Growth and Therapeutic Properties of *Agaricus blazei*

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By

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This is dedicated to Mum, Oma, Nana, and Opa

Abstract

Agaricus blazei is an immensely popular edible medicinal mushroom in Japan, mainly due to traditional beliefs that it has antitumour properties, and the ability to stimulate the immune system. The majority of scientific research carried out on *A. blazei* has demonstrated that polysaccharides extracted from mushrooms fruiting bodies are the active agents for the purported anticancer properties. Limited research has been undertaken with regards to liquid-cultured mycelium and liquid culture filtrates of *A. blazei* and this investigation involved screening these products for novel medicinal properties.

The growth of the fungus was examined on solid agar for the propagation of mycelium which was used for inoculation of liquid cultures. Optimal fungal storage conditions were also determined. The optimum growth temperature and pH of *A. blazei* on yeast malt agar were 28-29 °C and pH 5-6, respectively. Storage of mycelial plugs at -80°C in 10% glycerol was found to maintain viability of the fungus for up to 36 months. Subsequent growth trials in liquid media found that a temperature of 30°C over a pH range of 4-8 were optimal for mycelial growth. Glucose as a carbohydrate source produced the most mycelium, while sucrose was most favourable for exopolysaccharide production. The exopolysaccharides produced were identified as mannan-protein complexes.

Organic solvent extracts of liquid-cultured mycelia were examined, and antibacterial activity was identified against *Branhamella cattarhalis* and some Gram-positive bacteria, particularly for dichloromethane and ethyl acetate extracts. *In vitro* cytotoxic activity was observed against cervical cancer and lymphoma cell lines, particularly for hexane and dichloromethane extracts. Protein extracts of *Agaricus blazei* liquid-cultured mycelium were found to have antiviral effect against simian rotavirus, although the cause of the observed effect was likely due to the presence of a trypsin inhibitor in the extract. Cytotoxic activity of the protein extract was also identified against lymphoma cells *in vitro*. Exopolysaccharides from liquid culture filtrate of *A. blazei* did not have an effect on rotavirus, but had a marked cytotoxic effect on lymphoma cells, and to a lesser extent of cervical cancer cells. Thus, the cytotoxic effect was found to be specific for tumour cells.

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Declaration

I hereby declare, that to the best of my knowledge, this thesis contains neither material which has been accepted for the award to the candidate of any other degree or diploma, or any material previously published or written by another person, except where due reference is made in the text of the thesis. Where the work is based on joint research or publications, the thesis discloses the relative contributions of the respective workers or authors.

Danielle R. Tilmanis

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List of Abbreviations

Standard chemical symbols and SI units are used without definition.

°C	Degrees Celsius
ω-6APFA	Omega -6 polyunsaturated fatty acid
%V	Percentage cell viability
1xEPS	Exopolysaccharide extract precipitated with an equal volume of ethanol
2xEPS	Exopolysaccharide extract precipitated with twice the volume of ethanol
5-FU	5-fluorouracil
ABM	*Agaricus blazei* Murrill
ABWE	Water extract of *Agaricus blazei*
ACM	Australian Collection of Micro-organisms
ADH	Alcohol dehyrogenase
AGS	Human stomach cancer cell line
ASC	*Agaricus blazei* spent compost
ATCC	American Type Culture Collection
BHI	Brain heart infusion broth
BM	Basal medium
BRM	Biological response modifier
BSA	Bovine serum albumin
CC_{50}	Cytotoxic concentration 50
CDA	Czapek dox agar
CDA	Corn meal agar
CMM	Complete mushroom media
CO_2	Carbon dioxide
CPE	Cytopathic effect
DCM	Dicholoromethane
DEAE	Diethylaminoethyl
dH_20	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EC_{50}	Effective concentration 50
EDTA	Ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
EPS	Exo-polysaccharide
FBS	Foetal bovine serum

FMDV	Foot and mouth disease virus
F-PDA	Fresh potato dextrose agar
GABA	Gamma-aminobutyric acid
GLUC	Glucose liquid media
HBA	Horse blood agar
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IC_{50}	Inhibitory concentration 50
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
MEA	Malt extract agar
MEB	Malt extract broth
MEM	Minimum Essential medium
MeOH	Methanol
MeOH-P	Methanol extract precipitate
MeOH-S	Methanol extract supernatant
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimal Inhibitory Concentration
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NA	Nutrient agar
NB	Nutrient broth
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
PAGE	Polyacrlamide gel electrophoresis
PBS	phosphate buffered saline
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PMP	PDB-malt-peptone broth
p-s	penicillin-streptomycin solution
PSK	Krestin
PSPC	Polysaccharide-protein complex

RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMC	Rat peritoneal mast cells
RPMI	RPMI 1640 cell media formulation
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
SF-MEM	serum-free minimum essential media
SF-MEM-T	serum-free minimum essential media + porcine trypsin
SMA	Sabouraud maltose agar
SUCR	Sucrose liquid media
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor alpha
WA	Wort agar
WEE	Western equine encephalitis
YMA	Yeast malt agar
YMB	Yeast malt broth

Chapter One

Introduction

1.1 Natural products in drug discovery

Natural products have played, and continue to play, an important role in the drug discovery process, particularly in the areas of cancer and infectious disease treatment (Cragg *et al*., 1997). The last thirty years in particular, has seen an increase in the herbal medicine industry and the use of natural products for treating disease (Hamburger & Hostettmann, 1991). The stimulus for this interest has been the recognition of the value of traditional medical systems, as many sources of such natural products are historical in nature, or are known from folklore (Pandey, 1998). Sources of natural products, most notably plants and plant products, are vast. Compounds from tree bark, leaves, vegetables, and Chinese and Indian herbs and have been shown to be effective in treating an array of diseases and aliments (Pandey, 1998). Due to the fact that there is a constant search for effective new drugs, and the requirement for novel approaches due to the increasing number of pathogenic microorganisms displaying resistance to the drugs that are currently available, the vast biodiversity of the natural world represents a great untapped reservoir for novel drugs awaiting discovery (Farnsworth, 1995).

