lucidum (Portugal, Europe)	Fruiting bodies	*Staph aureus, B. cereus, Micrococcus flavus, L. monocytogenes, P. aeruginosa, S. typhimurium, E. coli, Entero cloacae*	Glucoronide (Heleno et al., 2013)
G. atrum (China, Asia)	Fruiting bodies	*Staph aureus* subsp. *Aureues*, *E. coli*, *B. substilis*, *Proteusbacillus vulgaris*	EtOH extract (Li et al., 2012)
G. lucidum (India, Asia)	Mycelia (Shake flask)	*E. coli* MTCC741*, Staph aureus* MTCC96*, Proteus sp., B. subtilis* MTCC121*, P. aeruginosa* MTCC741*, Klebsiella sp., B. cereus*	EtOH extract (Skalicka-Wozniak et al., 2012)
G. lucidum *G. applanatum* *G. austral* (Nigeria, Africa)	Fruiting bodies	*E. coli*, *P. aeruginosa*, *Pro mirabilis*, *K. pneumonia*, *Staph aureus*, *B. cereus*, *Actinomycetes* spp.	EtOH, MetOH and H_2O extract (Jonathan and Awotona, 2013)

G. mazandaran *G. lipsiense* *G. multicornum* *G. lucidum* (Iran, Middle East)	Fruiting bodies	*Pro mirabilis* MTCC 1420, *Candida albicans* MTCC 1637, *B. subtilis* NCIM 2011	Sesquiterpene (Sharifi et al., 2012)
G. lucidum (Poland, Europe)	Fruiting bodies	*Staph epidermis* ATCC 12228, *Staph aureus* ATCC 25923, *B. subtilis* ATCC 6633, *Micrococcus luteus* ATCC 10240, *E. coli* ATCC 25922, *K. pneumonia* ATCC 13883, *P. aeruginosa* ATCC 13883, *Pro mirabilis* ATCC 12453	Hot H_2O-MetOH extract (Kamra and Bhatt, 2012)
G. lucidum (India, Asia)	Fruiting bodies	*B. subtilis, Enterococcus faecalis, L. monocytogenes, Strep mutans, K, pneumonia, Pro vulgaris, S. typhimurium, P. aeruginosa*	Aqueous and MetOH extract (Kamra and Bhatt, 2012)
G. lucidum *G. praelongum* *G. resinaceum* *(*India, Asia)	Fruiting bodies	MRSA	Sesquiterpene (Ferreira et al., 2015b)
G. formosanum (Taiwan, Asia)	Mycelia (Bioreactor)	*L. monocytogenes*	EtOH extract (Ellington et al., 2010)
G. lucidum (China, Asia)	Fruiting bodies	*Erwinia carotovora*, *Penicillium digitatum*, *Botrytic cinerea*, *B. cereus*, *B. substilis*, *E. coli*, *Aspergillus niger*, *Rhizopus nigricans*	Hot H_2O extract (Daligault et al., 2014)

G. lucidum (India, Asia)	Fruiting bodies	MRSA	Hot H_2O extract (Prasad and Wesely, 2008)
G. applanatum (India, Asia)	Fruiting bodies	*Acitenobacter aerogenes, Acrobacter aerogenes, Arthrobacter citreus, B. brevis, B. substilis, Corynebacterium insidiosum, E. coli, Pro vulgaris, Clostridium pasterurianum, Micrococcus roseus, Mycobacterium phlei, Sarcina lutea, Sta aureus*	Not available (Slany et al., 2012)
G. colossum *G. resinaceum* *G. lucidum* *G. boninense* (Nigeria, Africa)	Fruiting bodies	*B. substilis* IMI347329, *P. syringae* ATCC19310	MetOH extract (Ofodile et al., 2005)
G. pfeifferi (German, Europe)	Fruiting bodies	*Staph aureus* ATCC 6538, *Staph aureus* ATCC 25923, *Staph aureus* ATCC 25913, *Staph aureus* SG 511, *B. subtilis* SBUG 14, *Micrococcus flavus* SBUG 16, *E. coli* SBUG 13, *Pro mirabilis* SBUG 47, *Serratia marcescens* SBUG 9	Ganomycin (Mothana et al., 2000)
G. lucidum (Korea, Asia)	Fruiting bodies	*Micrococcus luteus* ATCC 9341, *Pro vulgaris* ATCC 27853, *E. coli* ATCC 25922, *B. anthracis* ATCC 6603, *B. cereus* ATCC 27348, *B. substilis* ATCC 6633, *Staph aureus* ATCC 25923, *K. oxytoca* ATCC8724, *K. pneumoniae* ATCC 10031, *S. tompson* ATCC 10256, *S. typhi* ATCC 6229, *S. typhimurium* ATCC 14028, *Serratia marcescens* ATCC 27117,	Aqueous-extract (Yoon et al., 1994)

5.10 Antifungal activity of GS on *A. niger* A60

The antifungal screening was done on the supplied *A. niger* A60 as the previous work on *G. lucidum* extract from fruiting bodies showed it has the highest antifungal activity on *A. niger* (Heleno et al., 2013). Therefore, the antifungal activity of the current glucan sulphate (GS) on *A. niger* A60 is reported in Table 5.5 with their growth morphology (elongated fungal mycelial filaments in Fig. 5.2). GS showed antifungal activity against the test fungi with MIC of 60 mg/mL and MFC of 100 mg/mL. Other compounds present in *Ganoderma* species have also been reported as antifungal, such as three triterpenes (Applanoxidic acids A, C and F) sourced from decayed wood of *G. annulare* (Smania et al., 2003), fruiting bodies extract of *G. lucidum* (Heleno et al., 2013) and Ganodermin sourced from *G. lucidum* fruiting bodies (Wang and Ng, 2006). Nevertheless, as far as the present author is aware this is the first report on antifungal activity of any possible sulphated polysaccharides (GS) derived from extended batch cultures of *G. lucidum* pellets due to sulphation effect (Chapter 4).

Table 5.5 Antifungal activity (MIC and MFC, mg/mL) of glucan sulphate derived from extended batch cultures *G. lucidum* BCCM 31549 mycelium.

Fungi	GS level (mg/mL)		Bifonazole 2 mg/mL	DMSO
	MIC	MFC		
Aspergillus niger A60	60	100	(+) control	(-) control

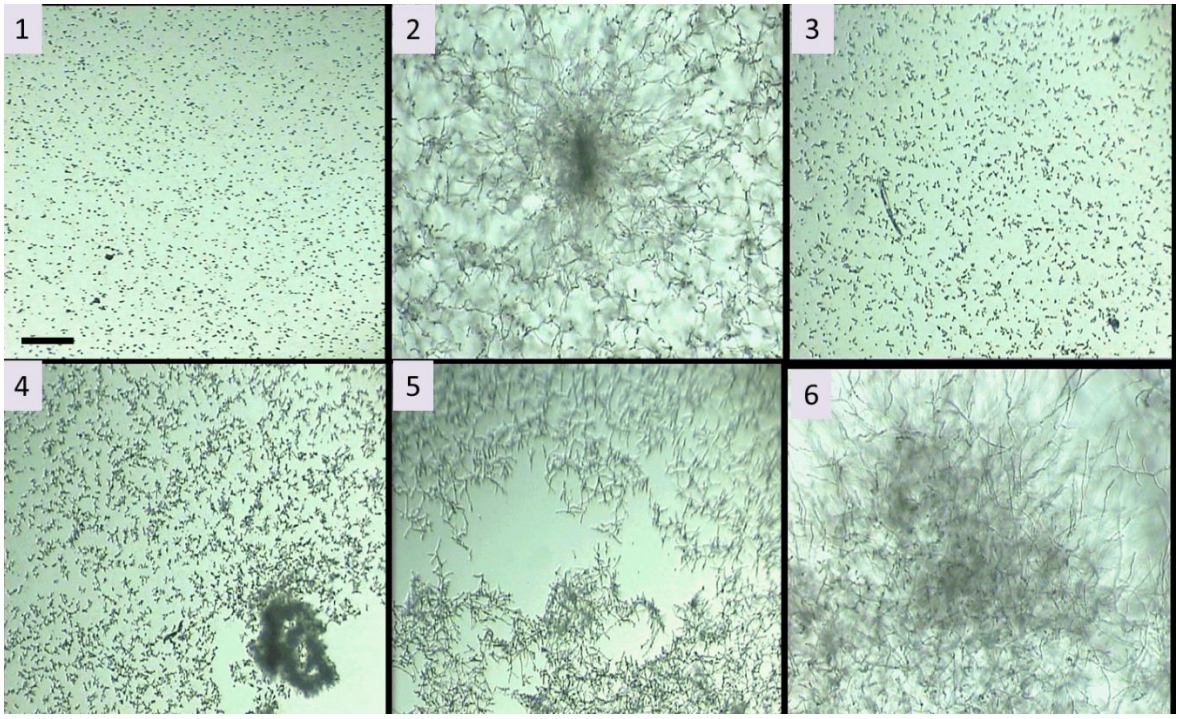


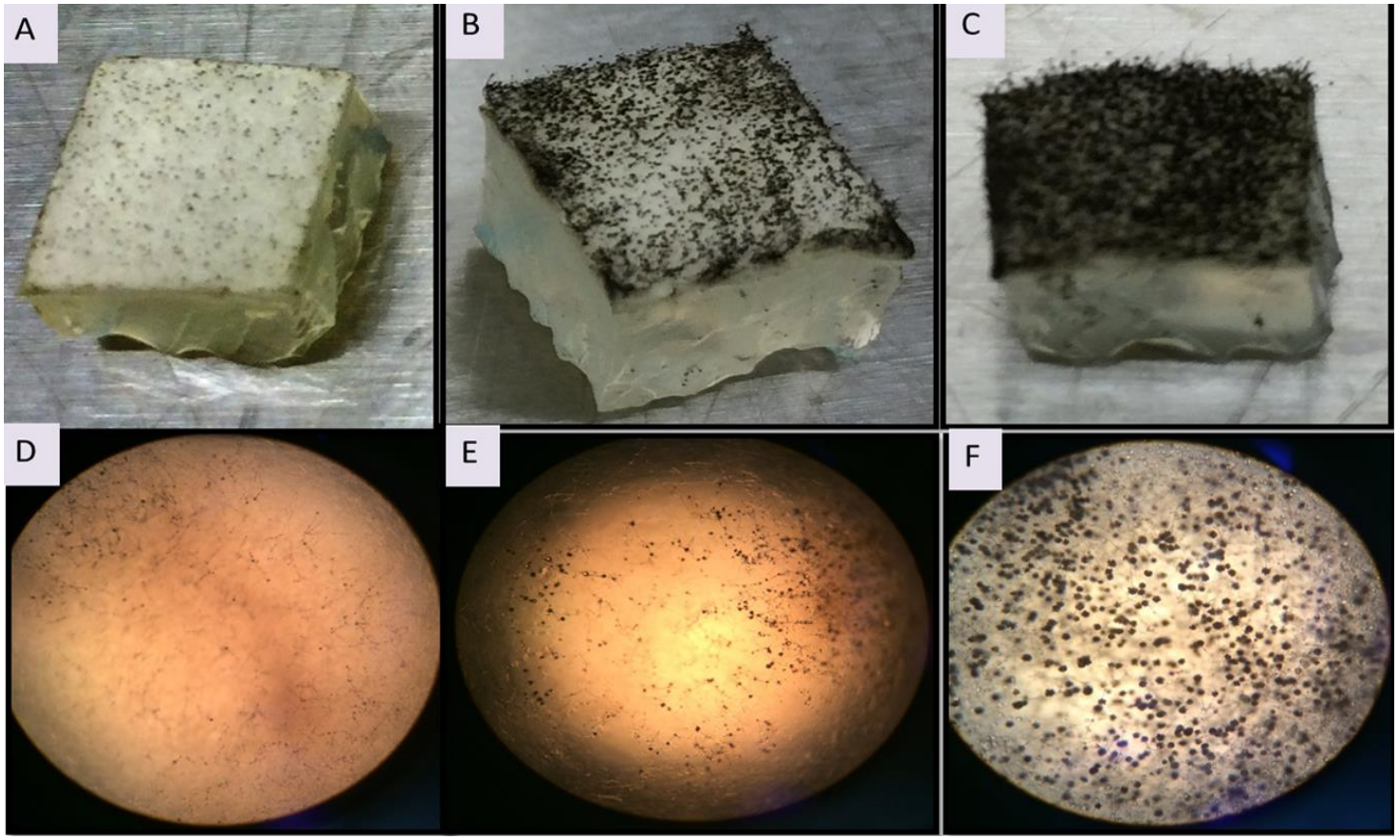
Figure 5.2 Growth morphology of *A. niger* A60 antifungal inhibition by glucan sulphate (GS) derived from extended batch cultures of *G. lucidum* BCCM 31549 pellets. (1) Positive control reaction with Bifonazole at 2 mg/mL; (2) Negative control reaction with 5% DMSO solution; (3-6) Positive antifungal treatment reactions with glucan sulphate (GS: mg/mL) at (3) 200, (4) 100, (5) 60, and (6) 30. Images were taken at 4-fold magnification using a Compound Microscope. Bar = 150 µm

* Heavier filaments growth indicates lesser antifungal reactions

5.10.1 Demelanising responses of GS on *A. niger* A60

Melanin plays an important role in fungal virulence and has been denoted to as 'fungal armour' due to the ability to shield against a broad range of toxics (Gomez and Nosanchuk, 2003). Therefore, by breaking the fungal armour (demelanisation) would suppress the growth of desired pathogenic fungi (such as *A. niger*). The demelanising activity of GS was investigated using *A. niger* A60. The results were expressed as minimum demelanising concentration (MDCs), which were defined as sub-inhibitory

and sub-lethal concentration essential to aggravate demelanisation in the fungus during a 72 h time period. The sub-inhibitory level was attained at 30 mg/mL, whereas sub-lethal concentration was obtained at 60 mg/mL of GS (Fig. 5.3, image A-F).



** Black conidiophores indicate weaker demelanising reactions*

Figure 5.3 (A) Demelanised mycelium of *A. niger* A60 stimulated with GS at 60 mg/mL; (B) Demelanised mycelium of *A. niger* A60 stimulated with GS at 32.5 mg/mL; (C) Normal mycelium of *A. niger* A60 without stimulation; (D) Culture of *A. niger* A60 with few amount of heads, stimulated with GS at 65 mg/mL; (E) Culture of *A. niger* A60 with smaller amount of heads stimulated with GS at 30 mg/mL; and (F) Typical culture of *A. niger* A60 with numerous heads. (D-F) Images were obtained under light microscope. Glucan sulphate (GS) was derived from the extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

In a previous study, the coloured conidiophores of some *Aspergillus* species were revealed to comprise pigments belonging to the group of melanins: a green coloured chromo-protein and a black insoluble pigment (Eisenman and Casadevall, 2012). The production of melanin by *A. niger* contributes to the virulence of pathogens of humans as well as those of food crops (Rosa et al., 2010), providentially the current work may suggest the cause of positive demelanisation on these pigments.

It has been shown that melanin has a vital role in the protection of the fungus against immune effector cells; it is able to scavenge reactive oxygen species generated by alveolar macrophages and neutrophils of the host (Brakhage and Liebmann, 2005). Morphological changes during melanisation of *A. niger* are obvious in Fig. 5.3 and represent depigmentation; samples were treated with GS at MDC of 30 and 60 mg/mL, respectively. Observing morphological variations of conidiophores, it was evaluated that demelanised cultures of tested fungi interestingly possessed reducing number of heads (Fig. 5.3 A and B) in comparison to those in untreated culture (Fig. 5.3 C). The reduction of head number and demelanisation of *A. niger* A60 spores were observed under light microscope (Fig. 5.3 D and E) and matched to an untreated control (Fig. 5.3 F). Thus, we may presume that the GS of *G. lucidum* might directly be involved in the inhibition or modification of the mechanism of demelanisation. The results for demelanising activity are important for the current work, since MDC is sub-lethal to fungus, in contrast to inhibitory and fungicidal doses.

5.10.2 Comparison with previous antifungal reactions

When compared with the previous *A. niger* antifungal work by Heleno (2013), the current values for MIC, MFC, and MDC (Table 5.5 and Fig. 5.3) were higher with the same pigmentation, shape and size microscopically. However, Fagade and Oyelade (2009) in their study on testing ethanolic extracts of *G. lucidum* on various microorganisms found that the MIC of *Candida albicans* was 750 mg/mL which is higher compared to current MIC (60 mg/mL) on *A. niger* A60. These showed the selectivity of the GS on other potential untested fungi. It can be suggested that the GS may be suitable for treating Candidiasis (caused by *C. albicans*) infections compared to Aspergillosis (caused by *A. niger*).

Nevertheless, when compared with the earlier medicinal application which used antifungal toothpaste containing *G. lucidum* against *C. albicans* at sensitivity concentrations of (mg/mL): 2, 4, 8, 16, 31.25, 62.5, 125, 250, and 500 (Nayak et al., 2010), the current tested concentrations [mg/mL: 200, 125, 100, 60, 30, 20, 15, 10, 2, and 1] are approximately similar thus suggesting the suitability of GS for future bioproducts industry. To the best of our knowledge, the current GS derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium was the latest compound possessing demelanising activity against *A. niger* A60. Further improvement on this GS such as purification, fractionation, food additives, clinical and animal studies would be beneficial.

5.11 Cell culture

The cells that were used in this study are presented in Fig. 5.4 include two cancerous cells: (ZR-75-1) Human Caucasian Breast Carcinoma and (U937) Human Caucasian Histiocytic Lymphoma with one differentiated-macrophage D-U937 (Macrophages derived from Human Caucasian Histiocytic Lymphoma). The healthy cells were Human Prostate Normal Cell (PNT2A) and Macrophage Cell Line (RAW264.7). All of the cultured cells were at their optimised cell density according to the supplier's manual (Appendix 5B). The pictures were taken at 4X magnification.

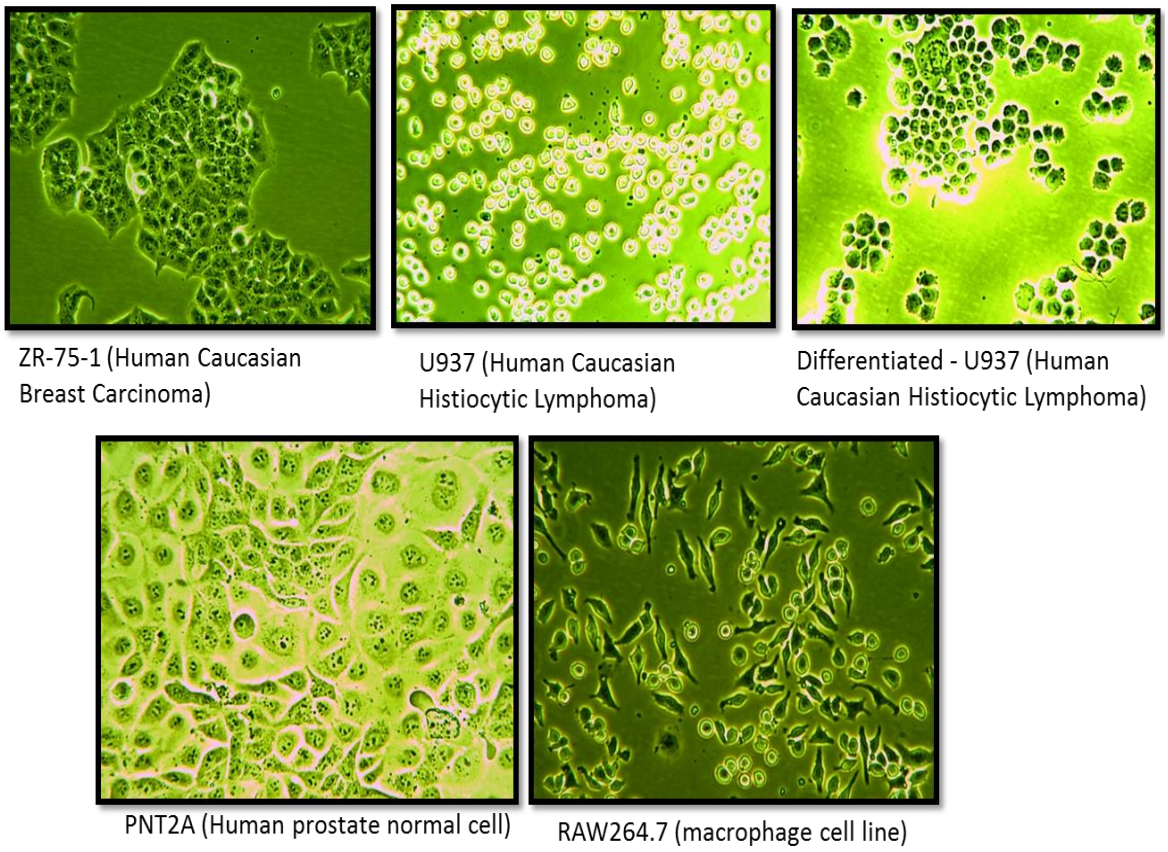
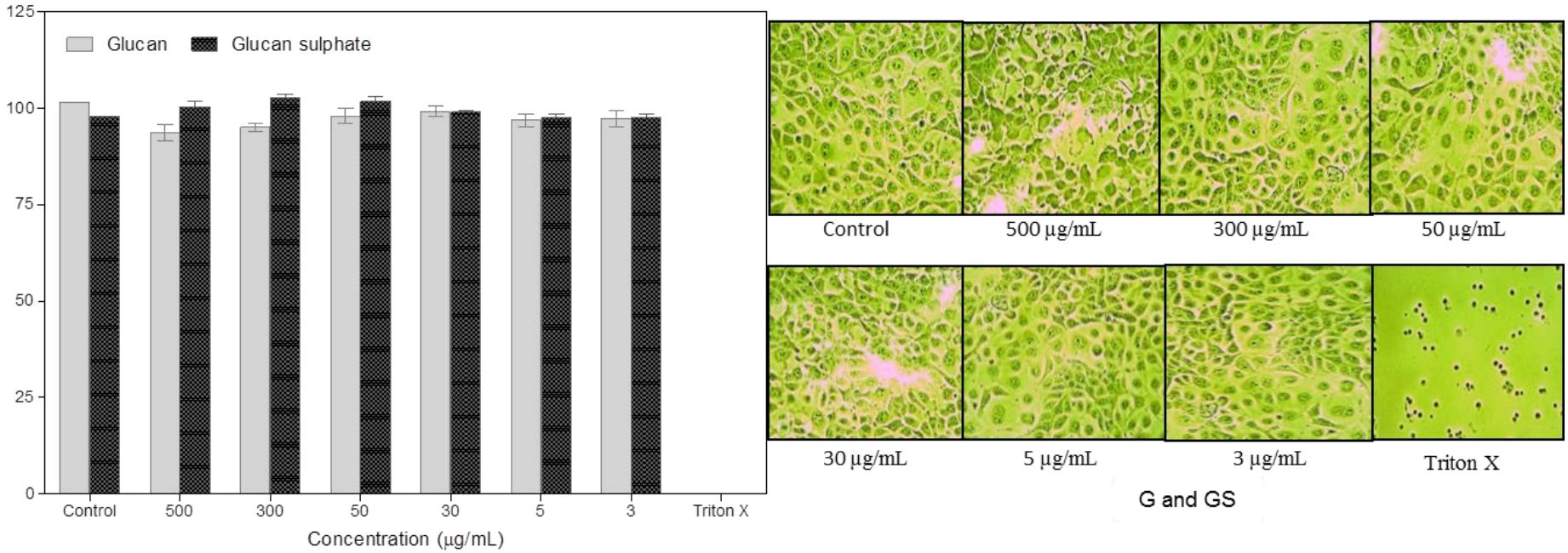


Figure 5.4 Diagram of used working cells in the current cell culture study. Images were taken at 4X and the cells were at their optimised growing density.