Many useful drugs have been obtained through screening of natural products (Shu, 1998). In addition to the original compounds from natural sources, modifications to natural products for the purpose of enhancing activity, or reducing side-effects or toxicity, became a major focus in the late 19th century, and this strategy continues today (Sneader, 1985; Newman *et al*. 2003). Aspirin, for example, was one of the earliest of these chemically modified natural products, while other significant compounds derived from renowned medicinal plants include alkaloids such as morphine, atropine and codeine, which are still in high use today (Foye *et al*., 1995). Clark *et al*. (1996) reported that on average, two or three antibiotics launched each year are derived from microorganisms. Newman & Cragg (2007), carried out a review of the new registered

chemical entities with medical indications (obtained from numerous drug registries and annual reports), over the period 1981-2006. They concluded that 65% were either biological in nature, from a natural source, derived from a natural product, or a natural product mimic. In particular, 47% of anticancer dugs from the 1940 s to date were reported to be either natural products or directly derived from natural products. In addition, the antiinfective area of drug development is very dependant on natural products and their structures (Newman & Cragg 2007, Harvey, 2008).

Plants have been a major focus for bioactive compound discovery from natural resources (Qin & Xu, 1998; Cowan, 1999). There are environmental concerns however, because the plants used traditionally as medicines are disappearing, causing ecologists to question the sustainability of using natural resources as a source of medicines (Nigg & Seigler, 1992; Cragg *et al*., 1993). Fungi can provide a more ecologically-friendly alternative to higher order plants. They can be artificially cultivated and are more sustainable due to the shorter time required for growth and, in addition to the potential bioactivity of structural compounds contained within their cell walls, they also produce a vast array of secondary metabolites that may be exploited for drug development.

1.2 Fungi as a source of bioactive compounds

Traditionally fungi have been included in the plant kingdom, but in 1969 fungi were classified as their own separate kingdom, Kingdom Fungi (Whittaker, 1969). They do not contain chlorophyll like green plants, and as a result do not manufacture their own food. Instead, they obtain nutrients by digesting organic matter and nutrients produced by other organisms (Whittaker, 1969; Arora, 1985). The Kingdom Fungi is divided into two divisions, Zygomycota (moulds) and Eumycota (septate fungi). The Eumycota can be further divided into four subdivisions Acomycota (cup fungi), Basidiomycota (club fungi), Deuteromycota (imperfect fungi) and Lichens.

Perhaps the most significant discovery of a bioactive compound from fungi was the antimicrobial activity observed in the fungus *Penicillium notatum* by Sir Alexander Fleming (Fleming, 1929). This led to the development of the antibiotic penicillin (Florey *et al*. 1949) and other β-lactam antibiotics which are still commonly used today.

In addition, statin drugs are commonly used for reducing risks associated with coronary heart disease (Quinn, 1999). These compounds, the first being mevastatin, were discovered from a species of *Penicillium* (Endo *et al*. 1976). This fungus belongs to the Deuteromycota or imperfect fungi, and since discovery and eventual use of these compounds as drugs, attention has been given to investigating bioactive properties and their production in other fungi from this subdivision (Fukushima *et al*., 1991; Al-Hilli & Smith, 1992; Lee *et al*., 1999; Rodrigues *et al.*, 2000). In the last 20 years however, it is another subdivision of fungi that has been the focus of much scientific investigation into its potential bioactive properties, the subdivision Basidiomycota, or more generally, mushrooms.

1.3 Medicinal Properties of Mushrooms

The term mushroom is often used to describe the reproductive structure or fruiting body of a fungus. Not all fungi produce fruiting bodies, for example moulds and yeasts, and the term is often applied in a more restricted sense to fleshy fungi like the cultivated mushroom, whose fruiting bodies bear spores on radiating blades called gills (Arora, 1986; Borchers *et al*., 1999). The fruiting bodies of Basidiomycetes fungi are the sites for the production of spores which are located on the exterior of microscopic club-shaped cells called basidia (Stamets & Chilton, 1983). Basidiomycetes fungi are also referred to as macrofungi as their fruiting bodies are large enough to be seen by the naked eye and can be picked by hand (Chang & Miles, 1992). Macrofungi can be divided into four groups: 1) Edible, e.g. *Agaricus*, *Lentinus*, *Pleurotus*; 2) Medicinal, e.g. *Ganoderma*; 3) Poisonous, e.g. *Amanita*; and 4) miscellaneous, the properties of these are not well defined.

Traditionally, mankind has regarded mushrooms as a special class of food, and for thousands of years humans have consumed mushrooms as part of a normal diet, and as a delicacy, due to their desirable flavour and aroma (Chang & Buswell, 1996). In addition, the medicinal properties of mushrooms have been recognized by many ancient and modern cultures. The ancient Greeks believed that mushrooms provided strength for warriors in battle, the Romans regarded them as the Food of the Gods , served only on festive occasions, while the Chinese have long regarded mushrooms as a health food

and an the elixir of life (Chang & Miles, 1989). Oriental cultures, in particular, have long recognised and embraced the use of mushroom species for their medicinal properties. Some of the ailments traditionally treated with mushrooms are haemorrhoids and stomach ailments (*Auricularia* spp.), gastric ulcers (*Hericium erinaceus*), while *Volvariella volvacea* has been thought to assist with lowering blood pressure wound healing (Chang & Buswell, 1996). The recent application of modern chemical analysis techniques has provided scientific support for these earlier empirical observations, with a vast number of studies over the last thirty years isolating and identifying the compounds responsible for the reported medicinal properties.

The main focus of medicinal mushroom research over the last three decades has been on the isolation of numerous polysaccharides and polysaccharide-protein complexes that have anticancer effects (Ikekawa *et al*., 1969; Fujii *et al*., 1978; Ito & Shimura, 1980; Kawagishi *et al*., 1990a; Mizuno *et al*., 1995; Mizuno, 1999; Wasser & Weis, 1999; Ooi & Liu, 2000; Reshetnikov *et al*., 2001; Gonzaga *et al*., 2009). The biological activity of these compounds has been reported to be immunomodulation, or enhancement of the host s immune system. They are often referred to as host defence potentiators, or biological response modifiers (BRM s) (Ooi & Liu, 2000). These polysaccharides are essentially β-glucans, and have been isolated from mushroom fruiting bodies, liquid cultured mycelium, and liquid culture filtrate (Wasser & Weis, 1999; Reshetnikov *et al*., 2001; Mizuno, 2002). Three drugs commercialised in Japan, and the first to be developed from medicinal mushrooms polysaccharides with immunomodulating activity, are Krestin (PSK) from *Coriolus versicolor* liquid cultured mycelium, Lentinan from the fruiting bodies of Shiitake (*Lentinus edodes)*, and Sonifilan (schizophyllum) from the culture fluid of *Schizopyllum commune* (Mizuno, 1993). Lentinan and Sonifilan are pure β-glucans, whereas Krestin is a protein-bound β-glucan (Ooi & Liu, 2000). Lentinan and Sonifilan are used for the treatment of stomach cancer and cervical cancers respectively, while Krestin is used to treat cancer of the digestive organs, lung cancer, and breast cancer (Mizuno, 1999).

A majority of research has focussed on antitumor polysaccharides, however there are many other medicinal properties attributed to substances found in the mushroom fruiting bodies, liquid cultured mycelium or culture fluid. These medicinal properties

include antiviral activity (Cochran, 1966; Suzuki *et al.*, 1979; Jong & Donovick, 1989; Chihara, 1992; Gao, 2003); antimicrobial activity (Tsukagoshi *et al*., 1984; Chihara, 1992; Hirasawa *et al*., 1999; Rosa *et al.*, 2003); antioxidant activity (Hu *et al*., 1992; Liu *et al*., 1997; Yang *et al*., 2002; anti-inflammatory activity (Czarnecki and Grzybek, 1995); hypocholesterolemic effects (Yamamura & Cochran 1976; Breene, 1990; Cheung, 1996); antidiabetic and hypoglycemic activity (Hikino *et al*., 1985; Tomoda *et al*., 1986); blood pressure regulation (Adachi *et al*., 1988; Kabir & Kimura, 1989; Wang *et al*., 1996b); hepatoprotective effects (Yeung *et al*., 1994; Ooi, 1996; Barbisan *et al*., 2002), and anti-thrombotic activity (Mizuno, 1995b).

1.4 Medicinal Properties of *Agaricus blazei*

Agaricus blazei is a mushroom of Brazilian origin that has been used traditionally as a functional food in its native habitat, the mountain district in Sao Paulo, south eastern Brazil, for many years (Mizuno, 1995). It was brought to Japan due to its alleged health effects, and is now widely used in oriental countries as a natural therapy for the prevention and treatment of cancer (Firenzuoli *et al*., 2007). It is immensely popular in Japan as a nutriceutical , a term which is defined as refined/partially defined food extractive, which is consumed in the form of capsules or tablets as a dietary supplement (Chang & Buswell, 1996). Mushroom nutriceuticals are widely used in China and Japan, and are exported to many other countries around the world. A simple search for the term *Agaricus blazei* on the internet will yield dozens of websites marketing these *Agaricus blazei* nutriceuticals, mainly in the form of tablets or teas containing pulverised dried fruiting bodies, or liquid extracts. There is little scientific basis for some of the therapeutic claims made about these products, such as the ability of *Agaricus blazei* to aid skin repair, assist collagen production, or improve erectile dysfunction. However, claims of the ability of *Agaricus blazei* to treat cancer and stimulate the immune system have been substantiated as a result of the amount of scientific research that has been carried out in the last 30 years.

Much research has been undertaken into the anticancer effects of polysaccharides and polysaccharide-protein complexes from *A. blazei* fruiting bodies, and there are substantial results to support the claims of anticancer activity. Polysaccharides from *A.*

blazei have been found to be effective *in vivo*, against a range of tumours including sarcomas or cancers of connective tissue, such as Sarcoma 180 and Meth A fibrosarcoma in mice (Kawagishi *et al*., 1989; 1990a; Mizuno *et al*., 1990a; 1990b; Itoh *et al*., 1994; Fujimiya *et al*., 1998a; 1998b; Ohno *et al*., 2001; Chen *et al*., 2006; Gonzaga *et al*., 2009). They have also been reported to be active against a range of mouse model carcinomas and cancers that effect epithelial cells, such as Ehrlich ascites carcinoma and Shionogi carcinoma (Ito *et al*., 1986; 1997). In addition, clinical studies in humans have reported that *A. blazei* can alleviate the adverse side effects of chemotherapy (Ahn *et al*., 2004; Okamoto, 2007).

A. blazei fruiting body polysaccharides has also demonstrated an effect on cancer cells *in vitro*, with reports of cytotoxicity against a range of tumour cells (Fujimiya *et al*., 1999; Ito & Itoh, 2000; Shen *et al*., 2001; Liu *et al*., 2006; Chen *et al*., 2007; Kim *et al*., 2009). The mode of action for the cytotoxic activity observed has been reported to be due to the induction of apoptosis (Shen *et al*., 2001; Liu *et al*., 2006; Kim *et al*., 2009). The anticancer effects of *A. blazei* have also been attributed immunomodulation effects, with many of the studies reporting *in vitro* effects (Sorimachi *et al*., 2001b; Hashimoto *et al*., 2002; Kuo *et al*., 2002; Yuminamochi, 2007). Many of these studies were carried out using extracts of *Agaricus blazei*, the active component of which has not been identified.