5.11.1 Cytotoxicity on healthy cells

The current extracted and processed sulphated β-glucan (GS) from β-glucan (G) was apparently reactive against pathogenic bacteria. Yet, to ensure whether G and GS might have clinical impact on healthy patient cells and before their introduction as new antimicrobial drugs, some preliminary assessment of the impact of such biomolecules upon normal host cells is of interest. Likewise, assessment of the effects of such derivatised polymers on tumours is of value given the widely reported impact of other fungal macromolecules on such cell types. Accordingly, in the present study cytotoxicity assays using alamarBlue® reagent were carried out on healthy human prostate cells (PN2TA). The *in vitro* effects of both GS and G on PN2TA were examined in the present study (Fig. 5.5).

In this study, a series of dose-response assays were performed to determine the cytotoxic effects in PN2TA. Once the cells exposed to different treatments of GS and G (3, 5, 30, 50, 300 and 500 µg/mL) for 24 h, and the alamarBlue® reagent assay displayed no loss of cell viability. Morphological observations of the treated cells in the Figure were the same as the control cells, therefore; these data indicated that GS and G did not exhibit cytotoxicity in normal human cell. When compared with the previous work by Li et al., (2012), the ethanol soluble extract from *G. atrum* did not reacted on the viability of healthy cells, thus confirming the clinical safety of *Ganoderma* spp extracts from the current strain.



**Both G and GS have the same morphological observation under the microscope at 10x magnification*

Figure 5.5 Cytotoxicity effects of both glucan (G) and glucan sulphate (GS) derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium in normal human Prostate-cell-line (PN2TA). After the cells were incubated with G and GS treatments [Control, 500, 300, 50, 30, 5, 3, Triton X µg/mL], the viability was measure by alamarBlue® assay. Each data was presented mean ± S.D, and the P value was > 0.05 when compared to control. If the error bars do not appear then, they are less than the size of the icon or symbol.

5.11.2 Cytotoxicity on cancerous cell lines

The cytotoxicity of G and GS against the development of cancer cells (U937 and ZR-75-1) were examined using the alamarBlue® reagent in this study. As revealed in Fig. 5.7 (U937 cells), GS displayed a dose-dependent antiproliferative reactions within the concentration range of 10 - 60 µg/mL and exhibited stronger inhibition than G (Fig. 5.6). In Fig. 5.7, GS showed the strongest inhibitory effect at 60 µg/mL with approximately 40% inhibition compared to 10% for G, as the Figure shows the fewest cell growth with ascending growth towards lower concentrations. As reported, it demonstrates that the inhibitory activity of cancer cell growth was enriched by the sulphation process (GS) as matched to the unprocessed glucan (G) (Bao et al., 2010; Wang et al., 2009).

Unfortunately, there were no anticancer reactions on breast cancer cells (ZR-75-1) for both G and GS compounds (Fig. 5.8), thus showing the selectivity of GS on U937 cancer cells. This shows the preferences of GS on the characteristic of U937 cells which is non-adherent compared to the adherent type of ZR-75-1 breast cancer cells. Yet, this is intriguing for the GS compound, as Singh et al., (2010) reported that cancer cells in a non-adherent behaviour are highly drug resistant compared to their adherent counterparts (less resistance).

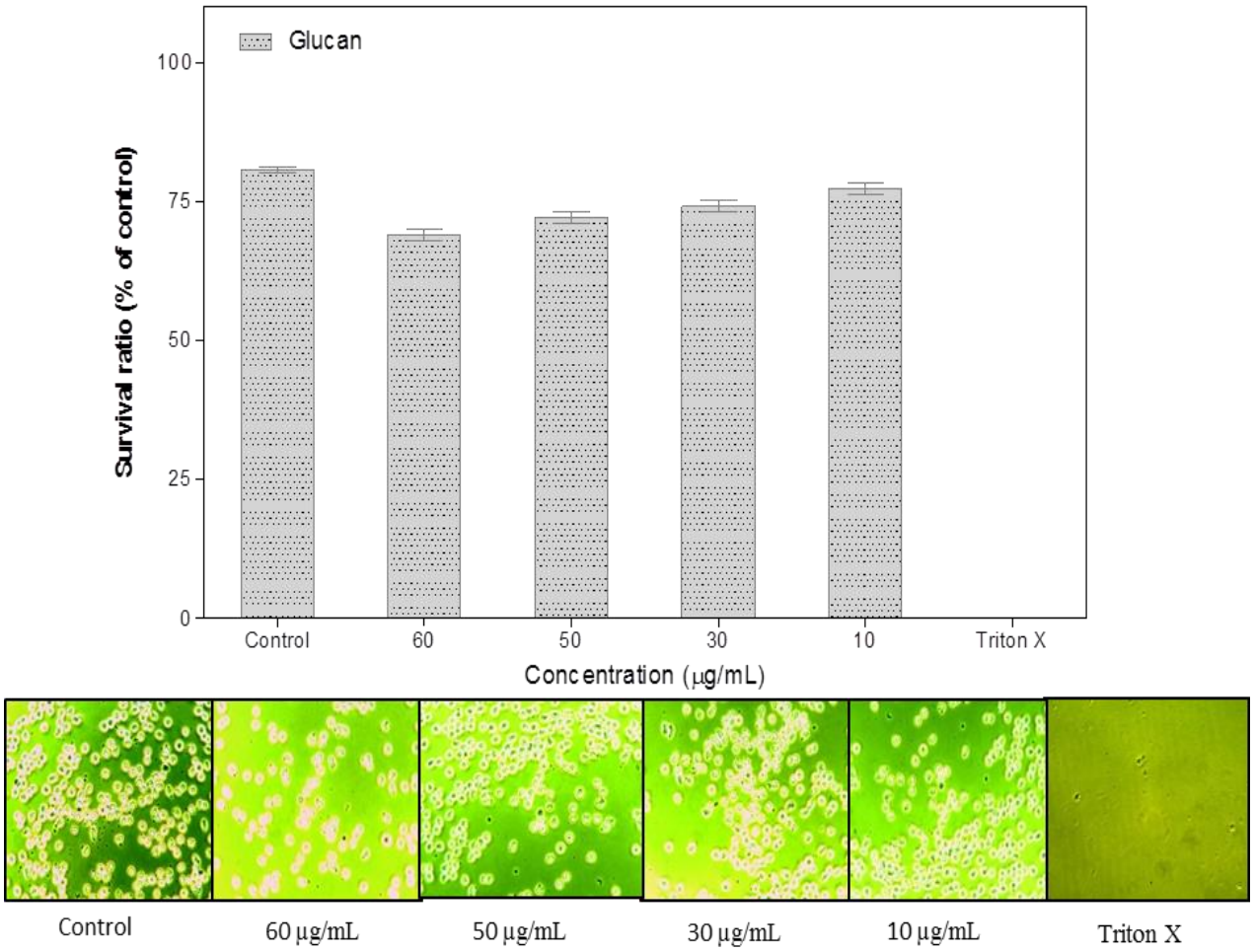


Figure 5.6 Cytotoxicity effects of glucan (G) derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium against cancerous human Caucasian-Histiocytic-lymphoma cell line U937 from a 37-year-old male patient. After the cells were incubated with G treatments [Control, 60, 50, 30, 10, Triton X µg/mL], the viability was measured by alamarBlue® assay. G had morphological observation under the microscope at 10x magnification. Each data was presented S.D ± mean, and the P value was < 0.05 when compared to control. If the error bars do not appear then, they are less than the size of the icon or symbol.

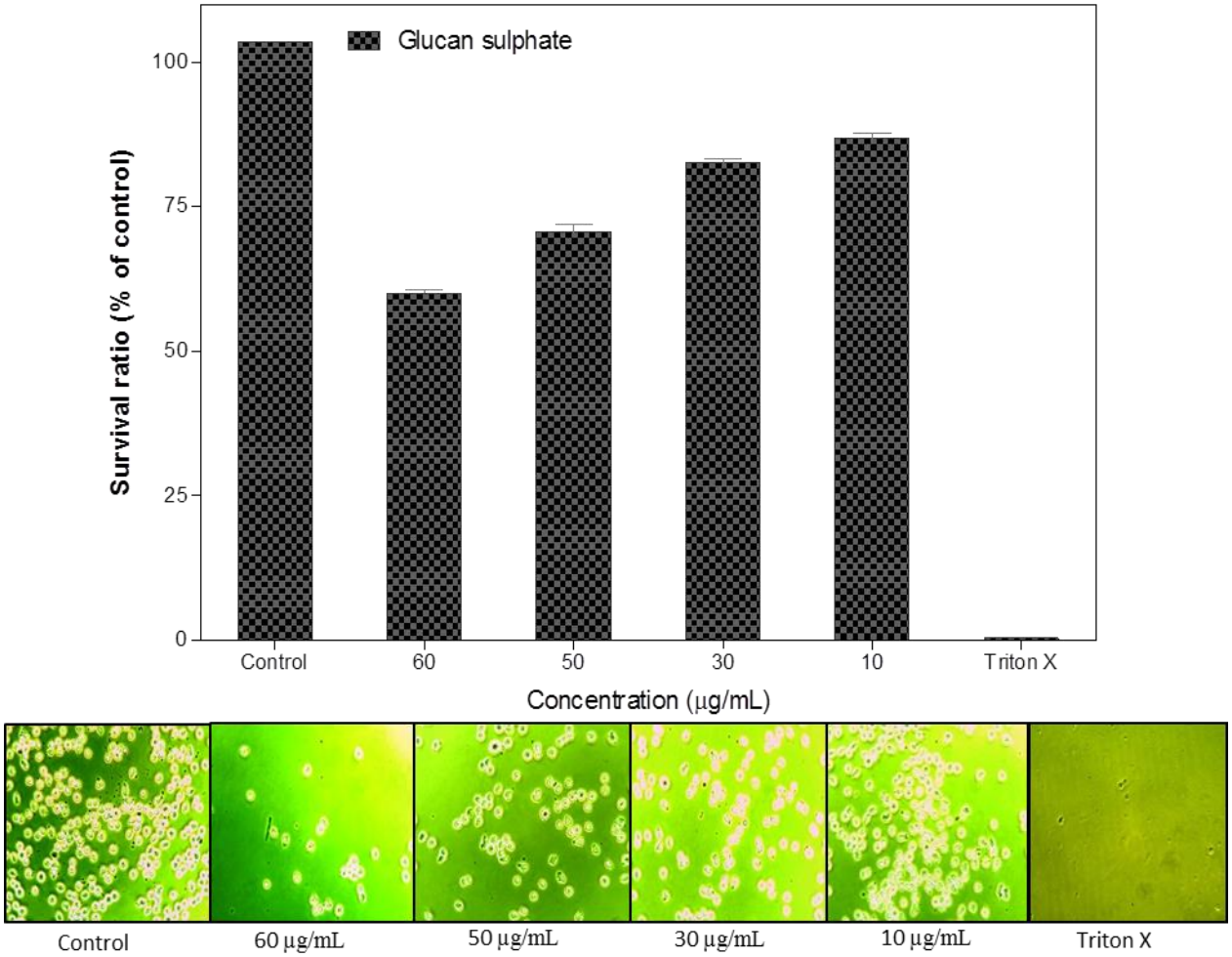
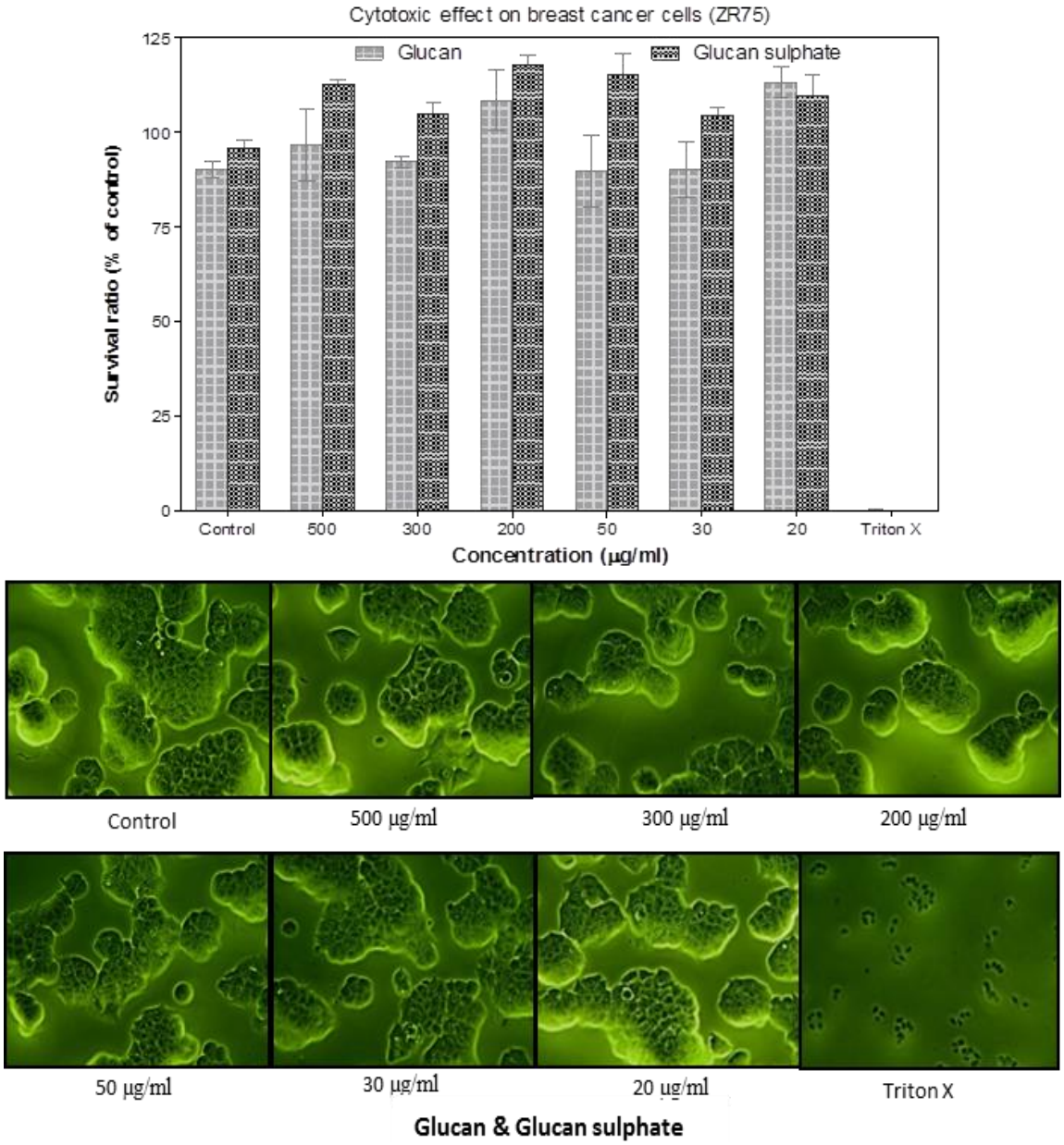


Figure 5.7 Cytotoxicity effects of glucan sulphate (GS) derived from glucan (G) against cancerous human Caucasian-Histiocytic-lymphoma cell line U937 from a 37-year-old male patient. After the cells were incubated with GS treatments [Control, 60, 50, 30, 10, Triton X µg/mL], the viability was measured by alamarBlue® assay. GS had morphological observation under the microscope at 10x magnification. Each data was presented S.D ± mean, and the P value was < 0.05 when compared to control. If the error bars do not appear then, they are less than the size of the icon or symbol.



** Negative reactions of both glucan and glucan sulphate resulted with the same morphological observation under the microscope at 10x magnification*

Figure 5.8 Cytotoxic of both glucan (G) and glucan sulphate (GS) derived from extended batch cultures of *G. lucidum* BCCM 31549 mycelium against breast cancer cells (ZR-75-1). After the cells were incubated with G and GS treatments (Control, 500, 300, 200, 50, 30, 20, Triton X µg/mL), the viability was measure by alamarBlue® assay. Each data was presented mean ± S.D, and the P value was < 0.05 when compared to control. If the error bars do not appear then, they are less than the size of the icon or symbol.

The current concentration of GS applied is considerably lower than that used in the earlier study on of sulphated glucan (sourced from *Hypsizigus marmoreus*) which showed only 39% of antiproliferative activity at 1000 µg/mL (Bao et al., 2010), thus further concentration increment for the current work (60 µg/mL with 40% inhibition) would be highly beneficial. As reported, the molecular weight (MW), chemical configuration, degree of branching, and structure of the polymeric backbone were crucial for antiproliferative activities stimulation for both glucan and sulphated glucan (Ma et al., 2013). Therefore, the biochemical aspects and mechanism of the antiproliferative reactions stimulated by GS from pellets of *G. lucidum* is still not fully unspoken and requests further study.

5.11.3 Effect of G and GS on NO production in macrophages

Macrophages are the main host protection against bacterial infection and cancer growth (Chiong et al., 2013; Liu et al., 2014), thus play an imperative role in the initiation of adaptive immune responses (Zhang et al., 2002) caused by a compound namely nitric oxide (NO). NO is one of the cytokines released by stimulating agents and is related to cytolytic function of macrophages against a variety of tumours (Daligault et al., 2014). NO is a gaseous molecule synthesised from L-arginine by nitric oxide synthase. It is a highly reactive free radical that can form a number of oxidation products, such as NO_2, NO_2-, N_2O_3, and S-nitrosothiols. NO participates in the physiology and pathophysiology of many systems (Diouf et al., 2009). In addition, it is an important mediator of the non-specific host defence against invading microbes and diseases. Thus, NO can be used as quantitative index of the macrophage activation.