More recently, an increased number of reports have been published regarding other medicinal effects of *A. blazei* such as antimutagenicity (Martins de Oliveira *et al*., 2002; Guterrez *et al*., 2004; Angeli *et al*., 2006; 2009), and antioxidant activity (Ker *et al*., 2005; Li *et al*., 2007; Soares *et al.,* 2009).

While the majority of reports regarding the medicinal properties of *Agaricus blazei* have focussed on substances isolated from fruiting bodies, there are a few reports of activity from liquid cultured mycelium and culture filtrate, although the range of activity in extracts and compounds from these sources has not been widely studied. A complete review of extracts and compounds from different sources of *A. blazei* and their reported medical properties is given in Chapter 2.

1.5 Aims of this Investigation

The main aim of this research was to investigate liquid cultured mycelium and liquid culture filtrates of *A. blazei* as a potential source for bioactive compounds.

There were several objectives for this investigation. Firstly, to examine the growth of *A. blazei* on solid agar in order to establish appropriate growth parameters such as temperature, pH, and media formulation for mycelial growth (Chapter 4). Healthy mycelial growth on agar is important for the requirement of establishing suitable short- and long-term storage methods for the fungus throughout the course of the investigation, and also for the preparation of inocula for liquid cultivation studies.

A second objective was to compare and establish suitable growth parameters for liquid cultivation of *A. blazei* (Chapter 5), and to examine the bioactive properties of any liquid culture products of *A. blazei*, as they have not been as widely examined as those from fruiting bodies of the fungus. Therefore, an investigation of the growth conditions was undertaken to optimise the production and yield of biomass and exopolysaccharides in order to obtain sufficient material for further bioactivity studies.

In addition, extracts obtained via solvent extraction of the liquid-cultured mycelium will be screened for novel bioactive properties (Chapter 6). The compounds predicted to be extracted via solvent extraction, particularly with non-polar solvents, are likely to be smaller steroidal compounds rather than larger molecules such as polysaccharides, which have been more extensively studied. The antibacterial activity of other mushroom species has been widely examined, though few reports of such activity are available for *A. blazei*.

There have been few, if any, reports of bioactivity in protein extracts from *A. blazei*, and therefore protein extracts from *A. blazei* mycelium will be screened to determine whether they possessed cytotoxic or antiviral activity (Chapter 7). Other mushroom species have been examined for this activity and found to contain substances with these medicinal effects, therefore similar experiments were carried out to determine whether these properties also existed in *Agaricus blazei* mycelium extracts.

The final objective was to screen exopolysaccharides from *A. blazei* liquid culture filtrate for cytotoxic activity and antiviral activity (Chapter 8). There have been few reports of bioactivity in *A. blazei* exo-polysaccharide extracts from liquid culture media, with the majority of research concentrating on antitumour and immunomodulation effects of these compounds. Therefore, the potential of culture filtrate as a source of polysaccharides with other bioactive properties was examined.

The review in Chapter 2 focuses on *Agaricus blazei*, and includes a history of the mushroom, followed by an overview of the current short-term and long-term storage methods for fungal mycelium. An outline of cultivation methods for the fungus is provided, including a discussion of the effect of growth parameters on the production of bioactive compounds using these cultivation methods. The compounds responsible for the bioactive properties observed in *Agaricus blazei* are discussed, and an overview of the reported medicinal properties of the mushroom to date is given.

Chapter Two

Literature review

2.1 History of *Agaricus blazei*

Agaricus blazei Murill (ABM) is known in Japan as Himematsutake, Agarikusutake, or Kawarihiratake. In Brazil it is referred to as Cogmelo de Deus, God s mushroom , Cogmelo do Sol, Sun mushroom , and Cogumelo de Vida mushroom of life . In China it is known as Ji Song Rong, and it is also commonly known as Royal Sun Agaricus, and almond mushroom. It was first discovered in Florida, USA, in 1944, but its main natural habitat is a small village called Piedade, in the mountainous district in the province of Sao Paulo, Brazil (Mizuno, T., 2002). An indication of its medicinal properties was first realised in 1960 by Takatoshi Furumoto, a grower and researcher living in the area. He observed that there was an extremely low occurrence of adult diseases in the Piedade region, a region were *Agaricus blazei* was regularly consumed by locals as regular part of their diet (Borzacchini, 1995).

Native to Brazil, it was imported to Japan in 1965, and identified as *Agaricus blazei* Murrill in 1967 by the Belgian botanist Heinemann (Mizuno, T. 1995a). It was first cultured at Iwade Mycological Institute in Japan in 1975 (Sorimachi & Koge, 2008), and since 1978 a vast amount of time and money has been invested towards the development a suitable artificial large scale cultivation of *A. blazei* for commercial purposes (Mizuno, T. 1995a). This initially proved difficult, because of the need to mimic the optimal growth conditions experienced in the mushroom s native habitat. In Piedade, temperatures of 25°C to 35°C were typically experienced during the day, and 20°C to 25°C at night, with an average humidity of 80%. Through much effort, these problems were overcome, and *A. blazei* is now cultivated in Japan were its fruiting bodies and health products are well-marketed, and very popular as a health supplement. As a result of this, in addition to the cost and duration for cultivation, *A. blazei* mushrooms are quite expensive.

Medicinal mushrooms have an established history of use in traditional oriental therapies. *Agaricus blazei* has been traditionally believed to relieve physical and emotional stress, stimulate the immune system, improve the quality of life in diabetics, reduce cholesterol, prevent osteoporosis, treat circulatory and digestive problems and most importantly, fight cancer (Mizuno, T. 1995a). After the arrival of *A. blazei* in Japan, Dr Shoji Shibata, a university professor, and Dr Tetuo Ikegawa of the National Cancer Centre first investigated the pharmacological effects of the mushroom, and reported their results at a convention of the Japan Pharmacological Association and the Japan Cancer Association. Their studies revealed that glucans contained in the mushroom significantly activated the immune system of mice (Borzacchini, 1995).

Since then, *A. blazei* has received much attention by research groups keen to examine the spectrum of biological activity of the mushroom both *in vivo* and *in vitro* (Sorimachi & Koge, 2008). The most notable conclusion resulting from research carried out over the last 30 years, is that *A. blazei* fruiting bodies have a high content of polysaccharides that demonstrate antitumour and immunomodulatory activity. This conclusion supports the traditional belief that *A. blazei* possesses health-giving properties. More recently, other bioactive properties of *A. blazei* have been identified as a result of scientific research, including antimutagenic and antioxidant activity.

2.2 Systematics of *Agaricus blazei*

Agariaceae is the type family of the order Agaricales Clem. of the subclass of Higher Basidiomycetes (Singer, 1986). It contains 918 species belonging to 51 genera (Kirk *et al*. 2001). Agaricus species are saprophytes and are widely distributed over geographical regions ranging from the tropics to the boreal regions, in a variety of habitats from alpine meadows, to salty and sandy seashores, to conifer woodlands (Geml *et al*. 2004). Of the 918 species of Agaricaceae, only a small number have been screened for their medicinal and nutritional properties, although it is internationally known as a source of biotechnological components (Didukh *et al*. 2003). The economically significant member of the family is *Agaricus bisporus*, commonly called the white button mushroom, which is cultivated on an immense industrial scale, with an

output yield amounting to 32% of total world mushroom production in 1997 (Miles & Chang, 1997). More recently, its worldwide production has decreased slightly due to the increased interest and production of medicinal mushrooms such as *Lentinus edodes* (the Shiitake mushroom), and *Pleurotus ostreatus* (the oyster mushroom), although it remains one of the world s six most important mushroom species (Chang, 1999). *Agaricus blazei* is gaining popularity and has established cultivation centres in China and Brazil, although the primary market is Japan where it has developed into a $600 million industry since 1995 (Mizuno, 2000).

The association of the name *Agaricus blazei* Murill with the Brazilian mushroom has been attributed to P. Heinemann (Kerrigan, 2005) however more recent data would suggest that the mushroom found in Brazil and Japan could be phylogenetically and biologically the same species as *Agaricus subrufescens* Peck from North America (Kerrigan, 2005). Recently, Wasser *et al*. (2002a) published an analysis indicating that the Brazilian *Agaricus blazei* Murill, differs from *A. blazei* ss. Heinemann, and concluded that the endemic North American species, *A. blazei* ss. Murrill, and the widely cultivated *A. blazei* ss. Heinemann are two different species. They proposed that *A. blazei* ss. Heinemann should be thought of as a new species *Agaricus brasiliensis*. This botanical dispute is as yet unresolved, so for the purposes of this study, *A. blazei* ss. Heinemann, *A. blazei* ss. Murrill, and *Agaricus brasiliensis* were investigated, and literature pertaining to both species was considered.

2.3 Preservation and Maintenance of Fungi

Appropriate storage methods for fungi are of extreme importance in order to maintain genetic stability of the pure mycelium (Chang & Miles, 1989). It is important to preserve and maintain the fungus throughout the course of investigation. If a strain is cultivated continuously for a long period of time, it may lose the genetic characteristics that it originally possessed (Oei, 1996). It is therefore necessary to store the strain under investigation in an appropriate manner, so that fresh cultures can routinely be resuscitated while the investigation is being carried out. This ensures that all samples used are genetically identical to the original culture. Cells in fungi can degenerate due to the lack of oxygen, lack of nutrients, infections, changes in substrate pH, or the

accumulation of undesirable metabolites (Oei, 1996). While there are generally accepted good practices for the storage of fungi, optimal storage conditions and storage time for a particular fungal species can also vary depending on the strain (Mayzumi *et al*., 1997). Cultures of edible mushrooms can be preserved as either spores or vegetative mycelia, although the latter is more common, due to time consuming fertility checks being required when spore cultures are stored (Chang & Miles, 1989). There are many methods available for the storage of fungi, depending on whether short-term (up to six months) or longer term storage is required.

The most common short-term storage method for fungal mycelium involves periodic agar to agar transfer. It requires few specialised laboratory facilities, is cheap, and requires only a short period of time for the generation of fresh cultures of new mycelium. The method involves subculturing mycelial plugs on stored on solid agar at 2-8°C every 3 to 12 months (Chang & Miles, 1989). While most fungal cultures can be maintained by this method by subculturing at two to six month intervals (Ando, 1997) some species of fungi are more susceptible to degeneration using this method and must be subcultured at two to four week intervals (Onions, 1983). This method does, however have its disadvantages, and is not advised for long term storage. The process of repeated subculturing can pose a problem due to the increased likelihood of contamination, and the possibility of the alteration of fungal characteristics due to spore production, pathogenicity or metabolite production (Ando, 1997). In addition, the storage of mycelium under refrigeration may result in degradation of the mycelium given that the fungus may still grow at these low temperatures (Oei, 1996).

Storage of mycelium on agar slants under mineral oil has long been regarded as an appropriate method for short term and long term storage (Wernham, 1946) and cultures can be stored for periods of as long as 25 years (Onions, 1983). Cultures can be stored using this method at room temperature or 4°C (Oei, 1996), although the depth of the oil layer is crucial for survival and genetic integrity of the fungal mycelium. If the oil layer is too deep, the diffusion of oxygen and carbon dioxide is restricted (Onions, 1983).Conversely, if the oil layer is too shallow, exposed hyphae may cause the mycelium to dry out. One major disadvantage of this method is that recovery of viable mycelium can be a slow procedure after extended periods of storage of fungal cultures.