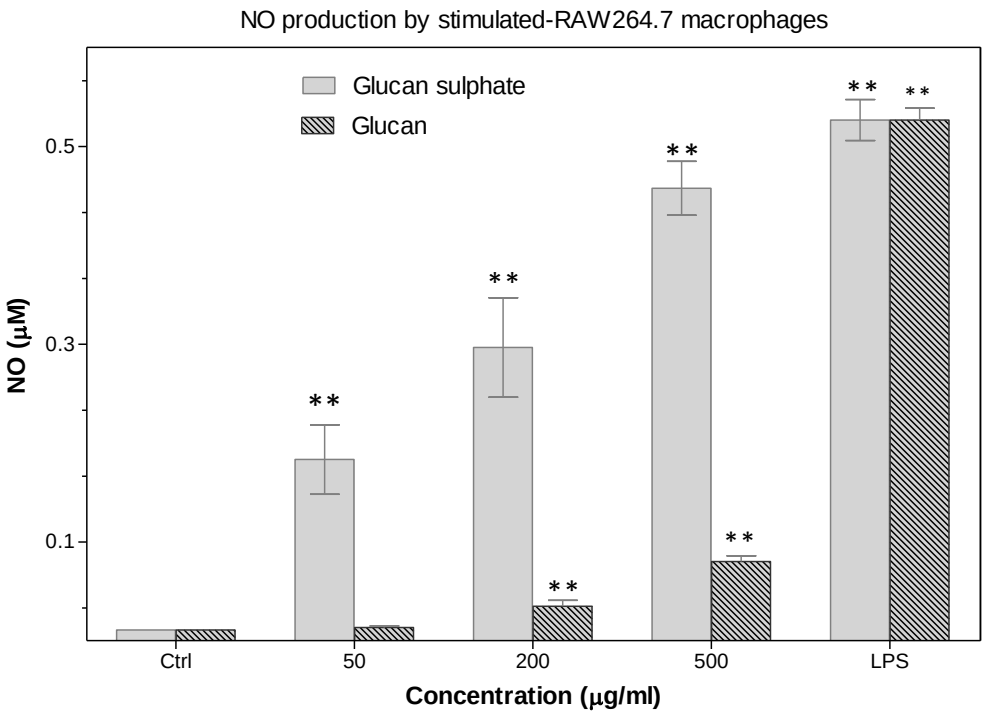


Figure 5.9 Effect of glucan (G) and glucan sulphate (GS) on NO production by macrophages RAW264.7. The cells were treated with different concentrations of compounds or LPS (2 µg/mL) for 48 h and culture supernatants were analysed for NO production. Values are the means ± S.D. of triplicate determinations. ** $P < 0.05$, significant difference from the control group.

Consequently, macrophage-derived NO synthesis could contribute to the antitumour immune response *in vivo* (Yim et al., 1993). From literature, the fruiting body extracts of *G. lucidum* have already shown effect on stimulating macrophages to release NO (Liu et al., 2014) , however the effects on its liquid-mycelial extract both on sulphated (GS) and non-sulphated glucan (G) have not been reported yet. As shown in Fig. 5.9, the current work shows positive NO production from RAW264.7 cells stimulated with GS [500, 200, and 50 µg/mL] for 48 h increased significantly comparing with the untreated controls. In part, GS (0.45 µM, 0.29 µM, and 0.18 µM) was more

reactive than the G (0.08 µM, 0.035 µM, and 0.013 µM) in initiating the macrophages to release NO. As observed, the level of NO secreted from RAW264.7 cells in the presence of the G was very low (< 0.1 µM), indicating its weak ability to trigger macrophages compared to GS. Besides, stimulating with GS increased the NO release to levels comparable to those detected in the positive control (LPS), suggesting that GS might have strong immunomodulatory activity (Bao et al., 2010). These indicated that the GS had obvious effect on stimulating macrophages to release NO.

For clarification, there was no macrophage reactions on the solvent (0.1M NaOH) used for both G and GS indicating the reactions had originated independently from the compounds themselves (Fig 5.10). Meanwhile, the stimulated-macrophage morphological reactions have shown that the GS-stimulated macrophage was similar morphologically with LPS at concentration of 500 µM and 200 µM. The G-stimulated macrophage was barely responded based on their morphology while the solvent proved to be unreactive towards the macrophage cells.

The GS tested in this work was comparable with the previous work as they reported that *G. lucidum* polysaccharides (non-sulphated) sourced from mycelia did stimulate macrophage cells to produce NO (Shi et al., 2013). However, the current compound (GS) in this study was obtained through newly implemented repeated-batch strategy (Chapter 3) with sulphation application (Chapter 4) while Shi (2013) did not explain in detail their fermented mycelia production. Therefore, this has added the value of the current work.

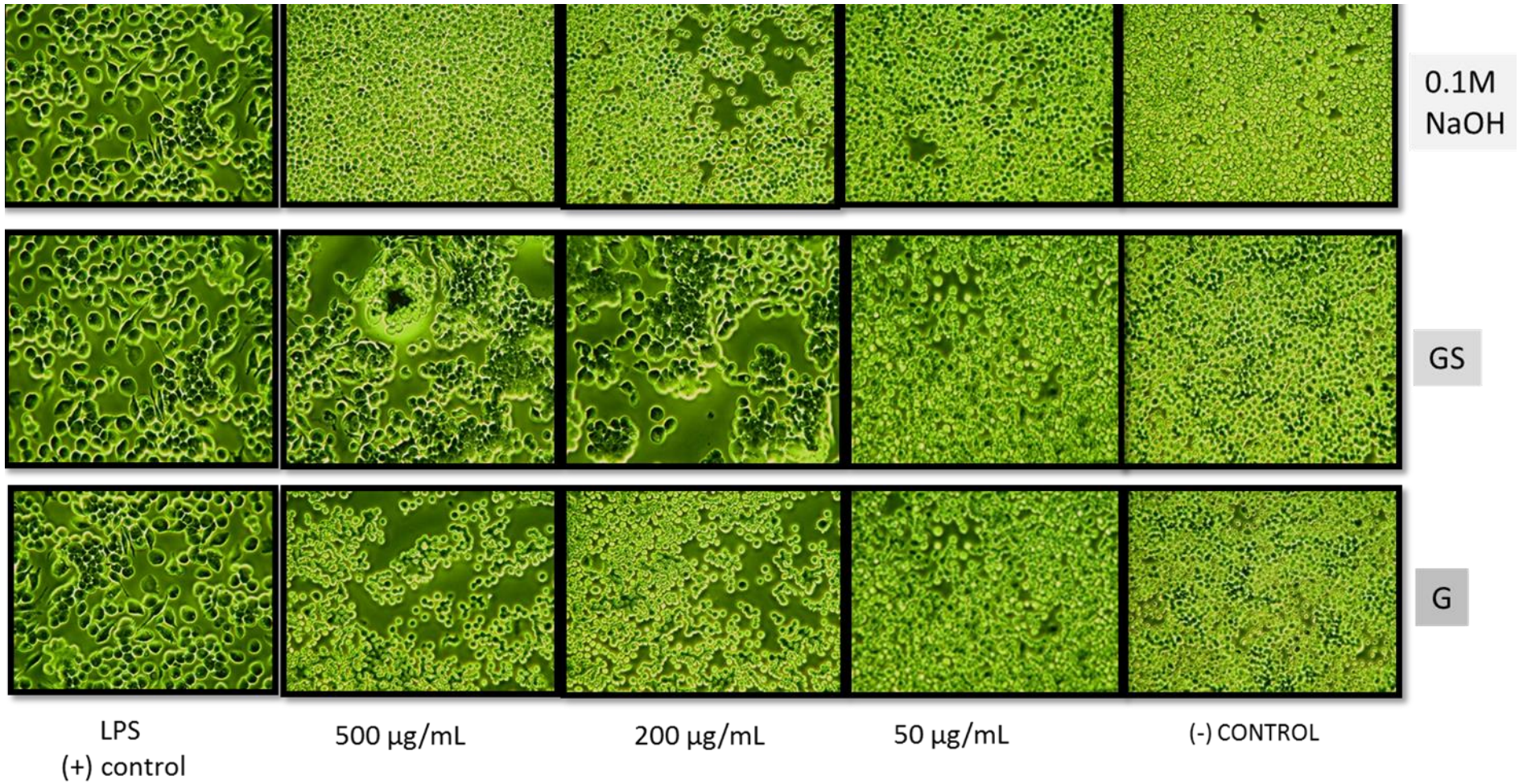


Figure 5.10 Reactions diagram on NO production by stimulated-RAW264.7 macrophages with glucan (G) and glucan sulphate (GS). The 0.1M NaOH was the solvent (indicating negative reaction) for both compounds indicating the positive reaction was independently from the G and GS. All images were at 10x magnification.

To summarise, glucan sulphate extracted from *G. lucidum* BCCM 31549 mycelium, not only has strong antimicrobial, antifungal and considerable cytotoxicity on cancer cells, but also exhibits a substantial immunomodulatory activity by inducing production of NO. Besides, it triggered the possibility of the current research interest in producing immunomodulatory-antibiotics polymer which are defined as antibacterial or antiviral agents which have potential immunomodulating properties (Alkuraishy et al.; Datta et al., 2015; Labro, 2000).

5.12 Relationship of antimicrobial, antiproliferative, antifungal and immunomodulation of GS

Despite impressive therapeutic progresses in the battle against infections, microorganisms are still a threat to mankind. With hundreds of available antibacterial molecules, major concerns remain about the emergence of resistant and multidrug-resistant and multidrug-resistant pathogens, thus the battle is not won. Approximately 15 million people die each year due to infectious diseases which nearly all live in developing countries (Allison et al., 2015). With the improvement on microorganisms' survival rate, more study must be dedicated on the development of host immune defences and antimicrobial drugs that act synergistically to effectively combat new infections. Therefore, many immunomodulating drugs are now on the market and are given in combination with antimicrobial agents.

As mentioned by Llaurado et al., (2015), we have now entered the third era of anti-infective strategy, which centres on the chemistry between active molecules and the immune system. Our current results have pointed out the potential immunomodulating properties of anti-infective agents, beginning with antibacterial, antifungal, immunomodulatory and antiproliferative agents, thus introducing a novel

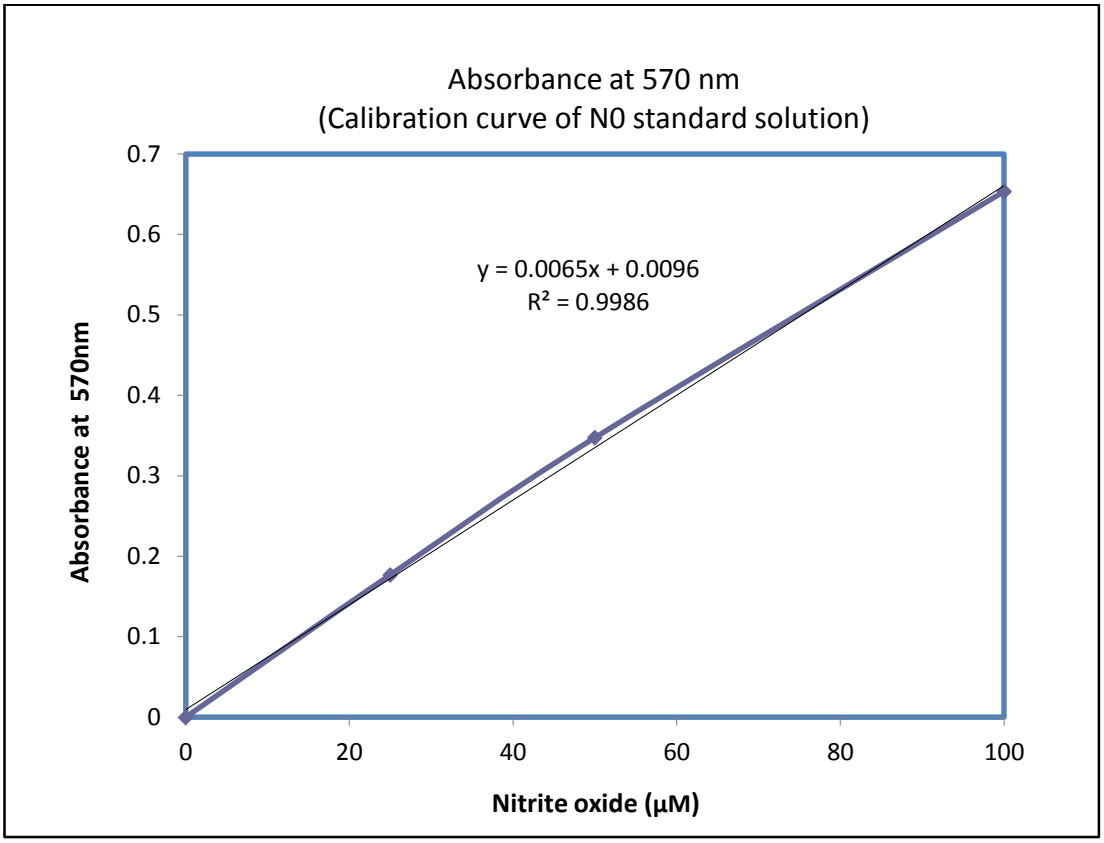
‘’quad-functional’’ approach to the nutraceutical potential of the mushroom species, mainly *G. lucidum* mycelia-sourced polysaccharides. To date, the closest comparison for the current *G. lucidum* reaction was on the hot-water mycelial extract of oyster mushroom possessing ‘’bifunctional’’ approach (antimicrobial-immunomodulatory) (Llaurado et al., 2015).

At present, between 80% and 85% of all edible-medicinal mushroom products are derived from fruiting bodies and only 15% are constructed on extracts from mycelia. The present study suggests that not only *G. lucidum* fruiting bodies but also their mycelia may be a good renewable and easily accessible resource for developing functional foods or even pharmaceutical agents.

5.13 Conclusions

The antimicrobial activity of the glucan sulphate (GS) from *G. lucidum* was effective against tested microbes in both disk-diffusion and *in vitro* assays. In addition, cytotoxicity of GS was evaluated with normal human cells and no such effects were noted at the levels tested in this study. The GS may also have potential in anticancer work based on its inhibition on Human-Caucasian-Histiocytic-Lymphoma cancer cells (U937). GS also shows antifungal-demelanising activity on *A. niger* A60 and has successfully stimulate macrophages to produce NO. These GS activities indicate that sulphate substitution on the glucan not only improved solubility, they also had impact on therapeutic activities, suggesting that sulphation was an effective way to enhance these activities and that the sulphated compound might have a role as a natural additive in many foods with multi-functional benefits (preservative, antifungal, anti-cancer, immune-stimulation).

5.15 Appendix



Appendix 5A: Calibration standard curve of Nitrite Oxide in µM

Appendix 5B: Cell details

	ZR-75-1	U937	PN2TA	RAW264.7
Biological source	Breast from human	Lymphoblast lung from human	Prostate from human	Blood from mouse
Growth mode	Adherent	Suspension	Adherent	Semi-adherent
Karyotype	2n=46, modal no.72	Not specified	Not specified	Not specified
Morphology	Epithelial	Lymphoblast	Epithelial	Macrophage
Cell line origin	Human Caucasian breast carcinoma	Human Caucasian histiocytic lymphoma	Human prostate normal, immortalised with SV40	Mouse monocyte macrophage
Culture medium	RPMI 1640 + 2 mM Glutamine + 1mM Sodium Pyruvate (NaP) + 10% Foetal Bovine Serum (FBS)	RPMI 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS)	RPMI 1640 + 2mM Glutamine + 6 -10% Foetal Bovine Serum (FBS)	DMEM + 2 mM Glutamine + 10% FBS/ FCS (FBS)
Subculture routine	Sub-confluent cultures were split (70-80%) 1:3 and seeded at 3-5x10,000 cells/cm^2 using 0.25% trypsin or trypsin/EDTA; 5% CO_2; 37°C	Cultures were maintained between 2-9x100,000 cells/ml; 5% CO_2; 37°C	Sub-confluent cultures were split (70-80%) 1:3 to 1:10 and seeded at 2-4x10,000 cells/cm^2 using 0.25% trypsin or trypsin/EDTA; 5% CO_2; 37°C	Sub-confluent cultures were split (70-80%) 1:2 to 1:8 and seeded at 2-4x10,000 cells/cm^2; 5% CO_2; 37°C. Cells were removed mechanically
Cell line description	Cells were derived from a malignant ascitic effusion in a 63 year old female Caucasian with infiltrating	Cells were derived from malignant cells of a pleural effusion of 37 year old caucasian male with diffuse histiocytic lymphoma.	Cells were obtained from a prostate of a 33 year old male at post mortem.	Cells were established from an ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV). Growth of cells were inhibited by LPS

CHAPTER 6

EXOPOLYSACCHARIDE PRODUCTION BY *Ganoderma lucidum* BCCM 31549 IMMOBILISED ON POLYURETHANE FOAM

6.0 Introduction

In recent times, a vast range of valuable biopolymers has been extensively exploited from fermented medicinal mushrooms. These mushrooms vary in their structure at different times in their life cycle, are morphologically complex, and present different growth forms in the surface and liquid cultivation. In nature, they have various growth media, physical culture conditions and environment (Rogalski et al., 2006). During their submerged liquid fermentation, the typical morphology ranges from dispersed mycelial filaments (hyphae) to densely interwoven mycelial masses as reported by Papagianni (2004), with the morphology playing a fundamental role in determining the overall bioproduct productivity. Fungal morphology manipulation has resulted in an increased bioproduct yield (Schügerl et al., 1998), concentration (Wagner et al., 2004) and productivity (Fazenda et al., 2010) in a range of fungal bioprocesses.

Mycelial filaments generated from mushrooms naturally tend to aggregate due to hyphal entanglement, which can due to continued growth generate a dense form around the core, a pellet. Pellet growth has some advantages, for example, reduced viscosity in the liquid phase, but the main drawback with pellet culture is the lack of oxygen diffusion caused by internal mass transport resistance that may lead to reduced biosynthetic activity (Wittier et al., 1986). There are no reasonable means by which the structure of the pellets can be controlled to reduce the diffusion resistance within the structure. In this regard, the recognition of the fundamental role of fungal morphology in determining performance in mycelial fermentation has led to a search for alternative culture methods to such as immobilisation strategy.

An immobilised mycelial system might be an effective means of improving exopolysaccharide productivity. This is because it has the potential to increase the mycelium amount per bioreactor volume by the mycelia being deposited on, in and even adherent to the chosen support matrices. As stated above, the adsorption by passive adhesion to surfaces would be preferable because it would provide a direct contact between the nutrients and the adhered cells thus reducing any diffusion problems (Brouers et al., 1989; Loyarkat et al., 2015). A variety of matrices, such as agar carrageenan, calcium alginate gels, polyacrylamide, and polyurethane foams have been used, but the nature of the mycelia to be immobilised, substrates and bioproducts produced, and the culture conditions are vital considerations for the choice of the immobilisation matrix and procedures. Adsorption to surfaces or porous materials (a particular type of physical attachment) has been one of the most widely studied methods for the immobilisation of microorganism. It represents a particular form of cellular adhesion based on the ability of certain fungi to fix themselves to solid surfaces (Moonmangmee et al., 2002) with improved physiological conditions (Rogalski et al., 2006).

By attaching the hyphae of *G. lucidum* onto the solid surfaces (carrier), the culture time may be extended. Immobilisation allows for easy separation of mycelial mass from the bulk liquid, minimising contamination, improves operational stability and mycelial viability, and reduces production costs compared to freely suspended fermentation processes (Ariyajaroenwong et al., 2012; Tripathi et al., 2010) . Most

carriers are low-cost, and other advantages would are freedom from toxicity problems, reusability, mechanical strength and robustness (Loyarkat et al., 2015).

Based on repeated-batch fermentation (Chapter 3), one of the factors that reduces the effectiveness of repeated-batch application on *G. lucidum* is the wall-growth problem both in the shake flask and bioreactor. Typically in liquid low shear systems *G. lucidum* often forms pellets' perhaps indicating a self-immobilisation approach. Nonetheless, this self-immobilisation is inadequate if the pellet becomes too dense and starts to cling to the bioreactor walls, thus reducing oxygen transfer from the outer into the inner core of the pellet (internal mass transport resistance) (Prasad et al., 2005). Possible improvement of this can be achieved by using external immobilisation materials such as polyurethane and polyethene foam, as mycelial entrapment avoids mass transfer restrictions (Moonmangmee et al., 2002). Eventually, this would improve the reliability and perhaps allow a continuous immobilised fermentation strategy.

A large number of literature reports are focused on the enhancement of bioproducts production using the immobilised approach (De Ory et al., 2004; Nakamura et al., 1999) including use of adsorption to surfaces, and encapsulation within polyurethane foam (PUF) during the immobilisation (De Ory et al., 2004; Wagner et al., 2003). Immobilisation of *G. lucidum* CCRC 36123 was done by Yang et al., (2000) in the shake flask using PUF and they indicated that the mycelial morphology adopted on a solid support was favourable for both fungal growth and polysaccharide formation though this strategy has not yet been used for repeated-batch fermentation.

The aim of this research was to study the ability of PUF as a low-cost carrier for the immobilisation of *G. lucidum* for exopolysaccharide (EPS) production and to investigate carrier and culture stability in repeated-batch fermentation (RBF) to improve EPS productivity. The effects of the carrier, broth replacement ratio, and broth replacement time were also investigated. To the best of our knowledge, the use of a physical carrier for repeated-batch immobilisation strategy on *G. lucidum* BCCM 31549 remains unreported.

6.1 Materials and methods

6.1.1 White rot fungi

G. lucidum BCCM 31549 used in the present study was described in Chapter 3, 4 and 5.

6.1.2 EPS production medium

In the present investigations, *G. lucidum* BCCM 31549 was studied to produce EPS in submerged agitation culture using cotton-plugged 500 mL Erlenmeyer flask containing 200 mL of production medium according to the RBF strategy in Chapter 3.

6.1.3 Analytical methods

The dry cell weight (DCW) was measured by subtracting the dry weight of PUF from the total dry weight of immobilised cells and the PUF. The foam matrices filled immobilised cells were carefully drained, washed, and dried to constant weight at 80 ^{o}C (Yang et al., 2000). The glucose concentration was measured by YSI 2950D

Biochemistry Analyser (YSI, Hampshire. UK). The EPS production was estimated using the method from Chapter 3.

6.1.4 Immobilisation

Polyurethane Foam (PUF) (Plastifoam, Manchester, UK) was used as a carrier. Prior to use, the PUF was cut into cube-shaped pieces of 5 mm x 5 mm x 5 mm by a sharp scalpel (Blades No.11 Sigma-Aldrich, UK) which were soaked in distilled water, pre-weighed and sterilised for 15 minutes at 121 ^{o}C three times. The distilled water was replaced every time and the PUF cubes were then placed in methanol for 24 h, washed twice with distilled water, and dried in a hot air oven at 50 ^{o}C until the moisture had been completely removed from the cubes. For the immobilisation of *G. lucidum* on PUF cubes, the pre-weighed PUF (8 cubes) were added under conditions A, B, C, and D into 500 mL Erlenmeyer flasks and sterilised at 121 ^{o}C for 15 minutes. The flasks were inoculated as described in Chapter 3.

6.1.4.1 PUF characteristics

The key features of PUF are summarised in Table 6.1 with its micrograph (Fig. 6.1). PUF was chosen as a carrier for this study as it allows an enormous number of immobilised cells in the shortest time (De Ory et al., 2004), is an excellent fungal mycelial immobiliser (Nakamura et al., 1999), improves bioproducts production (Prasad et al., 2005), and enhances *G. lucidum* batch fermentation growth (Yang et al., 2000).

Table 6.1 Summary of the key features for PUF.

Details	Polyurethane foam
Material	Commercial PUF
Shape	Cubes
Size	5 mm x 5 mm x 5 mm
Current supplier	Plastifoam, Manchester, UK
Primary benefit	Adhesion both at the surface and within the pores compared to only surface adsorption

*Characteristics were obtained from De ory (2004).

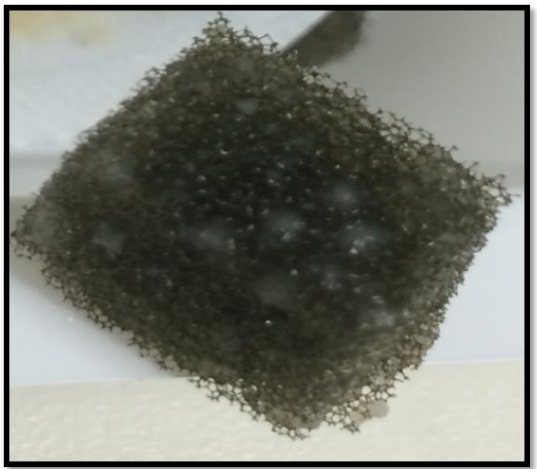


Figure 6.1 Picture indicates 5 mm x 5 mm x 5 mm of PUF cube from the current work using 10 Megapixel Resolution Nikon digital cameras (3X magnification).

6.1.5 Batch fermentation method

The inoculum preparations were the same as described in Chapter 3. Fermentation was carried out in a Rotary Shaker Incubator (New Brunswick, USA). The immobilised *G. lucidum* fungal cells were inoculated into sterile fresh complete media in the 500 mL flask. The fermentation was operated at 30 ^{o}C, initial pH 4 and 100 rpm. The samples were collected at 3-day intervals for analysis. Samples were drawn periodically during the fermentation, and the EPS production was analysed. The dry weight of

immobilised fungal mycelium on PUF was determined every 3-4 days using separate sets of fermented flasks. Kinetic calculations were the same as Chapter 3.

6.1.6 Repeated batch fermentation (RBF) strategy

The RBF was initially done in batch mode. After 12 to 13 days (depending on the broth replacement time), 60%, 80% and 100% of the volume of the whole broth was drained. Then, the same amount of the complete sterile medium (60%, 80% and 100%) was immediately added to initiate the next cycle. Seven successive cycles were performed. During each cycle, the fermented broth was assayed for EPS production as shown in the batch process.

6.1.7 Statistical analysis

All analyses were carried out in triplicate and the respective mean ± S.D using the software, GraphPad Prism 5 (Version 5.01) as mentioned in Chapter 3.

6.2 Results and Discussion

6.2.1 Carrier

With no carrier present, *G. lucidum* mycelia tended to form pellets (Chapter 3), resulting in poor substrate and oxygen transfer, thus resulting in deprived fermentation as mentioned by Yang et al., (2005). In contrast to suspension cultures, immobilised cell fermentation offers many benefits such as preventing mycelia to form pellets, easy to separate and a way to attempt repeated batch fermentation strategy. Yang et al., (2005) stated that the bioproducts activity of immobilised fermentation were better than suspended cultures.

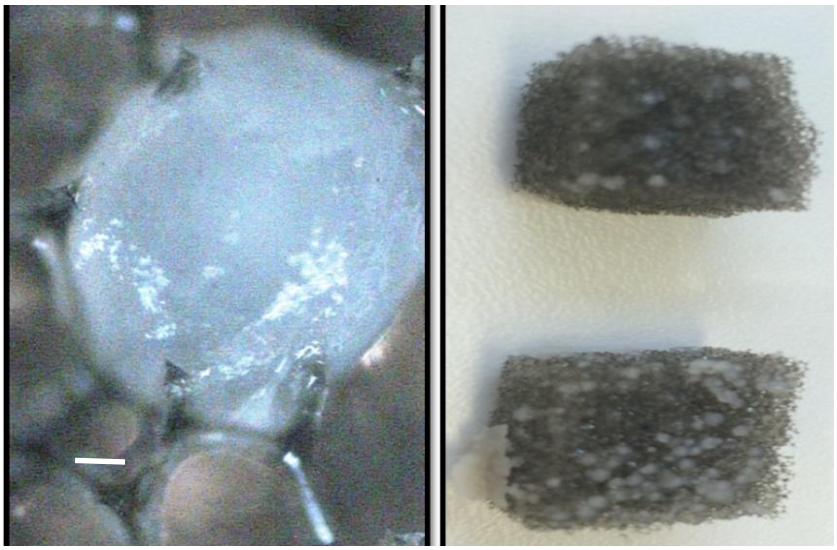


Figure 6.2 Immobilised *G. lucidum* cells on PUF cubes (5 mm^3): Condition A, initial pH 4, temperature 30 ^oC, 100 rpm, and 8 days grown culture in the shake flask. The left image was taken at 4-fold magnification. Bar = 150 µm. The right image was taken using a Nikon Digital Camera at 10x Megapixel Resolution.

In the present study, the growth of *G. lucidum* on PUF is shown in Fig. 6.2. It can be seen that PUF cubes adsorbed or entrapped the growing mycelia easily as the

mesh of polyurethane foam was colonised by the fungus both at the surface and within the pores. Based on this preliminary information and substantial relevant literature, PUF was subsequently used as the carrier in the current RBF studies.

6.2.2 Batch fermentation with PUF cubes

Batch fermentation of *G. lucidum* with the introduction of PUF cubes was done to study their growth pattern mainly on EPS production. Four different conditions were used (condition A, B, C, and D) to choose the most appropriate parameters and medium composition for RBF.

To summarise, Fig. 6.3 below shows the fungal cultures in immobilised-batch fermentation using condition A, B, C, and D. The *G. lucidum* fermentation was observed until day 16, and both EPS and biomass were analysed.

Table 6.2 Kinetics of immobilised-batch fermentation of *G. lucidum* BCCM 31549.

Condition set	Initial inoculum % (v/v)	Glucose level (g/L)	EPS concentration (g/L)	EPS productivity, P_{EPS} (g/L day^{-1})	Time (day)
A	10	50	0.58	0.044	13
B	20	30	0.40	0.029	14
C	10	30	0.51	0.039	13
D	20	50	0.22	0.017	13

*Temperature 30 ^{o}C, 100 rpm, initial pH 4 in 500 mL Erlenmeyer flask. Other conditions were the same as Figure 6.3

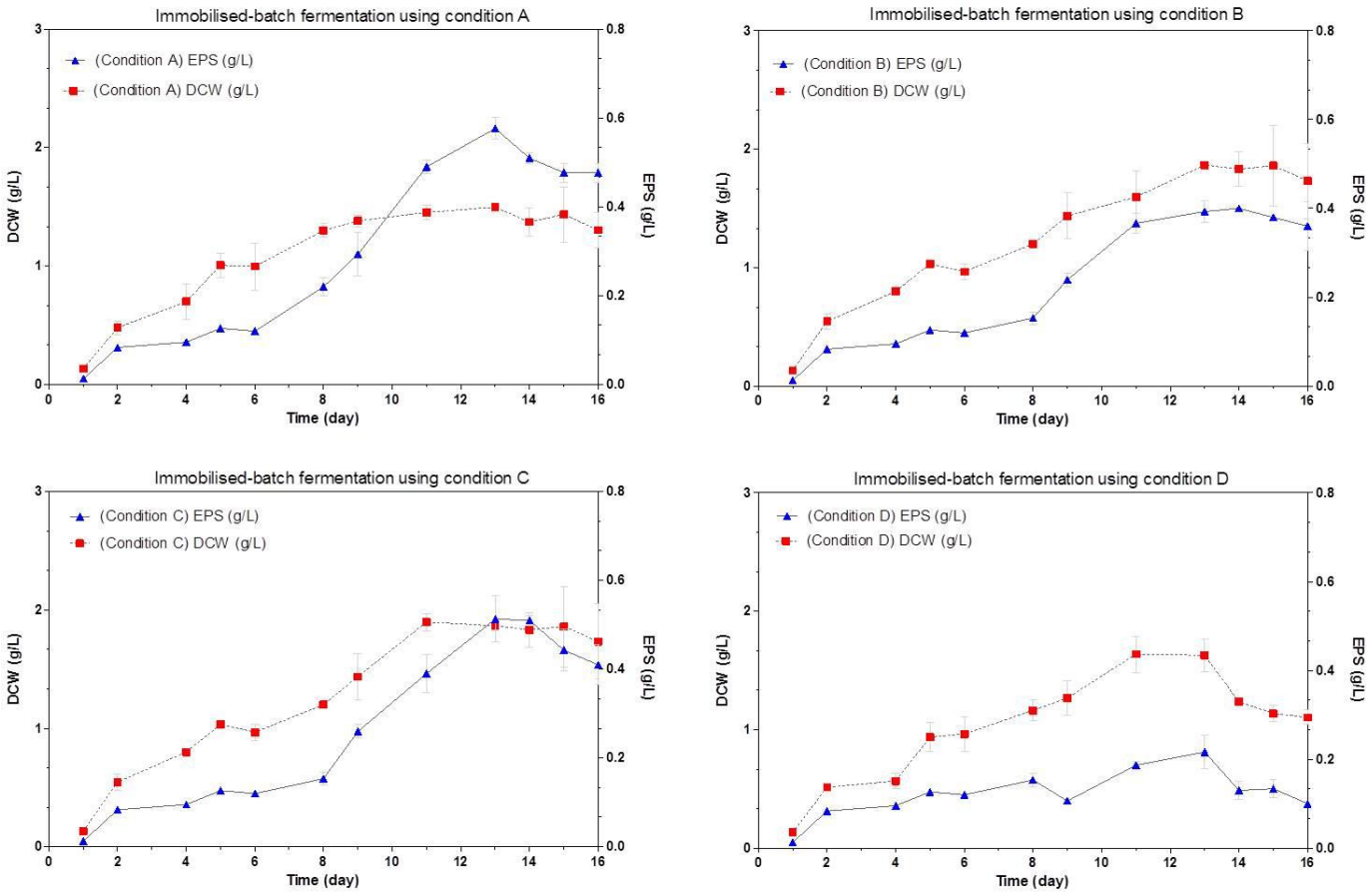


Figure 6.3 *G. lucidum* BCCM 31549 immobilised-batch fermentation in the shake flask using condition A [50 g/L of Glucose, 10% (v/v) inoculum], condition B [30 g/L of Glucose, 20% (v/v) inoculum], condition C [30 g/L of Glucose, 10% (v/v) inoculum], and condition D [50 g/L of Glucose, 20% (v/v) inoculum]. Parameters were at (g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_20$ 0.5, YE 1, NH_4Cl 4], 100 rpm, initial pH 4, and temperature 30 ^oC.

*1 way ANOVA has been carried out for each row with the P value of < 0.05. Bonferroni's post-test shows that more than one pair were significantly different ($P < 0.05$)

For the kinetic parameters (Table 6.2), the EPS concentration and productivity were the highest for Condition A (0.58 g/L, 0.044 g/L day^{-1}), followed by Condition C (0.51 g/L, 0.039 g/L day^{-1}), Condition B (0.40 g/L, 0.029 g/L day^{-1}), and Condition D (0.22 g/L, 0.017 g/L day^{-1}). As a result, condition A was preferred for immobilised-RBF strategy in the following experiments.

6.2.3 Repeated batch fermentation using PUF cubes

One of the advantages of immobilised fungal cells is the possible repeated utilisation of mycelia during fermentation. Since immobilised fungal cells are removed periodically, the EPS productivity (g/L day^{-1}) can be enhanced considerably. The broth replacement time and broth replacement volume were studied to optimise the RBF of immobilised *G. lucidum* BCCM 31549 in the following experiments. Condition A was chosen for RBF strategy as it had been tested on extended batch culture (Appendix 6.6.1) and managed to produce the most consistent EPS productivity in seven batches compared to others.

6.2.3.1 The broth replacement volume

The broth replacement volume was examined by replacing 120 mL (60%), 160 mL (80%) and 200 mL (100%) during the RBF cycles. For that, 120, 160, and 200 mL of fermented broth was withdrawn respectively from three 500 mL Erlenmeyer flasks after 13 days fermentation, and then the same volume of newly prepared medium of condition A was added into the flask. Samples were withdrawn at 2-3 days intervals and

analysed for EPS production in Fig. 6.4 below. Aseptic conditions were strictly preserved throughout the process especially during the broth replacement procedure.

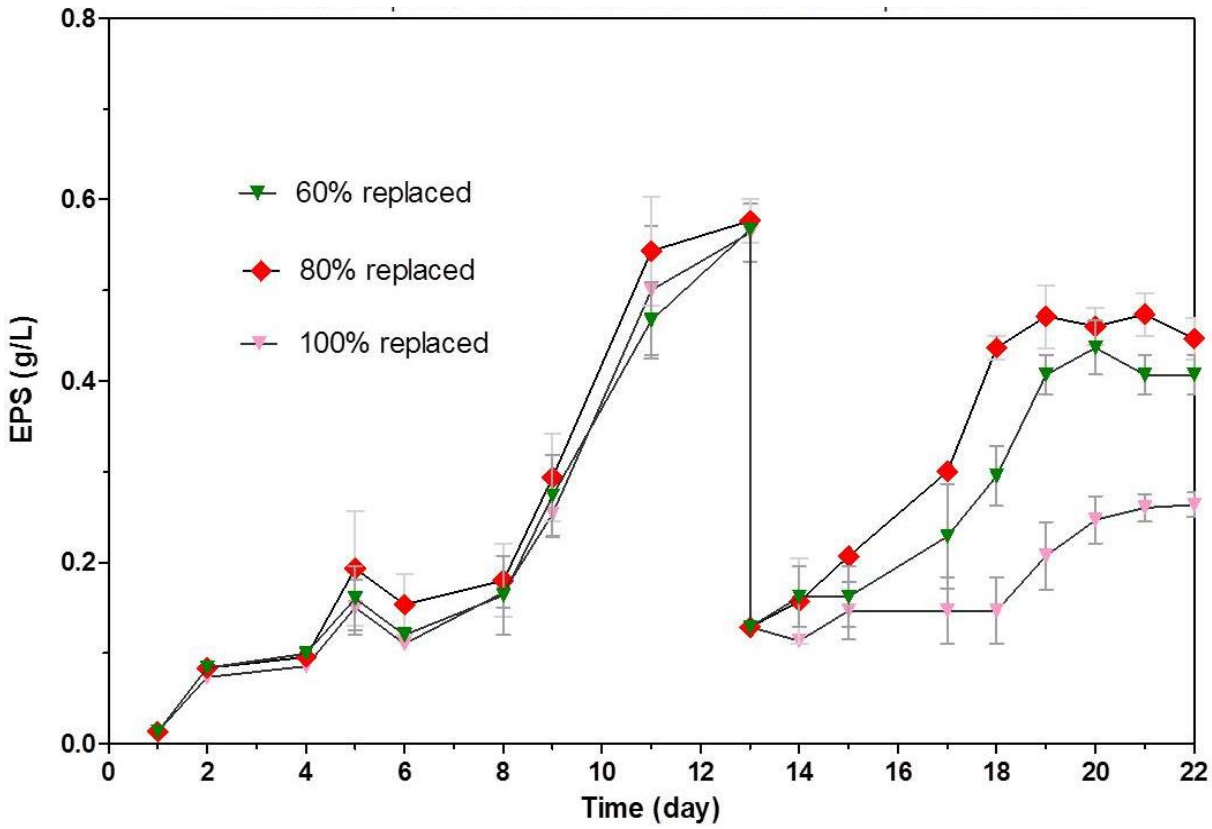


Figure 6.4 Effect of broth replacement volume % (v/v) during immobilised RBF using *G. lucidum* BCCM 31549 in the shake flask: temperature 30 ^{o}C; rotation speed 100 rpm; using Condition A as described in Fig. 6.3.

From the growth curve, a fairly acceptable EPS concentration as that before replacement (0-13 days) could be achieved in 6 days during 80% (v/v) broth replacement compared to 60% (v/v) and 100% (v/v). The fermentation time was reduced which led to the improvement of EPS productivity (g/L day^{-1}). When the whole broth was replaced entirely (200 mL), the growth of mycelia was too vigorous. Thus, EPS production was restrained. Therefore, 80% (v/v) replaced volume was used in the

following experiments considering the culture time and end product. The chosen replaced volume was the same as Chapter 3. It can be suggested that this percentage would be ideal for repeated-batch procedure as 100% (v/v) would be too high and 60% (v/v) might be too low for fungal aggregation and adherence onto the PUF cubes.

6.2.3.2 The broth replacement time point

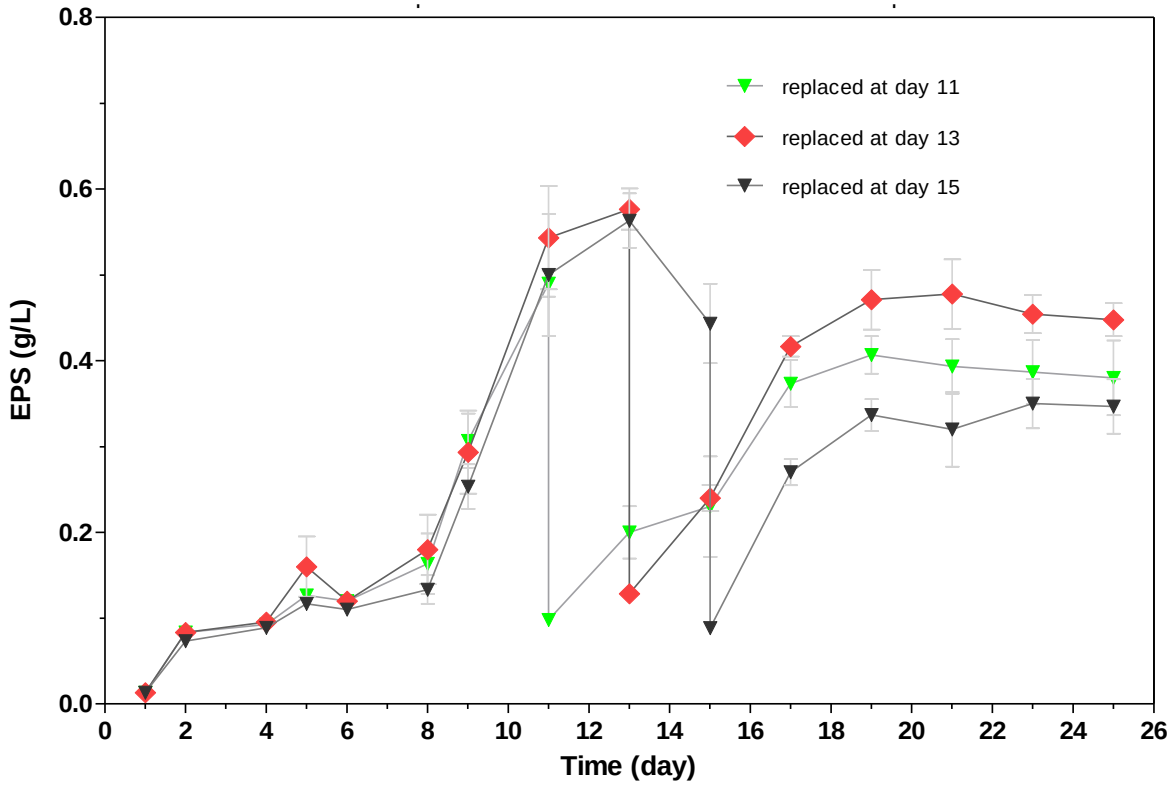


Figure 6.5 Effect of the broth replacement time (day) on immobilised RBF using *G. lucidum* BCCM 31549 RBF in the shake flask: temperature 30 ^{o}C, 100 rpm, 80% (v/v) replaced volume using condition A and parameters as described in Fig. 6.3.

To choose the broth replacement time point prior RBF implementation on the immobilised fungal cell, 160 mL (80%) of the fermented culture was replaced at day 11

(the end of logarithmic growth phase), day 13 (transition phase), day 15 (stationary phase), respectively, as shown in Fig. 6.5.