Freezing fungal mycelium is the most common method for long term preservation, with storage of mycelium in liquid nitrogen being the most widely used method for long term storage of fungal species (Smith, 1982). This is an expensive storage method, however cryogenic freezing is a preferred for storage of fungal mycelium because growth of the fungus is completely retarded (Smith, 1983; Oei, 1996). However, the variability in the responses of strains of the same species to this storage method has been demonstrated by Smith (1993), and it is therefore a requirement that fungal strains should be investigated as to their resilience to this storage method before using this method for long term preservation. Another storage method is that of freezing the cultures at -80°C (Ito & Yokoyama (1983; Ando, 1997; Kitamoto *et al*. 2002). Ito & Yokoyama (1983) have described an easy and effective method of storing mycelial plugs in a 10% glycerol solution at -80°C, which has been shown to successfully maintain nearly eight hundred strains of various species of fungi for at least a year (Ando, 1997). Kitamoto *et al*. (2002) found that 66 different species of fungi could remain viable for 5 years using this method. The use of glycerol ensures that cultures do not dry out, and a fast recovery time is observed for the growth of viable mycelium following long term storage, compared to the other methods described for preservation of frozen fungal mycelia.

2.4 Cultivation of *Agaricus blazei*

2.4.1 Cultivation for fruiting body formation

The native habitat of *Agaricus blazei* is a specific region of Brazil, where the natural environment is optimal for the formation of mushroom fruiting bodies. This area is characterised by typically higher temperatures and humidity than is usually required for fruiting body production of other mushrooms (Iwade & Mizuno, 1997) and requires markedly different nutritional requirements to that of its mass produced close relative, *Agaricus bisporus* (Bechara *et al*., 2008). Efforts have been made to optimise the growth conditions for artificial cultivation of mushroom bodies, particularly in Japan

where demand for *A. blazei* fruiting bodies is high (Iwade & Mizuno, 1997; Higaki *et al*., 1997; Takei *et al*. 2001; Yoshimoto *et al*., 2005).

An important first step in the artificial production of *Agaricus blazei* is the identification and preparation of a suitable compost in which the fruiting bodies will grow. The compost substrate must be of the appropriate carbon to nitrogen ratio, and provide nutrients required for mycelial growth and eventual mushroom production. The most suitable compost medium for *Agaricus blazei* is based on rice straw or bagasse, the squeezed residue of sugar cane (Iwade & Mizuno, 1997; Takei *et al*., 2001). Yoshimoto *et al*., (2005) examined the pharmacological effects of *A. blazei* fruiting bodies grown on different compost substrates including the top shoots of sugar cane, rice straw, wheat straw, broad leaf tree bark and substrate previously used for *Pleurotus ostreatus* cultivation. Fruiting bodies grown on the top shoots of sugar cane demonstrated more anti-platelet aggregation activity, and inhibited IL-8 gene expression stimulated by TNF-α, than the fruiting bodies grown on the other substrates examined. A nitrogen source is also required, commonly ammonium sulphate, although alternative nitrogen sources such as soya bean meal and urea have also been used, although substrates containing higher proportions of urea have been shown to be less productive for growth and quality of *A. blazei* fruiting bodies (Kopytowsji & Minhoni, 2004). A post fermentation step is also recommended to destroy various germs and harmful insects by increasing the temperature of the compost to 60°C, and raising the humidity to 80-90%, to create a favourable environment for the propagation of *A. blazei* hyphae (Iwade & Mizuno, 1997).

The prepared mushroom compost is then inoculated with spawn, mushroom mycelium growing on a grain medium such as rye grain, in a sterile atmosphere containing about 50% moisture. The exact method for producing spawn will depend on the facilities available to the mushroom cultivator. Healthy spawn free from infection and contamination is distributed evenly throughout the mushroom compost.

Once the hyphae have had time to sufficiently propagate throughout the mushroom bed, a soil covering or casing layer is then distributed over the bed. This should be free-flowing fresh soil, low in fertiliser content, with good water retention and air

permeability (Iwade & Mizuno, 1997). The mushroom bed is then watered every 7-10 days to prevent excessive drying of the soil covering. Fruiting bodies develop 40-50 days after the inoculation of spawn. The optimum temperature during this developmental period is 22-25°C, and a humidity of 70-85%, with proper ventilation to ensure oxygen is provided to the growing fruiting bodies, while carbon dioxide can be effectively cleared (Iwade & Mizuno, 1997). Once fruiting body formation is initiated, their full development should be complete in 10 days. Mushrooms should be picked when they are five centimetres high and the pileus has not yet begun to open.

2.4.2 Cultivation on agar media

Mushroom mycelium is commonly grown on solid agar medium for a number of applications, including the production of inoculum for the preparation of spawn, production of inoculum for liquid media, for short term storage via periodic subculturing, and to examine growth characteristics of different species of fungi such as suitable carbon and nitrogen sources, optimal growth temperature, and optimal pH for mycelial growth. Given the characteristic radial growth of fungi on agar, the diameter of the colony is typically used as a reliable measure of growth (Brancato & Golding, 1953).

Fungal growth typically occurs in three stages. The first is a lag or inductive phase for the development of the necessary digestive enzymes for growth in the solid or liquid substrate. This is followed by a logarithmic growth phase where rapid growth of mycelium is observed as the fungus utilises the nutrients in the growth substrate, and finally a stationary phase where the growth rate either reaches a plateau or decreases mainly due to the exhaustion of the nutrients required for growth. This may also occur as a result of a build up of unfavourable metabolites which may cause an inhibitory pH environment for fungus growth (Lin *et al*., 1973).

Different fungal species have particular optimal culture conditions, either on agar, or liquid media, and preliminary investigations of these must be carried out prior to extensive studies of other aspects of a particular species such as the production of bioactive compounds. *Agaricus blazei* obtained from different global sources has been

shown to exhibit different growth characteristics, and taxonomic investigations of various strains of *A. blazei* highlighting these differences has been reported by Wasser *et al*. (2002).