There were clear differences among the three experiments on the production of EPS with day 13 strategy showed the highest values, while substituting the culture medium at day 15 resulted in unfavourable growth for extended batch culture. Also, the EPS level after the broth replacement procedure (day 19 onwards) was nearly equal to the batch value at day 13. Therefore, day 13 was chosen for the following experiment.

6.2.4 Immobilised-RBF in the shake flask

Based on the chosen optimal conditions from the preceding experiments, RBF was carried out in the shake flask using PUF cubes as the carrier (Fig. 6.6). 160 mL (80%) fermented broth was withdrawn aseptically from 500 mL Erlenmeyer flask after 13 days of fermentation. Then the same volume of fresh medium (condition A composition) was added. Fermented fungal samples from the shake flask were analysed at 6-day intervals for EPS concentration and productivity. Immobilised fungal cells in the shake flask could generate EPS for up to 55 days (R1 to R7), with a mean productivity of at least 0.35 g/L day^{-1} (R3) in Fig. 6.6 below. Though, RBF experienced a slight decrease in productivity during the first three cycles (R2-R4) before it stabilised (R5), thus suggesting a slow EPS release from the PUF cubes. On average, the EPS productivity of immobilised-RBF was 50% more than the batch for seven consecutive cycles. Also, fermentation time was reduced from 13 days to 6 days, and the medium usage was also reduced due to the broth replacement volume procedure (Section 6.2.3.1).

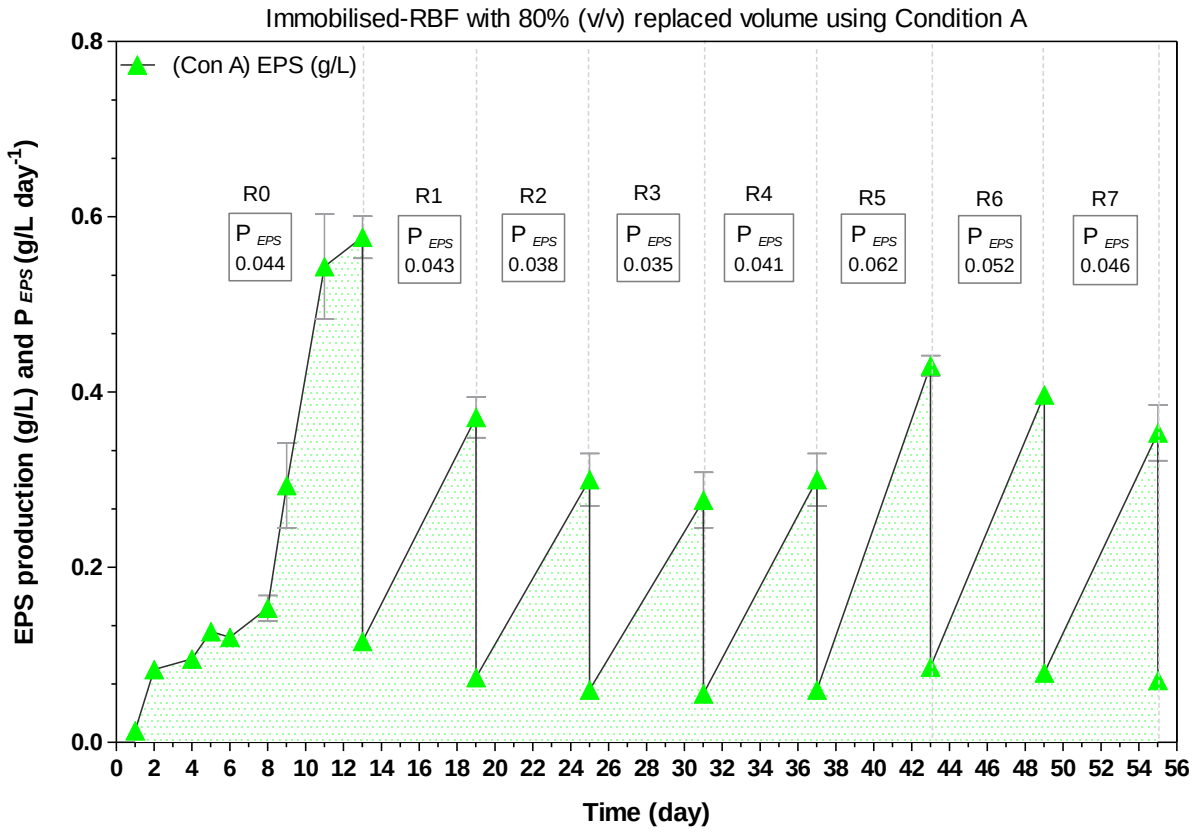


Figure 6.6 Immobilised repeated-batch fermentation of *G. lucidum* BCCM 31549 in the shake flask: Temperature 30 ºC; 100 rpm; initial pH 4; replaced volume of 160 mL; condition A. T-test shows the P value of 0.0016 from the column statistics. P_{EPS} = EPS productivity.

The current immobilised fungal cells have generated a significant, consistent amount of bioproduct despite extended cultures, and thus represents an advance compared to the immobilisation approach by Yang (2005) and Loyarkat et al., (2015). Meanwhile, the current work on fungal cells involved seven subsequent batches, thus was better compared to the work by Rogalski (2006) that had only managed to extend three subsequent batches using the same PUF cubes as a carrier.

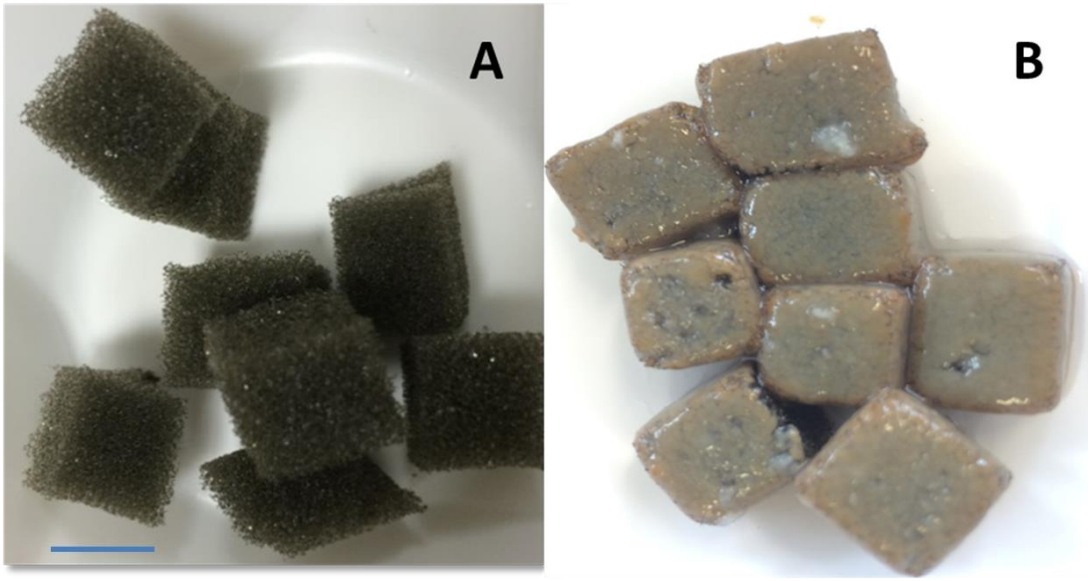


Figure 6.7 PUF cubes before (A) and after (B) immobilised-RBF for the seven successive batches in the shake flask. Temperature 30 ^{o}C; 100 rpm; initial pH 4; replaced volume of 160 mL: condition A. Images were taken using a Nikon Digital Camera 10x Megapixel of Resolution, Bar = 5 mm.

Macroscopic observation of the carriers containing the immobilised mycelia before (A) and after inoculation (B) for seven successive RBF cycles remain unchanged in general dimensions indicating no significant damage as can be seen in Fig. 6.7, pointing out that the PUF cubes were suitable to be used as the carrier for immobilisation matrix. The PUF cubes (B) were entirely penetrated and their surface concealed with a dense layer of fungal hyphae. Also, it has been proposed that such immobilisation may be stimulated by electrostatic forces between the cell membrane and the PUF cubes as described by Ariyajaroenwong (2012). Likewise, these electrostatic forces might be triggered by polymucosaccharide (a compound that act as a glue to help colonise the solid surfaces) for fungal cells adhesion (Moonmangmee et al., 2002).

Another advantage of immobilisation may be that the contact area strength between fungal cells and oxygen has reduced the shear stress on the cultures as proposed by Yup Jang et al., (2002). The current work represents an advance on previous comparable studies into immobilised cultures by use of a repeated-batch strategy with the PUF cubes as shown in Fig. 6.6 (B).

6.3 Comparison of immobilised-RBF with RBF in the shake flask

The parameters of repeated-batch EPS fermentation by the immobilised *G. lucidum* mycelia in Table 6.3 below were compared with freely suspended cultures (pellet formation) as reported in Chapter 3 in the shake flask. It was found that the fermentation time in freely suspended culture system (35 days) was seven days shorter than that of the immobilisation system (42 days). However, the average EPS yield of both systems in seven successive batches were quite similar, even though the average EPS concentration (0.27 ± 0.05 g/L) and EPS productivity (P_{EPS}: 0.045 ± 0.001 g/L day^{-1}) were markedly higher for immobilisation system compared to the freely suspended culture system (EPS concentration: 0.18 ± 0.01 g/L; P_{EPS}: 0.029 ± 0.002).

Table 6.3 Comparison of immobilisation and freely suspended culture in *G. lucidum* repeated-batch fermentation using shake flask.

Batch Number	Parameters (mean ± SD)				Reference
	EPS production, (g/L)	EPS productivity, P_{EPS} (g/L day^{-1})	Yield $_{EPS}$ (EPS yield on sugar), (g $_{EPS}$/g $_{GLU}$)	Total fermentation time (day)	
1	0.26 ± 0.02	0.043 ± 0.04	0.073 ± 0.02	6	
2	0.23 ± 0.05	0.038 ± 0.03	0.039 ± 0.01	6	
3	0.21 ± 0.03	0.035 ± 0.06	0.024 ± 0.05	6	This study
4	0.24 ± 0.01	0.041 ± 0.04	0.025 ± 0.02	6	
5	0.37 ± 0.02	0.062 ± 0.04	0.033 ± 0.06	6	
6	0.31 ± 0.01	0.052 ± 0.03	0.024 ± 0.02	6	
7	0.27 ± 0.01	0.046 ± 0.02	0.025 ± 0.04	6	
Average immobilisation culture	0.27 ± 0.05	0.045 ± 0.001	0.035 ± 0.004	42	This study
Average freely suspended culture	0.18 ± 0.01	0.029 ± 0.002	0.034 ± 0.01	35	Chapter 3

* Fermentations were carried out in the shake flasks with the conditions and medium compositions of [(g/L): Glucose 50, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, initial pH 4, 10% (v/v) initial inoculum and temperature at 30 ^oC for both immobilised and freely suspended cultures. RBF was done at 80% (v/v) broth replacement ratio and during the transition phase.

* Source of variation as P value summary: Interaction (0.0435), Column Factor (<0.0001), Row Factor (0.0034) generated from two-way ANOVA and Bonferroni's post-test.

The current immobilisation procedure was beneficial for polysaccharide production from *G. lucidum*; however Yang et al., (2000) did not focus on the repeated batch strategy used in the present study. Meanwhile, the current RBF immobilisation and culture strategy was efficient for *G. lucidum* mycelium adherence, and also showed an EPS productivity 1.55 times higher compared to freely suspended culture and 1.4 times greater than the levels reported by Rogalski (2006) on immobilisation of the Basidiomycete *Nematoloma frowardii*.

The higher EPS levels in immobilised system compared to freely suspended culture may be attributed to the static immobilised fungal culture on PUF cubes, which allows the contact area between hyphae and oxygen to be increased without mixing disruption. The increased surface area of fungal filaments on PUF cubes tends to reduce the mass transfer limitations compared to freely suspended culture, which improved access to substrate.

Hence, by introducing immobilised-RBF using this fungal species, it has created an alternative cultivation strategy for *G. lucidum* in a liquid environment. Also, this immobilisation idea may provide an alternative way to aid the recovery of the bioproducts for other Basidiomycete species.

6.4 Reported work on fungal immobilisation

As reported in Table 6.4 below, the application of RBF with immobilisation was mostly done using PUF cubes for Basidiomycetes.

Table 6.4 Comparison of the current immobilised-RBF with the literature.

Microorganism	Bioproducts	PUF cubes size (dimension)	Reactor and working volume	Repeated-batch strategy		Reference
				Total batches (cycles)	Total fermentation time (days)	
Basidiomycetes						
Ganoderma lucidum BCCM 31549	EPS	(5 x 5 x 5 mm)	• 500 mL Erlenmeyer flask (200 mL working volume)	7	55	This study
Ganoderma lucidum CCRC 36123	EPS	(4 x 4 x 2 cm)	• 250 mL Erlenmeyer flask (100 mL working volume)	NA	NA	(Yang et al., 2000)
Pleurotus ostreatus strain 1804	Laccase	(1 x 1 x 1 cm)	• 250 mL Erlenmeyer flask (100 mL working volume) • Packed-bed bioreactor (280 mL working volume)	NA	NA	(Prasad et al., 2005)
Bjerkandera adusta IFO 4983	Lignin	(1 x 1 x 1 cm)	• 300 mL Erlenmeyer flask (30 mL working volume)	NA	NA	(Nakamura et al., 1999)

Nematoloma frowardii ATCC 201144	MnP	(5 x 5 x 5 mm)	• 250 mL Erlenmeyer flask (100 mL working volume) • 3-L Fixed-net aerated bioreactor (2-L working volume)	NA 3	NA 44	(Rogalski et al., 2006)
Zygomycetes						
Rhizopus arrhizus BUCT	Lipase	(5 x 5 x 5 mm)	• 250 mL Erlenmeyer flask (100 mL working volume) • 5-L unspecified fermentor (2.7-L working volume)	8 6	5.83 8.125	(Yang et al., 2005)

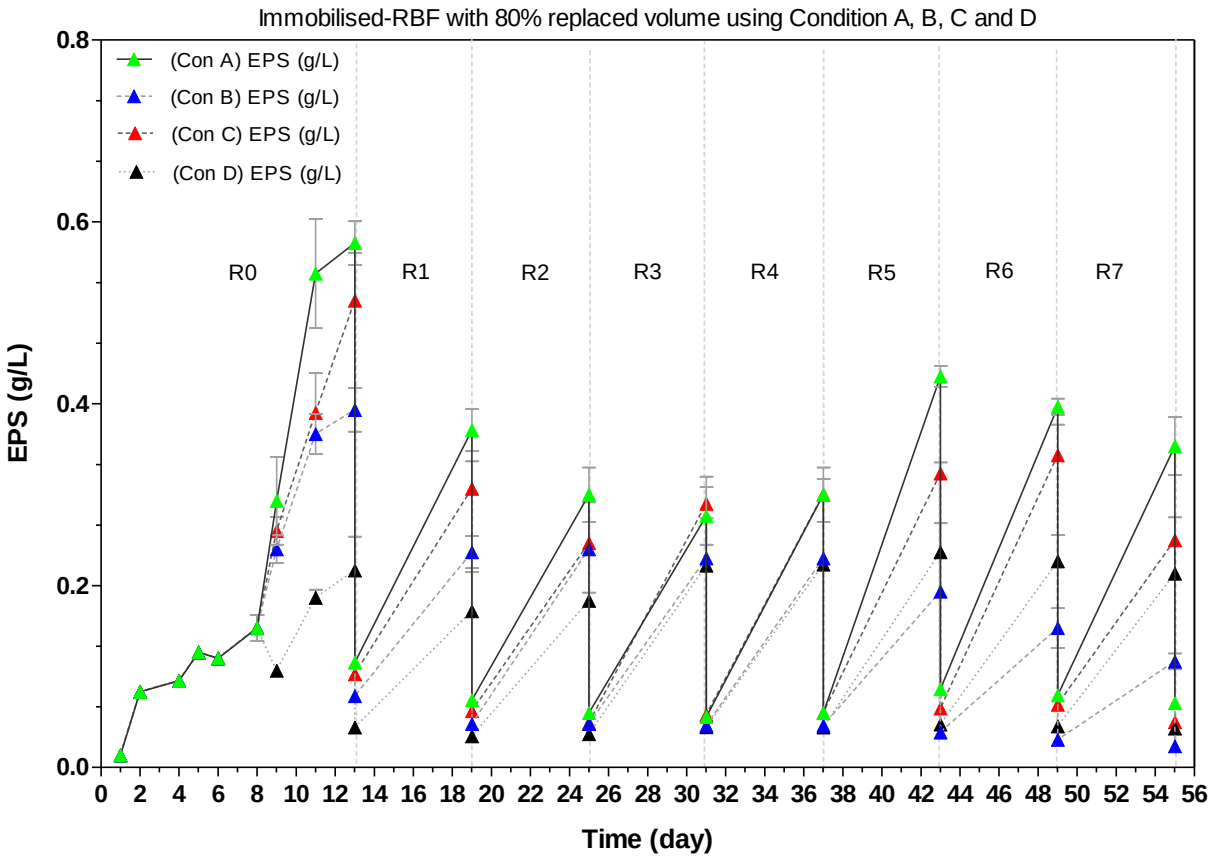
- NA = Not available

The dimension of PUF cubes were mostly below 1 cm^3 dimension, and this may ensure a sufficient surface area for immobilisation of fungi. When compared, the current work was the first to implement a repeated-batch strategy for *G. lucidum*. Nevertheless, the closest comparison for this immobilised repeated-bath strategy were on *Nematoloma frowardii* ATCC 201144 (Basidiomycetes) at three batches (44 days) and *Rhizopus arrhizus* BUCT (Zygomycetes) at eight batches (5.83 days) compared to seven batches of the current *G. lucidum* BCCM 31549 (55 days). Yang et al., (2000) stated that the idea of continuous operation proved to be immature on *G. lucidum* CCRC 36123, yet the current data disproved them that immobilisation has provided an alternative way for the recovery of product. On the whole, recent immobilisation procedure may be enhanced in a bioreactor that suits PUF cubes.

6.5 Conclusions

Immobilised mycelia of *G. lucidum* BCCM 31549 using PUF cubes had a high ability for continuous EPS production. Seven batches could be fermented consecutively in the shake flask for 55 days (7 batches). The time for repeated-batch fermentation was reduced substantially and the EPS production increased relative to a single batch strategy. The immobilised *G. lucidum* mycelia on the 5 x 5 x 5 mm^3 polyurethane foam could be reused at least for seven successive batches without any losses of EPS efficiencies.

6.6 Appendix



Appendix 6.6.1 Comparison of *Ganoderma lucidum* BCCM 31549 immobilised repeated-batch fermentation in the shake flask using condition A [50 g/L of Glucose, 10% (v/v) inoculum], condition B [30 g/L of Glucose, 20% (v/v) inoculum], condition C [30 g/L of Glucose, 10% (v/v) inoculum], and condition D [50 g/L of Glucose, 20% (v/v) inoculum]. Fermentation parameters were at (g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_47H_2O$ 0.5, YE 1, NH_4Cl 4], 100 rpm, initial pH 4, and temperature 30 ^oC.

*1 way ANOVA has been carried out for each row with the P value of < 0.0001. Since the result from one-way ANOVA analysis is significant, a posthoc analysis namely Bonferroni's Multiple Comparison Test was carried out to identify which pair(s) is/are statistically different (Richard and Dean, 2002). Bonferroni's post-test shows the (Con A) EPS (g/L) was significantly different (P < 0.05) vs (Con B) EPS (g/L), vs (Con D) EPS (g/L) and vs (Con C) EPS (g/L), respectively.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

The work presented in this thesis is focused on the development of the optimised conditions for exopolysaccharide (EPS) production in submerged culture using extended batch culture of *Ganoderma lucidum*. The produced EPS was further processed after primary recovery to enhance its solubility in aqueous solution. The characteristics of this processed EPS were assessed using chemical analysis and bioassays. In addition, the possibility of producing the EPS using entrapped or immobilised *G. lucidum* biomass was investigated.

Implementing the improvised strategy taken from the literature, where the actual interest lay in the production mostly by Ascomycota, rather than slow-growing Basidiomycota species, the initial attempts with shake flask fermentation did not appear promising, but rather challenging. Determining the blending time of mycelia prior inoculation was challenging for developing a primary fermentation process, understanding its physiology, and optimising it so it could be used industrially for EPS production. Also, with time constraints and limited resources it was only possible to carry out a modest number of experiments that addressed the research hypothesis.

7.1 Background on *Ganoderma lucidum*

- Natural compounds from the phylum of Basidiomycota (*G. lucidum*) are the popular choice to battle against resistant infections.
- If *Ganoderma* derived materials are to be used to promote health as described above, they would need to be produced in bulk, quickly, cheaply and to a consistent quality.

- Bioreactor submerged liquid fermentation as shown in the present study was capable of delivering *Ganoderma* biomass and EPS efficiently and these bioreactor-derived materials are safe.

7.2 Repeated batch fermentation

- The repeated batch fermentation (RBF) strategy has successfully produced fungal mycelial biomass and EPS in a very strictly regulated manner at high productivity rates compared to batch fermentation.
- The problematic lag phase and seed culture preparation were reduced in length or eliminated; harvesting volume doubled, yield of product increased, and medium consumption was reduced in an RBF relative to batch.
- 80% broth replacement volume and transition phase (time point) were optimised.
- Dispersed mycelial filaments with ovoid-shaped pellets are the typical morphological characteristics associated with EPS (β-glucan) production.
- N-limiting medium (N levels were kept to minimum) in an unbaffled 2.5-L bioreactor stimulated EPS formation during RBF compared to in baffled condition.

Future work

- Future developments of RBF are crucial for other Basidiomycota species to produce potentially interesting biopolymers at a consistently acceptable rate both industrially and economically.
- For the first approach, greater bioreactor volume must be used for the RBF strategy starting at 5-L to 10-L. The larger vessels may reduce the

problematic wall-growth generated by *G. lucidum* during extensive cultivation in small (2.5-L) vessels.

- Other bioreactor impellers, like pitched-blade or marine-propellers, are worth testing in comparison with the current Rushton turbine.
- The repeated fed-batch fermentation (RFBF) is recommended for *G. lucidum*, as this strategy remained unreported for this species.

7.3 Sulphation of the produced glucan

- The extracted β-glucan in the present study was difficult to dissolve in aqueous solution, which leads to difficulties in product analysis, formulation and delivery.
- The current study has managed to alter the molecule's hydrophobicity thus making it water-soluble as proved by compositional analysis and spectroscopy.
- The sulphated derivative of native glucan was identified as (1, 3)-β-D-glucan.

Future work

- Carbon NMR analysis are vital for more detailed chemical structure.
- Molecular weight identification would determine either low or high masses that may contribute to their therapeutic activities.
- A study on sulphation of lentinan, pullulan, and mannan is highly recommended for food industry and biomedical-applications of these biopolymers.

7.4 Therapeutic activities of glucan sulphate

- Sulphation was an effective approach to improve *in vitro* studies of antibacterial, antifungal, antiproliferative and immunomodulatory (NO stimulation) activity of the sulphated (1,3)-β-D-glucan.
- Glucan sulphate maybe safe in *in vivo* studies due to its demonstrated lack of toxicity towards a normal human prostate cell line (PN2TA). GS also showed antimicrobial-antifungal-immunomodulatory activities derived from a single compound.

Future work

- Future trials *in vivo* are necessary before the introduction of human trials.
- Glucan sulphate may be used as food additive, biofilms or nutritional supplements after appropriate studies.

7.5 Improvement of repeated-batch strategy via immobilisation

- During cultivation, *G. lucidum* mycelia tended to cling to any possible supports (sampling line, probes, impeller, and vessel wall).
- Fungal cells tended to grow well in the porous structure of PUF cubes and the RBF using immobilised fungal cells was an efficient method for production of β-glucan with a high yield.
- This study could be beneficial for other medicinal mushroom fermentation.

Future work

- The upscaling procedure should be done for this technique using an appropriate bioreactor system.
- Different amount of PUF cubes with different sizes should be tested on the immobilised RBF to determine the relationship of EPS production with carrier dimensions.
- N limitation in the immobilised cells should be tested with the RBF strategy as it may improve the yield.

CHAPTER 8

CONFLICT OF INTEREST AND REFERENCES

8.1 Conflict of Interest

Given Name: WAN ABD AL QADR IMAD

Surname: WAN MOHTAR

Date: 29th January 2016

Author of the thesis: Yes

Thesis Title: PRODUCTION AND BIOACTIVITY OF *Ganoderma lucidum* BCCM 31549 EXOPOLYSACCHARIDE USING SUBMERGED LIQUID FERMENTATION

Disclosure of Potential Conflicts of Interest: The author has nothing to disclose

8.2 REFERENCES

Abouelenien, F., Nakashimada, Y., Nishio, N., 2009. Dry mesophilic fermentation of chicken manure for production of methane by repeated batch culture. J Biosci Bioeng 107, 293-295.

Alkuraishy, H. M., Al-Gareeb, A. I., Alrifai, S. B., Awad, M. S., 2013. Immunomodulator effects of azithromycin, ciprofloxacin and ceftriaxone on cross-reaction between widal test and anti-helicobacter pylori Igm in acute typhoid fever. Int J Sci Res 4, 2541-2546.

Allison, R. D., Tong, X., Moorman, A. C., Ly, K. N., Rupp, L., Xu, F., Gordon, S. C., Holmberg, S. D., Investigators, C. H. C. S., 2015. Increased incidence of cancer and cancer-related mortality among persons with chronic Hepatitis C infection, 2006-2010. J Hepatol.

Alvarez-Suarez, J. M., Tulipani, S., Díaz, D., Estevez, Y., Romandini, S., Giampieri, F., Damiani, E., Astolfi, P., Bompadre, S., Battino, M., 2010. Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. Food Chem Toxicol 48, 2490-2499.

Ariyajaroenwong, P., Laopaiboon, P., Jaisil, P., Laopaiboon, L., 2012. Repeated-batch ethanol production from sweet sorghum juice by *Saccharomyces cerevisiae* immobilized on sweet sorghum stalks. Energies 5, 1215-1228.

Arora, D., Shepard, G. H., 2008. Mushrooms and economic botany 1. Eco Bot 62, 207-212.

Bao, H., Choi, W.-S., You, S., 2010. Effect of sulfated modification on the molecular characteristics and biological activities of polysaccharides from *Hypsizigus marmoreus*. Biosci Biotech Biochem 74, 1408-1414.

Bao, X. F., Wang, X. S., Dong, Q., Fang, J. N., Li, X. Y., 2002. Structural features of immunologically active polysaccharides from *Ganoderma lucidum*. Phytochem 59, 175-181.

Bartnicki-Garcia, S., 1970. Cell wall composition and other biochemical markers in fungal phylogeny. Phytochem Phylogeny, 81-103.

Berovic, M., Habijanic, J., Zore, I., Wraber, B., Hodzar, D., Boh, B., Pohleven, F., 2003. Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. J Biotech 103, 77-86.

Birhanli, E., Yesilada, O., 2006. Increased production of laccase by pellets of *Funalia trogii* ATCC 200800 and *Trametes versicolor* ATCC 200801 in repeated-batch mode. Enzyme Microb Tech 39, 1286-1293.

Birhanli, E., Yesilada, O., 2010. Enhanced production of laccase in repeated-batch cultures of *Funalia trogii* and *Trametes versicolor*. Biochem Eng J 52, 33-37.

Brakhage, A., Liebmann, B., 2005. *Aspergillus fumigatus* conidial pigment and cAMP signal transduction: significance for virulence. Med Mycol 43, 75-82.

Brouers, M., Dejong, H., Shi, D., Hall, D., 1989. Immobilized cells: An appraisal of the methods and applications of cell immobilization techniques. Algal Cyanobac Biotechnol, 272-293.

Carlisle, M. J., Watkinson, S. C., Gooday, G. W., 2001. "The Fungi". Academic Press, London, UK.

Cechinel-Filho, V., 2012. Plant bioactives and drug discovery: principles, practice, and perspectives. John Wiley & Sons, Inc., Hoboken, New Jersey.

Chan, W. K., Lam, D. T. W., Law, H. K. W., Wong, W. T., Leung Koo, M. W., Lau, A. S. Y., Lau, Y. L., Chan, G. C. F., 2005. *Ganoderma lucidum* mycelium and spore extracts as natural adjuvants for immunotherapy. J Altern Complem Med 11, 1047-1057.

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 Microb Tech 38, 407-414.

Chen, J., Seviour, R., 2007. Medicinal importance of fungal b-(1/3), (1/6)-glucans. Mycol Res 111, 635-652.

Chen, S., Wang, J., Xue, C., Li, H., Sun, B., Xue, Y., Chai, W., 2010. Sulfation of a squid ink polysaccharide and its inhibitory effect on tumor cell metastasis. Carbohydr Polym 81, 560-566.

Chen, T.-Q., Wu, Y.-B., Wu, J.-G., Ma, L., Dong, Z.-H., Wu, J.-Z., 2014. Efficient extraction technology of antioxidant crude polysaccharides from *Ganoderma lucidum* (Lingzhi), ultrasonic-circulating extraction integrating with superfine-pulverization. J Taiwan Inst Chem E 45, 57-62.

Chen, X., Hu, Z. P., Yang, X. X., Huang, M., Gao, Y., Tang, W., Chan, S. Y., Dai, X., Ye, J., Ho, P. C. L., 2006. Monitoring of immune responses to a herbal immuno-modulator in patients with advanced colorectal cancer. Int Immunopharmacol 6, 499-508.

Chen, Y., Xie, M.-Y., Nie, S.-P., Li, C., Wang, Y.-X., 2008. Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. Food Chem 107, 231-241.

Cherry, R. S., Papoutsakis, E. T., 1988. Physical mechanisms of cell damage in microcarrier cell culture bioreactors. Biotechnol Bioeng 32, 1001-1014.

Chien, Y. L., Ho, C. T., Chiang, B. H., Hwang, L. S., 2011. Effect of fermentation time on antioxidative activities of *Ganoderma lucidum* broth using leguminous plants as part of the liquid fermentation medium. Food Chem 126, 1586-1592.

Chiong, H. S., Yong, Y. K., Ahmad, Z., Sulaiman, M. R., Zakaria, Z. A., Yuen, K. H., Hakim, M. N., 2013. Cytoprotective and enhanced anti-inflammatory activities of liposomal piroxicam formulation in lipopolysaccharide-stimulated RAW 264.7 macrophages. Intern J Nanomedicine 8, 1245.

Daligault, H., Davenport, K., Minogue, T., Bishop-Lilly, K., Bruce, D., Chain, P., Coyne, S., Frey, K., Jaissle, J., Koroleva, G., 2014. Draft genome assembly of *Klebsiella pneumoniae* type strain ATCC 13883. Genome Announc 2, e00939-00914.

Dashti, M. G., Abdeshahian, P., 2015. Batch culture and repeated-batch culture of *Cunninghamella bainieri* 2A1 for lipid production as a comparative study. Saudi J Biol Sci.

Datta, C., Das, D., Mondal, P., Chakraborty, B., Sengupta, M., Bhattacharjee, C. R., 2015. Novel water soluble neutral vanadium (IV)–antibiotic complex: Antioxidant, immunomodulatory and molecular docking studies. Eur J Medic Chem 97, 214-224.

De Ory, I., Romero, L. E., Cantero, D., 2004. Optimization of immobilization conditions for vinegar production. Siran, wood chips and polyurethane foam as carriers for *Acetobacter aceti*. Process Biochem 39, 547-555.

Deacon, J. W., 2005. Fungal biology. Blackwell Publishers, Cambridge, MA.

Deacon, J. W., 2013. Fungal Biology. John Wiley & Sons, Cambridge, MA.

Dennis, R. W. G., Ainsworth, G. S., 1962. Dictionary of the Fungi. Ainsworth & Bisby's, Wallingford: CABI Europe, UK.

Devlieghere, F., Vermeulen, A., Debevere, J., 2004. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiol 21, 703-714.

Ding, Z., Wang, Q., Peng, L., Zhang, L., Gu, Z., Shi, G., Zhang, K., 2012. Relationship between mycelium morphology and extracellular polysaccharide production of medicinal mushroom *Ganoderma lucidum* in submerged culture. J Med Plants Res 6, 2868-2874.

Diouf, P. N., Stevanovic, T., Boutin, Y., 2009. The effect of extraction process on polyphenol content, triterpene composition and bioactivity of yellow birch (*Betula alleghaniensis* Britton) extracts. Ind Crop Prod 30, 297-303.

Dudhgaonkar, S., Thyagarajan, A., Sliva, D., 2009. Suppression of the inflammatory response by triterpenes isolated from the mushroom *Ganoderma lucidum*. Int Immunopharmacol 9, 1272-1280.

Dutta, P., Tripathi, S., Mehrotra, G., Dutta, J., 2009. Perspectives for chitosan based antimicrobial films in food applications. Food Chem 114, 1173-1182.

Eisenman, H. C., Casadevall, A., 2012. Synthesis and assembly of fungal melanin. Appl Microbiol Biotechnol 93, 931-940.

Ellington, M. J., Hope, R., Livermore, D. M., Kearns, A. M., Henderson, K., Cookson, B. D., Pearson, A., Johnson, A. P., 2010. Decline of EMRSA-16 amongst methicillin-resistant *Staphylococcus aureus* causing bacteraemias in the UK between 2001 and 2007. J Antimicrob Chemoth 65, 446-448.

Eyal, J., 1991. Mushroom mycelium grown in submerged culture-Potential food applications. New York: Van Nostrand Reinhold.

Fagade, O., Oyelade, A., 2009. A comparative study of the antibacterial activities of some wood-decay fungi to synthetic antibiotic discs. Electron J Environ Agr Food Chem 8, 184-188.

Fan, L. F., Soccol, A. T., Pandey, A., Soccol, C. R., 2007. Effect of nutritional and environmental conditions on the production of exo-polysaccharide of *Agaricus brasiliensis* by submerged fermentation and its antitumor activity. Lwt-Food Sci Technol 40, 30-35.

Fang, Q.-H., Zhong, J.-J., 2002a. Submerged fermentation of higher fungus *Ganoderma lucidum* for production of valuable bioactive metabolites—ganoderic acid and polysaccharide. Biochem Eng J 10, 61-65.

Fang, Q. H., Tang, Y. J., Zhong, J. J., 2002. Significance of inoculation density control in production of polysaccharide and ganoderic acid by submerged culture of *Ganoderma lucidum*. Process Biochem 37, 1375-1379.

Fang, Q. H., Zhong, J. J., 2002b. Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. Process Biochem 37, 769-774.

Fang, Q. H., Zhong, J. J., 2002c. Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*. Biotechnol Progr 18, 51-54.

Fazenda, M. L., Harvey, L. M., McNeil, B., 2010. Effects of dissolved oxygen on fungal morphology and process rheology during fed-batch processing of *Ganoderma lucidum*. J Microbiol Biotechnol 20, 844-851.

Fazenda, M. L., Seviour, R., McNeil, B., Harvey, L. M., 2008. Submerged culture fermentation of ‘‘higher fungi’’: The macrofungi. Adv App Microbiol 63, 33-103.

Feng, S., Srinivasan, S., Lin, Y. H., 2012. Redox potential-driven repeated batch ethanol fermentation under very-high-gravity conditions. Process Biochem 47, 523-527.

Ferreira, I. C., Heleno, S. A., Reis, F. S., Stojkovic, D., Queiroz, M. J., Vasconcelos, M. H., Sokovic, M., 2015a. Chemical features of Ganoderma polysaccharides with antioxidant, antitumor and antimicrobial activities. Phytochem 114, 38-55.

Ferreira, I. C., Heleno, S. A., Reis, F. S., Stojkovic, D., Queiroz, M. J. R., Vasconcelos, M. H., Sokovic, M., 2015b. Chemical features of Ganoderma polysaccharides with antioxidant, antitumor and antimicrobial activities. Phytochem 114, 38-35.

Figlas, D., Curvetto, N., 2000. Medicinal mushroom reishi (*Ganoderma lucidum*). Main toxicity and allergenicity studies. Dosage, posology and side effects. Hongoscomestibles-latinoamerica, 1-13.

Fraga, I., Coutinho, J., Bezerra, R. M., Dias, A. A., Marques, G., Nunes, F. M., 2014. Influence of culture medium growth variables on *Ganoderma lucidum* exopolysaccharides structural features. Carbohydr Polym 111, 936-946.

Gadd, G. M., Sariaslani, S., 2014. Advances in applied microbiology. Academic Press.

Gaidhani, H., McNeil, B., Ni, X., 2005. Fermentation of pullulan using an oscillatory baffled fermenter. Chem Eng Res Des 83, 640-645.

Ganjali Dashti, M., Abdeshahian, P., Wan Yusoff, W. M., Kalil, M. S., Abdul Hamid, A., 2014. Repeated batch fermentation biotechnology for the biosynthesis of lipid and gamma-linolenic acid by *Cunninghamella bainieri* 2A1. Biomed Res Int 2014, 831783.

Gao, Y., Zhou, S., Huang, M., Xu, A., 2003. Antibacterial and antiviral value of the genus Ganoderma P. Karst. species (aphyllophoromycet-ideae): a review. Int J Med Mushrooms 5, 235–246.

Geml, J., Gravendeel, B., van der Gaag, K. J., Neilen, M., Lammers, Y., Raes, N., Noordeloos, M. E. (2014). The contribution of DNA metabarcoding to fungal conservation: diversity assessment, habitat partitioning and mapping red-listed fungi in protected coastal *Salix repens* communities in the Netherlands. PloS one, 9(6), e99852.

Gibbs, P., Seviour, R., Schmid, F., 2000. Growth of filamentous fungi in submerged culture: problems and possible solutions. Crc Rev Biotechnol 20, 17-48.

Gow, N. A. R., Gadd, G. M., 1995. ‘‘The growing fungus’’. Chapman & Hall, London, UK.

Gómez, B. L., & Nosanchuk, J. D. (2003). Melanin and fungi. Curr Opin Infect Dis, 16(2), 91-96.

Hajjaj, H., Macé, C., Roberts, M., Niederberger, P., Fay, L. B., 2005. Effect of 26-oxygenosterols from *Ganoderma lucidum* and their activity as cholesterol synthesis inhibitors. App Environ Microbiol 71, 3653-3658.

Han, M. D., Han, Y. S., Hyun, S. H., Shin, H. W., 2008. Solubilization of water-insoluble β-glucan isolated from *Ganoderma lucidum*. J Environ Biology 29, 237-242.

Harvey, L. M., McNeil, B., Berry, D. R., White, S. (1998). Autolysis in batch cultures of *Penicillium chrysogenum* at varying agitation rates. Enzyme Microb Tech, 22(6), 446-458

Heleno, S. A., Ferreira, I. C., Esteves, A. P., Ćirić, A., Glamočlija, J., Martins, A., Soković, M., Queiroz, M. J. R., 2013. Antimicrobial and demelanizing activity of *Ganoderma lucidum* extract, p-hydroxybenzoic and cinnamic acids and their synthetic acetylated glucuronide methyl esters. Food Chem Toxicol 58, 95-100.

Hernández-Lauzardo, A., Bautista-Baños, S., Velázquez-del Valle, M., Méndez-Montealvo, M., Sánchez-Rivera, M., Bello-Pérez, L., 2008. Antifungal effects of chitosan with different molecular weights on in vitro development of *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. Carbohydr Polym 73, 541-547.

Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., Huhndorf, S., James, T., Kirk, P. M., Lücking, R., Thorsten Lumbsch, H., Lutzoni, F., Matheny, P. B., McLaughlin, D. J., Powell, M. J., Redhead, S., Schoch, C. L., Spatafora, J. W., Stalpers, J. a., Vilgalys, R., Aime, M. C., Aptroot, A., Bauer, R., Begerow, D., Benny, G. L., Castlebury, L. a., Crous, P. W., Dai, Y.-C., Gams, W., Geiser, D. M., Griffith, G. W., Gueidan, C., Hawksworth, D. L., Hestmark, G., Hosaka, K., Humber, R. a., Hyde, K. D., Ironside, J. E., Kõljalg, U., Kurtzman, C. P., Larsson, K.-H., Lichtwardt, R., Longcore, J., Miadlikowska, J., Miller, A., Moncalvo, J.-M., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J. D., Roux, C., Ryvarden, L., Sampaio, J. P., Schüssler, A., Sugiyama, J., Thorn, R. G., Tibell, L., Untereiner, W. a., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M. M., Winka, K., Yao, Y.-J., Zhang, N., 2007. A higher-level phylogenetic classification of the Fungi. Mycol Res 111, 509-547.

Hsieh, C., 2004. Reusing soy residue for the solid-state fermentation of *Ganoderma lucidum*. Bioresource Technol 91, 105-109.

Hsieh, C., Hsu, T.-H., Yang, F.-C., 2005. Production of polysaccharides of *Ganoderma lucidum* by reusing thin stillage. Process Biochem 40, 909-916.

Hsieh, C., Tseng, M.-H., Liu, C.-J., 2006. Production of polysaccharides from *Ganoderma lucidum* (CCRC 36041) under limitations of nutrients. Enzyme Microb Tech 38, 109-117.

Huang, W.-C., Chen, S.-J., Chen, T.-L., 2008. Production of hyaluronic acid by repeated batch fermentation. Biochem Eng J 40, 460-464.

Huie, C. W., Di, X., 2004. Chromatographic and electrophoretic methods for Lingzhi pharmacologically active components. J Chromatogr B 812, 241-257.

Ibelgaufts, H., 1995. Dictionary of cytokines. VCH, Weinheim.

James, T. Y., Letcher, P. M., Longcore, J. E., Mozley-Standridge, S. E., Porter, D., Powell, M. J., Griffith, G. W., Vilgalys, R., 2006. A molecular phylogeny of the

flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). Mycologia 98, 860-871.

James, T. Y., Porter, D., Leander, C. A., Vilgalys, R., Longcore, J. E., 2000. Molecular phylogenetics of the Chytridiomycota supports the utility of ultrastructural data in chytrid systematics. Can J Bot 78, 336-350.

Jedinak, A., Thyagarajan-Sahu, A., Jiang, J., Sliva, D., 2011. Ganodermanontriol, a lanostanoid triterpene from *Ganoderma lucidum*, suppresses growth of colon cancer cells through ss-catenin signaling. Int J Oncol 38, 761-767.