There have been limited studies reporting the growth of *Agaricus blazei* on solid agar. Eguchi *et al.* (1994) examined the optimal growth conditions for the CJ-01 strain of *Agaricus blazei* Murril on solid agar. They reported that the optimal temperature and pH for dense radial growth of *Agaricus blazei* Murril CJ-01 on malt-yeast agar, was 26 °C and pH 6.0 respectively. Further examination of the effect of the various carbon and nitrogen sources on the radial growth of this strain of *A. blazei* indicated that glucose and sucrose were the most effective carbon sources, while casamino acid was the best nitrogen source for optimal growth. In, addition of the vitamin inositol to the solid medium enhanced the radial growth of *A. blazei*.

Wasser *et al*. (2002), used wort agar medium when studying the cultural characteristics of the *A. blazei* strain ATCC 76739 from Fungi Perfecti (USA). The media was adjusted to pH 6.5 and the plates incubated at 26°C for fungal growth. The ATCC recommends the use of yeast malt agar, for growth of this strain, at an incubation temperature of 24°C.

2.4.3 Cultivation in liquid media

In recent years, submerged fermentation of *Agaricus blazei* has emerged as an alternative and popular method for production of artificially cultured *A. blazei* fruiting bodies. This has been shown to be an expensive cultivation method, and hence the resulting mushrooms are also expensive (Lin & Yang, 2006; Zou, 2006; Firenzuoli, *et al*. 2007). Liquid culture provides a cheaper alternative for mycelium production for commercial purposes. The growth of mushroom mycelium in liquid culture has several advantages over the cultivation of fruiting bodies particularly when the cultivation is taking place for the extraction of bioactive compounds. In solid substrate cultivation methods, the preparation of the growth substrate and the subsequent production of mushroom fruiting bodies can take many months, depending on the species of mushroom; artificial cultivation of *Agaricus blazei* for example, requires several months

for the production of fruiting bodies (Iwade & Mizuno, 1997). In contrast, typical liquid culture cultivation of fungal mycelium require only between one to two weeks incubation of culture flasks for the production of sufficient biomass for extraction (Jia *et al*. 2002; Zou *et al*. 2002; Kim *et al*. 2004). Liquid culture methods also require less space, and provide a cultivation method that can be kept relatively free contamination (Bae *et al*. 2000). Moreover, liquid culture media can be easily altered in composition, and subjected to different environmental conditions in order to study the effect of these variables on mycelium and bioactive compound production.

Temperature is one of the most important environmental factors influencing the extent of mycelial growth of fungi (Brancato & Golding, 1953). Different research groups have studied liquid culture growth of *Agaricus blazei* at various temperatures, for example 27-28°C (Lin & Yang, 2006; Shu *et al*., 2007) and 30-32°C (Akazawa *et al*. 2002; Fan *et al*. 2003). The temperature required for optimal liquid culture growth of *Agaricus blazei* is typically higher than that required for other *Agaricus* species. This is consistent with the fact that the native habitat of *Agaricus blazei* is a region of Brazil where high temperatures and humidity are typically experienced.

The initial pH of the culture medium is another important parameter affecting mycelial growth of fungi in liquid culture. It may affect cell membrane function, cell morphology and structure and uptake of various nutrients (Fang & Zhong, 2002). While it has been demonstrated that mycelium from a number of fungal species can grow over a wide range of pH values (Lonergan *et al*. 1993) the initial pH of the culture medium affects the production of mycelial biomass (Fang & Zhong, 2002). In addition, Yang & Liau (1998) have shown that optimal pH for growth of one fungal species can depend on the components in the cultivation media. The optimal pH reported for liquid cultivation of *Agaricus blazei* varies between pH 5.0 and pH 6.0 (Zou *et al*. 2002; Kim *et al*. 2004; Fan *et al*. 2003; Lin & Yang, 2006; Hamedi *et al*. 2007), depending on the strain, growth temperature, and culture medium utilised.

The composition of the culture medium is extremely important for the growth of fungi as it provides the nutrients for proliferation of the fungal mycelium. Carbohydrates are an essential component and studies have been carried out to determine effective carbon sources for growth of *A. blazei* mycelium. Fan *et al*. (2003) examined the use of

glucose, fructose, maltose and sucrose as carbon sources for *A. blazei* biomass production, and found that fructose yielded the highest dry mycelial mass, although the consensus appears to be that glucose is the most appropriate carbon source for growth of *A. blazei* mycelium in liquid culture (Jia *et al*. 2002; Kim *et al*., 2004; Zou, 2006; Shu & Xu., 2007). It has been found that the most prolific growth of *A. blazei* mycelium occurs when yeast extract or peptone is used as a nitrogen source (Jia *et al*., 2002; Fan *et al*., 2003; Shu *et al*., 2004).

The length of specific growth phases for *A. blazei* in liquid culture has been reported to be influenced by the inoculum used. Yoneyama *et al*. (1997a) found that while *A. blazei* grew to a greater extent using aeration culture than shake culture in potato dextrose agar, a long inductive phase was observed when the liquid medium was inoculated with mycelium grown on solid agar. This long inductive phase could be shortened markedly by the use of mycelium grown in liquid media as the inoculum (Yoneyama *et al*. (1997b)). They also reported that *A. blazei* adapted quickly to respiration of dissolved oxygen from respiration of gaseous oxygen, as required for liquid culture growth, compared to *L. edodes* and *P. ostreatus*.

2.5 Effect of growth parameters on the production of bioactive substances

In the last five to six years, more attention has been given to the effect with which environmental growth parameters and culture medium ingredients play in determining the production and type of bioactive metabolites, particularly polysaccharides, by *A. blazei* in liquid culture (Fan *et al*. 2003; Shu *et al*., 2004; Kim *et al*. 2004; Zou, 2006; Hamedi *et al*. 2007; Liu *et al*. 2007). Since many of these metabolites are produced in small amounts, manipulation of the liquid culture media and growth parameters has the potential to increase the quality and quantity of bioactive species being produced.