Ji, X.-J., Zhang, A.-H., Nie, Z.-K., Wu, W.-J., Ren, L.-J., Huang, H., 2014. Efficient arachidonic acid-rich oil production by *Mortierella alpina* through a repeated fed-batch fermentation strategy. Bioresour Technol 170, 356-360.

Jonathan, S., Awotona, F., 2013. Studies on antimicrobial potentials of three Ganoderma species. Afr J Biomed Research 13, 131-139.

Kamra, A., Bhatt, A., 2012. Evaluation of antimicrobial and antioxidant activity of *Ganoderma lucidum* extracts against human pathogenic bacteria. Inter J Pharm Pharm Sci 4, 359-361.

Kao, C., Jesuthasan, A. C., Bishop, K. S., Glucina, M. P., Ferguson, L. R., 2013. Anticancer activities of *Ganoderma lucidum*: active ingredients and pathways. Funct Foods Health Dis 3, 48-65.

Karnjanapratum, S., Tabarsa, M., Cho, M., You, S., 2012. Characterization and immunomodulatory activities of sulfated polysaccharides from *Capsosiphon fulvescens*. Int J Biol Macromol 51, 720-729.

Kawagishi, H., Mitsunaga, S., Yamawaki, M., Ido, M., Shimada, A., Kinoshita, T., Murata, T., Usui, T., Kimura, A., Chiba, S., 1997. A lectin from mycelia of the fungus *Ganoderma lucidum*. Phytochem 44, 7-10.

Khalaf, A. I., Bourdin, C., Breen, D., Donoghue, G., Scott, F. J., Suckling, C. J., Macmillan, D., Clements, C., Fox, K., Sekibo, D. A., 2012. Design, synthesis and antibacterial activity of minor groove binders: the role of non-cationic tail groups. Eur J Med Chem 56, 39-47.

Kim, B. K., Cho, H. Y., Kim, J. S., Kim, H. W., Choi, E. C., 1993. Studies on constituents of higher fungi of Korea (LXVIII). Antitumor components of the cultured mycelia of *Ganoderma lucidum*. Korean J Pharmacol 24, 203-2012.

Kim, H. M., Paik, S. Y., Ra, K. S., Koo, K. B., Yun, J. W., Choi, J. W., 2006a. Enhanced production of exopolysaccharides by fed-batch culture of *Ganoderma resinaceum* DG-6556. J Microbiol 44, 233-242.

Kim, H. M., Park, M. K., Yun, J. W., 2006b. Culture pH affects exopolysaccharide production in submerged mycelial culture of *Ganoderma lucidum*. App Biochem Biotechnol 134, 249-262.

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 J Mycol 14, 49-59.

Kim, S. W., Hwang, H. J., Xu, C. P., Sung, J. M., Choi, J. W., Yun, J. W., 2003. Optimization of submerged culture process for the production of mycelial biomass and exo-polysaccharides by *Cordyceps militaris* C738. J Appl Microbiol 94, 120-126.

Kimura, Y., Taniguchi, M., Baba, K., 2002. Antitumor and antimetastatic effects on liver of triterpenoid fractions of *Ganoderma lucidum*: mechanism of action and isolation of an active substance. Anticancer research 22, 3309-3318.

Kirk, P. M., Cannon, P. F., David, J. C., Stalpers, J., 2001. "Ainsworth and Bisby's Dictionary of the Fungi".

Ko, H. H., Hung, C. F., Wang, J. P., Lin, C. N., 2008. Antiinflammatory triterpenoids and steroids from *Ganoderma lucidum* and *G. tsugae*. Phytochem 69, 234-239.

Kohut, G., Ádám, A. L., Fazekas, B., Hornok, L., 2009. N-starvation stress induced FUM gene expression and fumonisin production is mediated via the HOG-type MAPK pathway in *Fusarium proliferatum*. Int J Food Microbiol 130, 65-69.

Kozarski, M. S., Klaus, A. S., Niksic, M. P., Van Griensven, L. J. L. D., Vrvic, M. M., Jakovljevic, D. M., 2014. Polysaccharides of higher fungi: biological role, structure and antioxidative activity. Hem Ind 68, 305-320.

Kurosumi, A., Kobayasi, F., Mtui, G., Nakamura, Y., 2006. Development of optimal culture method of *Sparassis crispa* mycelia and a new extraction method of antineoplastic constituent. J Biochem Eng 30, 109-113.

Labro, M.-T., 2000. Interference of antibacterial agents with phagocyte functions: immunomodulation or “immuno-fairy tales”? Clinical Microbiol Rev 13, 615-650.

Laybourn-Parry, J., 2009. Microbiology. No place too cold. Science 324, 1521-1522.

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J., Yun, J. W., 2004. Submerged culture conditions for the production of mycelial biomass and exopolysaccharides by the edible Basidiomycete *Grifola frondosa*. Enzyme Microb Tech 35, 369-376.

Lee, J. M., Kwon, H., Jeong, H., Lee, J. W., Lee, S. Y., Baek, S. J., Surh, Y. J., 2001. Inhibition of lipid peroxidation and oxidative DNA damage by *Ganoderma lucidum*. Phytother Res 15, 245–249.

Lee, S., Park, S., Oh, J. W., Yang, C., 1998. Natural inhibitors for protein prenyltransferase. Planta Med 64, 303-308.

Lee, W. Y., Park, Y., Ahn, J. K., Ka, K. H., Park, S. Y., 2007. Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*. Enzyme Microb Tech 40, 249-254.

Leskosek-Cukalovic, I., Despotovic, S., Lakic, N., Niksic, M., Nedovic, V., Tesevic, V., 2010. *Ganoderma lucidum* - Medical mushroom as a raw material for beer with enhanced functional properties. Food Res Int 43, 2262-2269.

Li, W. J., Nie, S. P., Liu, X. Z., Zhang, H., Yang, Y., Yu, Q., Xie, M. Y., 2012. Antimicrobial properties, antioxidant activity and cytotoxicity of ethanol-soluble acidic components from *Ganoderma atrum*. Food Chem Toxicol 50, 689-694.

Li, X. B., Zhao, G. R., Yuan, Y. J., 2005. A strategy of phosphorus feeding for repeated fed-batch fermentation of penicillin G. Biochem Eng J 27, 53-58.

Li, Y.-Q., Wang, S.-F., 2006. Anti-hepatitis B activities of ganoderic acid from *Ganoderma lucidum*. Biotechnol Lett 28, 837-841.

Li, Y. B., Liu, R. M., Zhong, J. J., 2013. A new ganoderic acid from *Ganoderma lucidum* mycelia and its stability. Fitoterapia 84, 115-122.

Li, Z. J., Shukla, V., Fordyce, A. P., Pedersen, A. G., Wenger, K. S., Marten, M. R., 2000. Fungal morphology and fragmentation behavior in a fed-batch *Aspergillus oryzae* fermentation at the production scale. Biotechnol Bioeng 70, 300-312.

Liao, S. F., Liang, C. H., Ho, M. Y., Hsu, T. L., Tsai, T. I., Hsieh, Y. S., Tsai, C. M., Li, S. T., Cheng, Y. Y., Tsao, S. M., Lin, T. Y., Lin, Z. Y., Yang, W. B., Ren, C. T., Lin, K. I., Khoo, K. H., Lin, C. H., Hsu, H. Y., Wu, C. Y., Wong, C. H., 2013. Immunization of fucose-containing polysaccharides from Reishi mushroom induces antibodies to tumor-associated Globo H-series epitopes. Proc Natl Acad Sci USA 110, 13809-13814.

Lin, S. B., Li, C. H., Lee, S. S., Kan, L. S., 2003. Triterpene-enriched extracts from *Ganoderma lucidum* inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. Life Sci J 72, 2381-2390.

Lindequist, U., Niedermeyer, T. H., Julich, W. D., 2005. The pharmacological potential of mushrooms. Evid Based Complement Alternat Med 2, 285-299.

Liu, G. Q., Wang, X. L., Han, W. J., Lin, Q. L., 2012a. Improving the fermentation production of the individual key triterpene ganoderic acid me by the medicinal fungus *Ganoderma lucidum* in submerged culture. Molecules 17, 12575-12586.

Liu, J., Kurashiki, K., Shimizu, K., Kondo, R., 2006. Structure-activity relationship for inhibition of 5alpha-reductase by triterpenoids isolated from *Ganoderma lucidum*. Bioorg Med Chem 14, 8654-8660.

Liu, R. M., Zhong, J. J., 2011. Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells. Phytomedicine 18, 349-355.

Liu, Y., Zhang, J., Tang, Q., Yang, Y., Guo, Q., Wang, Q., Wu, D., Cui, S. W., 2014. Physicochemical characterization of a high molecular weight bioactive beta-D-

glucan from the fruiting bodies of *Ganoderma lucidum*. Carbohydr Polym 101, 968-974.

Liu, Y. J., Shen, J., Xia, Y. M., Zhang, J., Park, H. S., 2012b. The polysaccharides from *Ganoderma lucidum*: Are they always inhibitors on human hepatocarcinoma cells? Carbohydr Polym 90, 1210-1215.

Llaurado, G., Morris, H. J., Ferrera, L., Camacho, M., Castan, L., Lebeque, Y., Beltran, Y., Cos, P., Bermudez, R. C., 2015. In-vitro antimicrobial activity and complement/macrophage stimulating effects of a hot-water extract from mycelium of the oyster mushroom Pleurotus sp. Innov Food Sci Emerg 30, 177-183.

Lo, H. C., Tsai, F. A., Wasser, S. P., Yang, J. G., Huang, B. M., 2006. Effects of ingested fruiting bodies, submerged culture biomass, and acidic polysaccharide glucuronoxylomannan of *Tremella mesenterica* Retz.:Fr. on glycemic responses in normal and diabetic rats. Life Sci J 78, 1957-1966.

Lorenzen, K., Anke, T., 1998. Basidiomycetes as a source for new bioactive natural products. Curr Org Chem 2, 329-364.

Loyarkat, S., Cheirsilp, B., Prasertsan, P., 2015. Two-stage repeated-batch fermentation of immobilized *Clostridium beijerinckii* on oil palm fronds for solvents production. Process Biochem.

Ma, C. W., Feng, M. Y., Zhai, X. F., Hu, M. H., You, L. J., Luo, W., Zhao, M. M., 2013. Optimization for the extraction of polysaccharides from *Ganoderma lucidum* and their antioxidant and antiproliferative activities. J Taiwan Inst Chem E 44, 886-894.

Marzluf, G. A., 1997. Genetic regulation of nitrogen metabolism in the fungi. Microbiol Mol Biol Rev 61, 17-32.

McLaughlin, D. J., Hibbett, D. S., Lutzoni, F., Spatafora, J. W., Vilgalys, R., 2009. The search for the fungal tree of life. Trends Microbiol 17, 488-497.

McNeil, B., Harvey, L. M., 2008. Practical fermentation technology. John Wiley and Sons, United Kingdom, United Kingdom.

Min, B.-S., Gao, J.-J., Nakamura, N., Hattori, M., 2000. Triterpenes from the spores of *Ganoderma lucidum* and their cytotoxicity against meth-A and LLC tumor cells. Chem Pharm Bull 48, 1026-1033.

Min, B. S., Gao, J. J., Hattori, M., Lee, H. K., Kim, Y. H., 2001. Anticomplement activity of terpenoids from the spores of *Ganoderma lucidum*. Planta Med 67, 811–814.

Mirończuk, A. M., Furgała, J., Rakicka, M., Rymowicz, W., 2014. Enhanced production of erythritol by *Yarrowia lipolytica* on glycerol in repeated batch cultures. J Ind Microbiol Biotechnol 41, 57-64.

Mizuno, T., Wang, G. Y., Zhang, J., Kawagishi, H., Nishitoba, T., Li, J. X., 1995. Reishi, *Ganoderma lucidum* and *Ganoderma tsugae* - Bioactive substances and medicinal effects. Food Rev Int 11, 151-166.

Moeller, L., Grunberg, M., Zehnsdorf, A., Aurich, A., Bley, T., Strehlitz, B., 2011. Repeated fed-batch fermentation using biosensor online control for citric acid production by *Yarrowia lipolytica*. J Biotechnol 153, 133-137.

Moeller, L., Grünberg, M., Zehnsdorf, A., Strehlitz, B., Bley, T., 2010. Biosensor online control of citric acid production from glucose by *Yarrowia lipolytica* using semicontinuous fermentation. Eng Life Sci 10, 311-320.

Moonmangmee, S., Kawabata, K., Tanaka, S., Toyama, H., Adachi, O., Matsushita, K., 2002. A novel polysaccharide involved in the pellicle formation of *Acetobacter aceti*. J Biosci Bioeng 93, 192-200.

Moradali, M. F., Mostafavi, H., Hejaroude, G. A., Tehrani, A. S., Abbasi, M., Ghods, S., 2006. Investigation of potential antibacterial properties of methanol extracts from fungus *Ganoderma applanatum*. Chemoth 52, 241-244.

Morigiwa, A., Kitabatake, K., Fujimoto, Y., Ikekawa, N., 1986. Angiotensin converting enzyme-inhibitory triterpenes from *Ganoderma lucidum*. Chem Pharm Bull (Tokyo) 34, 3025-3028.

Mothana, R. A., Jansen, R., Julich, W. D., Lindequist, U., 2000. Ganomycins A and B, new antimicrobial farnesyl hydroquinones from the basidiomycete *Ganoderma pfeifferi*. J Nat Prod 63, 416-418.

Muzzarelli, R., Tarsi, R., Filippini, O., Giovanetti, E., Biagini, G., Varaldo, P., 1990. Antimicrobial properties of N-carboxybutyl chitosan. Antimicrob Agents Ch 34, 2019-2023.

Nakamura, Y., Sungusia, M. G., Sawada, T., Kuwahara, M., 1999. Lignin-degrading enzyme production by Bjerkandera adusta immobilized on polyurethane foam. J Biosci Bioeng 88, 41-47.

Nakamura, Y., Tagata, M., Tokuda, H., Yasukawa, K., Uchiyama, E., Suzuki, T., Kimura, Y., 2007. Anti-inflammatory and anti-tumor-promoting effects of triterpene acids and sterols from the fungus *Ganoderma lucidum* Chem Biodivers 4, 224-231.

Naritomi, T., Kouda, T., Yano, H., Yoshinaga, F., Shigematsu, T., Morimura, S., Kida, K., 2002. Influence of broth exchange ratio on bacterial cellulose production by repeated-batch culture. Process Biochem 38, 41-47.

Nasreen, Z., Kausar, T., Nadeem, M., Bajwa, R., 2005. Study of different growth parameters in *Ganoderma lucidum*. Micologia Aplicada Int 17, 5-8.

Nayak, A., Nayak, R. N., Bhat, K., 2010. Antifungal activity of a toothpaste containing *Ganoderma lucidum* against *Candida albicans*-an in vitro study. J Int Oral Health 2, 51-57.

Ngai, P. H. K., Ng, T. B., 2004. A mushroom (*Ganoderma capense*) lectin with spectacular thermostability, potent mitogenic activity on spleno- cytes, and antiproliferative activity toward tumor cells. Biochem Biophys Res Commun 314, 988–993.

Nitsche, B. M., Burggraaf-van Welzen, A. M., Lamers, G., Meyer, V., Ram, A. F., 2013. Autophagy promotes survival in aging submerged cultures of the filamentous fungus *Aspergillus niger*. Appl Microbiol Biotechnol 97, 8205-8218.

O'Donnell, K., Lutzoni, F. M., Ward, T. J., Benny, G. L., 2001. Evolutionary relationships among mucoralean fungi (Zygomycota): Evidence for family polyphyly on a large scale. Mycologia 93, 286-297.

Ofodile, L., Uma, N., Kokubun, T., Grayer, R., Ogundipe, O., Simmonds, M., 2005. Antimicrobial activity of some Ganoderma species from Nigeria. Phytother Res 19, 310-313.

Oh, J. Y., Cho, E. J., Nam, S. H., Choi, J. W., Yun, J. W., 2007. Production of polysaccharide–peptide complexes by submerged mycelial culture of an entomopathogenic fungus *Cordyceps sphecocephala*. Process Biochem 42, 352-362.

Oh, K. W., Lee, C. K., Kim, Y. S., Eo, S. K., Han, S. S., 2000. Antiherpetic activities of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* alone and in combinations with acyclovir and vidarabine. J Ethnopharmacol 72, 221-227.

Packer, H. L., Thomas, C. R., 1990. Morphological measurements on filamentous microorganisms by fully automatic image analysis. Biotechnology and Bioengineering 35, 870-881.

Pala, S. A., Wani, A. H., 2011. Mushrooms: The entities with multifarious medicinal properties. J Pharm Res 4.

Pan, D., Zhang, D., Wu, J., Chen, C., Xu, Z., Yang, H., Zhou, P., 2014. A novel proteoglycan from *Ganoderma lucidum* fruiting bodies protects kidney function and ameliorates diabetic nephropathy via its antioxidant activity in C57BL/6 db/db mice. Food Chem Toxicol 63, 111-118.

Pandey, A., 2003. Solid-state fermentation. Biochem Eng J 13, 81-84.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial process. Biotechnol Adv 22, 189-259.

Papinutti, L., 2010. Effects of nutrients, pH and water potential on exopolysaccharides production by a fungal strain belonging to *Ganoderma lucidum* complex. Bioresour Technol 101, 1941-1946.

Park, E. J., Ko, G., Kim, J., Sohn, D. H., 1997. Antifibrotic effects of a polysaccharide extracted from *Ganoderma lucidum*, glycyrrhizin, and pentoxifylline in rats with cirrhosis induced by biliary obstruction. Biol Pharm Bull 20, 417-420.

Park, J. P., Kim, Y. M., Kim, S. W., Hwang, H. J., Cho, Y. J., Lee, Y. S., Song, C. H., Yun, J. W., 2002. Effect of aeration rate on the mycelial morphology and exo-biopolymer production in *Cordyceps militaris*. Process Biochem 37, 1257-1262.

Paterson, R. R., 2006. Ganoderma - a therapeutic fungal biofactory. Phytochem 67, 1985-2001.

Patra, N., De, U., Kang, J. A., Kim, J. M., Ahn, M. Y., Lee, J., Jung, J. H., Chung, H. Y., Moon, H. R., Kim, H. S., 2011. A novel epoxypropoxy flavonoid derivative and topoisomerase II inhibitor, MHY336, induces apoptosis in prostate cancer cells. Eur J Pharmacol 658, 98-107.

Peng, Y., Zhang, L., Zhang, Y., Xu, X., Kennedy, J. F., 2005. Solution properties of water-insoluble polysaccharides from the mycelium of *Ganoderma tsugae*. Carbohydr Polym 59, 351-356.

Petre, M., Teodorescu, A., Tuluca, E., Bejan, C., Andronescu, A., 2010. Biotechnology of mushroom pellets producing by controlled submerged fermentation. Rom Biotechnol Lett 15, 50-55.

Pi, C.-C., Chu, C.-L., Lu, C.-Y., Zhuang, Y.-J., Wang, C.-L., Yu, Y.-H., Wang, H.-Y., Lin, C.-C., Chen, C.-J., 2014. Polysaccharides from *Ganoderma formosanum* function as a Th1 adjuvant and stimulate cytotoxic T cell response in vivo. Vaccine 32, 401-408.

Prasad, K. K., Mohan, S. V., Bhaskar, Y. V., Ramanaiah, S., Babu, V. L., Pati, B., Sarma, P., 2005. Laccase production using *Pleurotus ostreatus* 1804 immobilized on PUF cubes in batch and packed bed reactors: influence of culture conditions. J Microbiol-SEOUL 43, 301.

Prasad, Y., Wesely, W., 2008. Antibacterial activity of the bio-multidrug (*Ganoderma lucidum*) on Multidrug resistant *Staphylococcus aureus* (MRSA). Adv Biotechnol 10, 9-16.

Qu, L., Ren, L. J., Sun, G. N., Ji, X. J., Nie, Z. K., Huang, H., 2013. Batch, fed-batch and repeated fed-batch fermentation processes of the marine thraustochytrid *Schizochytrium* sp. for producing docosahexaenoic acid. Bioprocess Biosyst Eng 36, 1905-1912.

Rau, U., 2004. Glucans secreted by fungi. Turk Electron J Biotechnol 2, 30-36.

Ren, L., Reynisson, J., Perera, C., Hemar, Y., 2013. The physicochemical properties of a new class of anticancer fungal polysaccharides: a comparative study. Carbohydr Polym 97, 177-187.

Revankar, M. S., Lele, S. S., 2007. Synthetic dye decolorization by white rot fungus, Ganoderm sp. WR-1. Bioresour Technol 98, 775-780.

Reyes, C., Peña, C., Galindo, E., 2003. Reproducing shake flasks performance in stirred fermentors: production of alginates by *Azotobacter vinelandii*. J Biotechnol 105, 189-198.

Richard, A. J., Dean, W. W., 2002. Applied multivariate statistical analysis. Prentice Hall, London, UK.

Riley, G. L., Tucker, K. G., Paul, G. C., Thomas, C. R., 2000. Effect of biomass concentration and mycelial morphology on fermentation broth rheology. Biotechnol Bioeng 68, 160-172.

Rogalski, J., Szczodrak, J., Janusz, G., 2006. Manganese peroxidase production in submerged cultures by free and immobilized mycelia of *Nematoloma frowardii*. Bioresour Technol 97, 469-476.

Rosa, L. H., Almeida Vieira Mde, L., Santiago, I. F., Rosa, C. A., 2010. Endophytic fungi community associated with the dicotyledonous plant *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae) in Antarctica. FEMS Microbiol Ecol 73, 178-189.

Ruan, W., Popovich, D. G., 2012. *Ganoderma lucidum* triterpenoid extract induces apoptosis in human colon carcinoma cells (Caco-2). Biomed Prevent Nutr 2, 203-209.

Sanodiya, B. S., Thakur, G. S., Baghel, R. K., Prasad, G. B., Bisen, P. S., 2009. *Ganoderma lucidum*: a potent pharmacological macrofungus. Curr Pharm Biotechnol 10, 717-742.

Sasaki, T., Arai, Y., Ikekawa, T., Chihara, G., Fukuoka, F., 1971. Antitumor polysaccharides from some polyporaceae, *Ganoderma applanatum* (Pers.) Pat and *Phellinus linteus* (Berk. et Curt) Aoshima. Chem Pharm Bull 19, 821-826.

Schügerl, K., Gerlach, S., Siedenberg, D., 1998. Influence of the process parameters on the morphology and enzyme production of Aspergilli. Relation between morphology and process performances. Springer, pp. 195-266.

Schüβler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105, 1413-1421.

Seviour, R. J., McNeil, B., Fazenda, M. L., Harvey, L. M., 2011. Operating bioreactors for microbial exopolysaccharide production. Crit Rev Biotechnol 31, 170-185.

Shahidi, F., Arachchi, J. K. V., Jeon, Y.-J., 1999. Food applications of chitin and chitosans. Trends Food Sci Tech 10, 37-51.

Sharifi, A., Khoramrooz, S. S., Jahedi, S., Khosravani, S. A., 2012. Screening of antimicrobial activity of sesquterpenoid crude extract of *Ganoderma*. Life Sci J 9, 2516-2519.

Shi, M., Zhang, Z., Yang, Y., 2013. Antioxidant and immunoregulatory activity of *Ganoderma lucidum* polysaccharide (GLP). Carbohydr Polym 95, 200-206.

Shiao, M. S., 1992. Triterpenoid natural-products in the fungus *Ganoderma lucidum*. J Chinese Chem Soc 39, 669-674.

Shieh, Y. H., Liu, C. F., Huang, Y. K., Yang, J. Y., Wu, I. L., Lin, C. H., Lin, S. C., 2001. Evaluation of the hepatic and renal-protective effects of *Ganoderma lucidum* in mice. T Am J Chinese Med 29, 501-507.

Shih, I. L., Chou, B. W., Chen, C. C., Wu, J. Y., Hsieh, C., 2008. Study of mycelial growth and bioactive polysaccharide production in batch and fed-batch culture of *Grifola frondosa*. Bioresour Technol 99, 785-793.

Shih, I. L., Pan, K., Hsieh, C., 2006. Influence of nutritional components and oxygen supply on the mycelial growth and bioactive metabolites production in submerged culture of *Antrodia cinnamomea*. Process Biochem 41, 1129-1135.

Shu, C.-H., Wen, B.-J., 2003. Enhanced shear protection and increased production of an anti-tumor polysaccharide by *Agaricus blazei* in xanthan-supplemented cultures. Biotechnol Lett 25, 873-876.

Sies, H., 1997. Oxidative stress: oxidants and antioxidants. Exp Physiol 82, 291-295.

Singh, N., Chakrabarty, S., Liu, G., 2010. Multidrug resistance of non-adherent cancer cells. Nature Precedings.

Skalicka-Wozniak, K., Szypowski, J., Los, R., Siwulski, M., Sobieralski, K., Glowniak, K., Malm, A., 2012. Evaluation of polysaccharides content in fruit bodies and their antimicrobial activity of four *Ganoderma lucidum* (W Curt. Fr.) P. Karst. strains cultivated on different wood type substrates. Acta Soc Bot Pol 81, 17-21.

Slany, M., Jezek, P., Fiserova, V., Bodnarova, M., Stork, J., Havelkova, M., Kalat, F., Pavlik, I., 2012. *Mycobacterium marinum* infections in humans and tracing of its possible environmental sources. Can J Microbiol 58, 39-44.

Smania, A. J., Delle Monache, F., Smania, E. F. A., Cuneo, R. S., 1999. Antibacterial activity of steroidal compounds isolated from *Ganoderma applanatum* (Pers.) Pat. (aphyllophoromycetideae) fruit body. Int J Med Mushrooms 1, 325–330.

Smania, E. F., Delle Monache, F., Smania, A., Jr., Yunes, R. A., Cuneo, R. S., 2003. Antifungal activity of sterols and triterpenes isolated from *Ganoderma annulare*. Fitoterapia 74, 375-377.

Smith, J. E., Rowan, N. J., Sullivan, R., 2002. Medicinal mushrooms: a rapidly developing area of biotechnology for cancer therapy and other bioactivities. Biotechnol Lett 24, 1839-1845.

Sone, Y., Misaki, A., 1985. Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing culture of mycelium of *Ganoderma lucidum*. Agr Biol Chem Tokyo 49, 2641-2653.

Sone, Y., Okuda, R., Wada, N., Kishida, E., Misaki, A., 1985. Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing culture of mycelium of *Ganoderma lucidum*. Agr Biol Chem 49, 2641-2653.

Stamets, P., 1993. Growing gourmet and medicinal mushrooms: Shokuyō Oyobi Yakuyō Kinoko No Saibai: a companion guide to the mushroom cultivator. Ten Speed Press, Berkeley, CA.

Stanbury, P. F., 1988. Ferment Technol. Extraction 2, 1.1.

Stanbury, P. F., Whitaker, A., Hall, S. J., 1995. ‘‘Principles of fermentation technology’’. Butterworth-Heinemann, Oxford, UK.

Stanbury, P. F., Whitaker, A., Hall, S. J., 2013. Principles of Fermentation Technology. Elsevier Science LtD, Oxford, UK.

Subramaniyam, R., Vimala, R., 2012. Solid state and submerged fermentation for the production of bioactive substances: A comparative study. Int J Sci Nat 3, 480-486.

Suryanarayan, S., 2003. Current industrial practice in solid state fermentations for secondary metabolite production: the Biocon India experience. Biochem Eng J 13, 189-195.

Suzuki, T., Ohno, N., Adachi, Y., Cirelli, A. F., Covian, J. A., Yadomae, T., 1991. Preparation and biological activities of sulfated derivatives of (1-3)-beta-D-glucans. J Pharmacobiodyn 14, 256-266.

Tang, Y.-J., Zhong, J.-J., 2002. Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. Enzyme Microb Tech 31, 20-28.

Tang, Y.-J., Zhong, J.-J., 2003. Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of Ganoderma polysaccharide and ganoderic acid. Enzyme Microb Tech 32, 478-484.

Tang, Y. J., Zhang, W., Liu, R. S., Zhu, L. W., Zhong, J. J., 2011. Scale-up study on the fed-batch fermentation of *Ganoderma lucidum* for the hyperproduction of ganoderic acid and Ganoderma polysaccharides. Process Biochem 46, 404-408.

Tang, Y. J., Zhang, W., Zhong, J. J., 2009. Performance analyses of a pH-shift and DOT-shift integrated fed-batch fermentation process for the production of ganoderic acid and Ganoderma polysaccharides by medicinal mushroom *Ganoderma lucidum*. Bioresour Technol 100, 1852-1859.

Tang, Y. J., Zhu, L. W., Li, H. M., Li, D. S., 2007. Submerged culture of mushrooms in bioreactors - Challenges, current state-of-the-art, and future prospects. Food Technol Biotech 45, 221-229.

Tepwong, P., Giri, A., Ohshima, T., 2012. Effect of mycelial morphology on ergothioneine production during liquid fermentation of *Lentinula edodes*. Mycoscience 53, 102-112.

Thomas, C. R., Paul, G. C., 1996. Applications of image analysis in cell technology. Curr Opin Biotechnol 7, 35-45.

Treskatis, S. K., Orgeldinger, V., Wolf, H., Gilles, E., 2000. Morphological characterization of filamentous microorganisms in submerged cultures by on-line digital image analysis and pattern recognition. Biotechnol Bioeng 53, 191-201.

Tripathi, A., Sami, H., Jain, S. R., Viloria-Cols, M., Zhuravleva, N., Nilsson, G., Jungvid, H., Kumar, A., 2010. Improved bio-catalytic conversion by novel immobilization process using cryogel beads to increase solvent production. Enzyme Microb Tech 47, 44-51.

Tucker, K. G., Kelly, T., Delgrazia, P., Thomas, C. R., 1992. Fully-automatic measurement of mycelial morphology by image analysis. Biotechnol Prog 8, 353-359.

Tung, N. T., Cuong, T. D., Hung, T. M., Lee, J. H., Woo, M. H., Choi, J. S., Kim, J., Ryu, S. H., Min, B. S., 2013. Inhibitory effect on NO production of triterpenes from the fruiting bodies of *Ganoderma lucidum*. Bioorg Med Chem Lett 23, 1428-1432.

Ukai, S., Kiho, T., Hara, C., Kuruma, I., Tanaka, Y., 1983. Polysaccharides in fungi. XIV. Anti-inflammatory effect of the polysaccharides from the fruit bodies of several fungi. J Pharmacobiodyn 6, 983–990.

Vaidyanathan, S., Macaloney, G., Vaughan, J., McNeil, B., Harvey, L. M., 1999. Monitoring of submerged bioprocesses. Crit Rev Biotechnol 19, 277-316.

van der Hem, L. G., van der Vliet, J. A., Bocken, C. F., Kino, K., Hoitsma, A. J., Tax, W. J., 1995. Ling Zhi-8: studies of a new immunomodulating agent. Transplantation 60, 438-443.

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 Technol Biotech 41, 371-382.

Wagner, R., Mitchell, D. A., Sassaki, G. L., de Almeida Amazonas, M. A., 2004. Links between morphology and physiology of *Ganoderma lucidum* in submerged culture for the production of exopolysaccharide. J Biotechnol 114, 153-164.

Walker, C., Schüßler, A., 2004. Nomenclatural clarifications and new taxa in the Glomeromycota *Pacispora*. Mycol Res 108, 981-982.

Walser, P. J., Hollenstein, M., Klaus, M. J., Kes, U., 2001. Genetic analysis of basidiomycete fungi. In: Talbot, N. (Ed.), ‘‘Molecular and cellular biology of filamentous fungi: A practical approach’’. Oxford University Press, UK, pp. 59-90.

Wang, H., Ng, T. B., 2006. Ganodermin, an antifungal protein from fruiting bodies of the medicinal mushroom *Ganoderma lucidum*. Peptides 27, 27-30.

Wang, J., Zhang, L., 2009. Structure and chain conformation of five water-soluble derivatives of a β-d-glucan isolated from *Ganoderma lucidum*. Carbohyd Res 344, 105-112.

Wang, J., Zhang, L., Yu, Y., Cheung, P. C., 2009. Enhancement of antitumor activities in sulfated and carboxymethylated polysaccharides of *Ganoderma lucidum*. J Agr Food Chem 57, 10565-10572.

Wang, L., Ridgway, D., Gu, T., Moo-Young, M., 2005a. Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. Biotechnol Adv 23, 115-129.

Wang, S. Y., Hsu, M. L., Hsu, H. C., Tzeng, C. H., Lee, S. S., Shiao, M. S., Ho, C. K., 1997. The antitumour effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T-lymphocytes. Int J Cancer 70, 699-705.

Wang, Y.-J., Yao, S.-J., Guan, Y.-X., Wu, T.-X., Kennedy, J., 2005b. A novel process for preparation of (1→ 3)-β-D-glucan sulphate by a heterogeneous reaction and its structural elucidation. Carbohydr Polym 59, 93-99.

Wang, Y., McNeil, B., 1995. Production of the fungal exopolysaccharide scleroglucan by cultivation of *Sclerotium glucanicum* in an airlift reactor with an external loop. J Chem Technol Biotechnol 63, 215-222.

Wasser, S., 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl Microbiol Biotechnol 60, 258-274.

Wasser, S. P., 2010. Reishi. Encyclopedia of dietary supplements 2nd Edition. In: Coates, E., Betz, P. M., Blackman, J. M., Cragg, M. R., Levine, G. M., Moss, M., J. & White, Healthcare, J. D. I. (Eds.), vol. Chapter 78.

Watanabe, I., Miyata, N., Ando, A., Shiroma, R., Tokuyasu, K., Nakamura, T., 2012. Ethanol production by repeated-batch simultaneous saccharification and fermentation (SSF) of alkali-treated rice straw using immobilized *Saccharomyces cerevisiae* cells. Bioresour Technol 123, 695-698.

Wei, Z. H., Duan, Y. Y., Qian, Y. Q., Guo, X. F., Li, Y. J., Jin, S. H., Zhou, Z. X., Shan, S. Y., Wang, C. R., Chen, X. J., Zheng, Y., Zhong, J. J., 2014. Screening of Ganoderma strains with high polysaccharides and ganoderic acid contents and optimization of the fermentation medium by statistical methods. Bioprocess Biosyst Eng 37, 1789-1797.

Weng, Y., Xiang, L., Matsuura, A., Zhang, Y., Huang, Q., Qi, J., 2010. Ganodermasides A and B, two novel anti-aging ergosterols from spores of a medicinal mushroom *Ganoderma lucidum* on yeast via UTH1 gene. Bioorg Med Chem 18, 999-1002.

Wenyan, J., Jingbo, Z., Zhongqiang, W., Shang-Tian, Y., 2014. Stable high-titer n-butanol production from sucrose and sugarcane juice by *Clostridium acetobutylicum* JB200 in repeated batch fermentations. Bioresour Technol, 0960-8524.

White, S., McIntyre, M., Berry, D. R., McNeil, B., 2002. The autolysis of industrial filamentous fungi. Crit Rev Biotechnol 22, 1-14.

Williams, D. L., Pretus, H. A., McNamee, R. B., Jones, E. L., Ensley, H. E., Browder, I. W., 1992. Development of a water-soluble, sulfated (1-->3)-beta-D-glucan biological response modifier derived from *Saccharomyces cerevisiae*. Carbohydr Res 235, 247-257.

Wittier, R., Baumgartl, H., Lübbers, D. W., Schügerl, K., 1986. Investigations of oxygen transfer into *Penicillium chrysogenum* pellets by microprobe measurements. Biotechnol Bioeng 28, 1024-1036.

Wymelenberg, A. V., Minges, P., Sabat, G., Martinez, D., Aerts, A., Salamov, A., Grigoriev, I., Shapiro, H., 2006. Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. Fungal Gen Bio 43, 343-356.

Xiao, J. H., Chen, D. X., Wan, W. H., Hu, X. J., Qi, Y., Liang, Z. Q., 2006. Enhanced simultaneous production of mycelia and intracellular polysaccharide in submerged cultivation of *Cordyceps jiangxiensis* using desirability functions. Process Biochem 41, 1887-1839.

Xu, J. W., Xu, Y. N., Zhong, J. J., 2010. Production of individual ganoderic acids and expression of biosynthetic genes in liquid static and shaking cultures of *Ganoderma lucidum*. Appl Microbiol Biotechnol 85, 941-948.

Xu, Z., Chen, X., Zhong, Z., Chen, L., Wang, Y., 2011. *Ganoderma lucidum* polysaccharides: immunomodulation and potential anti-tumor activities. Am J Chin Med 39, 15-27.

Yang, F.-C., Liau, C.-B., 1998a. Effects of cultivating conditions on the mycelial growth of *Ganoderma lucidum* in submerged flask cultures. Bioprocess Eng 19, 233-236.

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

Yang, F. C., Liau, C. B., 1998b. Effects of cultivating conditions on the mycelial growth of *Ganoderma lucidum* in submerged flask cultures. Bioprocess Eng 19, 233-236.

Yang, F. C., Liau, C. B., 1998c. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. Process Biochem 33, 547-553.

Yang, F. C., Yang, M. J., Cheng, S. H., 2009. A novel method to enhance the mycelia production of *Ganoderma lucidum* in submerged cultures by polymer additives and agitation strategies. J Taiwan Inst Chem E 40, 148-154.

Yang, H., Min, W., Bi, P., Zhou, H., Huang, F., 2013. Stimulatory effects of Coix lacryma-jobi oil on the mycelial growth and metabolites biosynthesis by the submerged culture of *Ganoderma lucidum*. Biochem Eng J 76, 77-82.

Yang, H. l., Wu, T. x., Zhang, K. c., 2004. Enhancement of mycelial growth and polysaccharide production in *Ganoderma lucidum* (the Chinese medicinal fungus, Lingzhi) by the addition of ethanol. Biotechnol Lett 26, 841-844.

Yang, X., Wang, B., Cui, F., Tan, T., 2005. Production of lipase by repeated batch fermentation with immobilized *Rhizopus arrhizus*. Process Biochem 40, 2095-2103.

Yim, C. Y., Bastian, N. R., Smith, J. C., Hibbs, J. B., Jr., Samlowski, W. E., 1993. Macrophage nitric oxide synthesis delays progression of ultraviolet light-induced murine skin cancers. Cancer Res 53, 5507-5511.

Yoon, S. Y., Eo, S. K., Kim, Y. S., Lee, C. K., Han, S. S., 1994. Antimicrobial activity of *Ganoderma lucidum* extract alone and in combination with some antibiotics. Arch Pharm Res 17, 438-442.

Yup Jang, M., Ryul Ryu, W., Hwan Cho, M., 2002. Laccase production from repeated batch cultures using free mycelia of Trametes sp. Enzyme Microb Tech 30, 741-746.

Zaidman, B. Z., Yassin, M., Mahajna, J., Wasser, S. P., 2005. Medicinal mushroom modulators of molecular targets as cancer therapeutics. Appl Microbiol Biot 67, 453-468.

Zhang, G. L., Wang, Y. H., Ni, W., Teng, H. L., Lin, Z. B., 2002. Hepatoprotective role of *Ganoderma lucidum* polysaccharide against BCG-induced immune liver injury in mice. World J Gastroentero 8, 728-733.

Zhang, H. N., Lin, Z. B., 2004. Hypoglycemic effect of *Ganoderma lucidum* polysaccharides. Acta Pharmacol Sin 25, 191-195.

Zhang, J., Liu, Y.-j., Park, H.-s., Xia, Y.-m., Kim, G.-s., 2012. Antitumor activity of sulfated extracellular polysaccharides of *Ganoderma lucidum* from the submerged fermentation broth. Carbohydr Polym 87, 1539-1544.

Zhang, S., Nie, S., Huang, D., Huang, J., Wang, Y., Xie, M., 2013. Polysaccharide from *Ganoderma atrum* evokes antitumor activity via toll-like receptor 4-mediated NF-κB and mitogen-activated protein kinase signaling pathways. J Agr Food Chem 61, 3676-3682.

Zhang, Y., Chen, X., Qi, B., Luo, J., Shen, F., Su, Y., Khan, R., Wan, Y., 2014a. Improving lactic acid productivity from wheat straw hydrolysates by membrane integrated repeated batch fermentation under non-sterilized conditions. Bioresour Technol 163, 160-166.

Zhang, Y., Chen, X., Qi, B., Luo, J., Shen, F., Su, Y., Khan, R., Wan, Y., 2014b. Improving lactic acid productivity from wheat straw hydrolysates by membrane integrated repeated batch fermentation under non-sterilized conditions. Bioresource Technology.

Zhao, B., Wang, L., Li, F., Hua, D., Ma, C., Ma, Y., Xu, P., 2010. Kinetics of d-lactic acid production by *Sporolactobacillus* sp. strain CASD using repeated batch fermentation. Bioresour Technol 101, 6499-6505.

Zhao, S. F., Ye, G., Fu, G. D., Cheng, J. X., Yang, B. B., Peng, C., 2011a. *Ganoderma lucidum* exerts anti-tumor effects on ovarian cancer cells and enhances their sensitivity to cisplatin. Int J Oncol 38, 1319-1327.

Zhao, W., Xu, J. W., Zhong, J. J., 2011b. Enhanced production of ganoderic acids in static liquid culture of *Ganoderma lucidum* under nitrogen-limiting conditions. Bioresour Technol 102, 8185-8190.

Zhong, J. J., 2010. Recent advances in bioreactor engineering. Korean J Chem Eng 27, 1035-1041.

Zhou, X., Zhong, J. J., 2009. Effect of initial ammonium concentration on taxoid production and biosynthesis genes expression profile in suspension cultures of *Taxus chinensis* cells. Eng Life Sci 9, 261-266.

Zhu, L. W., Zhong, J. J., Tang, Y. J., 2010. Multi-fed batch culture integrated with three-stage light irradiation and multiple additions of copper ions for the hyperproduction of ganoderic acid and Ganoderma polysaccharides by the medicinal mushroom *Ganoderma lucidum*. Process Biochem 45, 1904-1911.

Zhu, M., Chang, Q., Wong, L. K., Chong, F. S., Li, R. C., 1999. Triterpene antioxidants from *Ganoderma lucidum*. Phytother Res 13, 529-531.

Znidarsic, P., Pavko, A., 2001. The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter. Food Technol Biotech 39, 237-252.