The production of polysaccharides by *A. blazei* in liquid culture has been studied by Fan *et al*. (2003). It was found that the parameters that increase polysaccharide production are, in general, different to those required for optimal biomass production. These

authors found that while fructose and peptone were the optimal carbon and nitrogen sources, respectively, for biomass formation, incorporating sucrose and yeast extract in the culture medium increased the level of polysaccharide production in liquid culture. Similar results were obtained by Zou (2006) who identified sucrose as the preferential carbon source for polysaccharide formation, as opposed to glucose for maximum biomass production. Shu & Xu (2007) however, found that the use of glucose as a carbon source for *A. blazei* liquid cultivation was beneficial for maximum levels of biomass and polysaccharide production. Starch has also been identified as the optimal carbon source for polysaccharide formation by *A. blazei* in liquid culture by Hamedi *et al*. (2007) and Liu *et al*. (2007).

The temperature at which liquid cultivation of *A. blazei* is performed has also been shown to influence the amount of polysaccharide production. Shu *et al*. (2007) demonstrated that while an incubation temperature of 28°C produced the most biomass under the conditions studied, a temperature of 30°C was optimal for maximum polysaccharide formation. A temperature of 24°C was found to be the optimal temperature for production of bioactive polysaccharides that stimulated the release of TNF-α in macrophage cells. Furthermore, they reported that polysaccharides isolated from fungi grown in a lower temperature range (20-24°C), had a higher β-glucan content, average molecular weight and greater bioactivity than the polysaccharides isolated from fungi grown in liquid media at higher temperatures (30-34°C). Hamedi *et al*. 2007, and Liu *et al*. 2007, have also reported that increased levels of polysaccharides result if the temperature of incubation is lower than that required for optimal biomass production.

It has been demonstrated that the pH of the culture media can influence the level of polysaccharide production by *A. blazei* in liquid culture. It can also influence the polysaccharide molecular weight distribution and level of bioactivity. Shu *et al*. (2004) examined polysaccharide formation in pH controlled batch cultures of *A. blazei*, and found that polysaccharides with higher molecular weights, β-glucan content and bioactivity (stimulation of TNF-α in macrophage cells) were obtained in cultures incubated under lower pH conditions (between pH 4.0-5.0). Conversely,

polysaccharides with lower molecular weight, β-glucan content, and bioactivity were obtained from cultures incubated under higher pH conditions (pH 6.0 to 7.0), however higher yields of polysaccharides were obtained under these pH conditions. pH values between 6.0- 7.0 have been reported to produce optimum levels of polysaccharide production, whereas optimum levels of biomass production were achieved at pH levels of 5.0-6.0 (Kim *et al.* 2004; Hamedi *et al*. 2007).

The polysaccharides produced by *Agaricus blazei* in liquid culture have been identified as beta-glucans (Fan *et al*., 2003; Shu *et al*., 2004, 2007), and mannan-protein complexes (Ito *et al*., 1980; Mizuno, 1995). While fruiting bodies have been the main focus of polysaccharide research on *A. blazei*, the polysaccharide content of liquid cultured mycelia has been reported to be nearly equal to that of fruiting bodies (Jia, *et al*. 2002).

The extent of production of other compounds secreted by *A. blazei* in liquid culture is also influenced by the composition of the growth media. Zou (2006) examined the production of polysaccharides and ergosterol by *A. blazei* in liquid media. Ergosterol is considered an important chemical in the pharmaceutical industry, as it is a precursor of vitamin D, can be converted to cortisone. It is usually commercially produced by yeast fermentation (Tianwei *et al*. 2003). *A. blazei* can produce high concentrations of this compound, and therefore this fungus may be considered a potential new natural source of ergosterol. A glucose carbon source was found to produce higher levels of ergosterol than sucrose, with the extent of production being concentration dependent, with glucose concentrations exceeding 40 g L^{-1} resulting in decreased levels of ergosterol being produced.

2.6 Bioactive compounds in *Agaricus blazei*

A large amount of the research has been carried out on various extracts of *Agaricus blazei*, and a range of bioactive compounds have been identified. The majority of these compounds include polysaccharides, polysaccharide-protein complexes, steroids,

nucleic acids and lipids. The polysaccharides and polysaccharide-protein complexes will be discussed here in detail.

2.6.1 Polysaccharides and polysaccharide protein complexes

Polysaccharides are found in all parts of the mushroom, including the fruiting bodies and liquid cultured mycelium. They have also been isolated from the culture medium itself when *A. blazei* is grown in liquid culture. The majority of the published literature reports data pertaining to the bioactive polysaccharides extracted from the fruiting bodies of *A. blazei*, however a smaller number of articles report these bioactive compounds being extracted from liquid cultured mycelium, and to a lesser extent, liquid culture filtrate.

The bioactivity of most of the polysaccharides extracted from *Agaricus blazei*, and indeed other mushroom species, is the enhancement of the body s immune response. This effect is generally accepted as the basis of the antitumour effects observed for these molecules, rather than having a direct effect on tumour cells, and therefore they are often referred to as biological response modifiers (BRM s) (Wasser & Weis, 1999b). Other activities often observed for BRM s, such as anti-inflammatory, hypotensive, and antiviral activity, highlights the potential of these molecules for development of new drugs (Mizuno *et al*. 1995a). Extensive reviews have been published regarding the antitumour effects of polysaccharides from *A. blazei* and other fungi and there modes of action (Mizuno *et al*. 1995; Mizuno, T. 1996, 1999; Wasser, 2002b).

2.6.2 Steroids and phenols

Kawagishi *et al*. (1988) isolated six steroids from an acetone extract of *A. blazei* fruiting bodies, three of which were found to be cytotoxic to cervical cancer cells (HeLa) and showed complete inhibition of cell growth at concentration of 8-63 μg mL^{-1}. One of compounds, Compound 2, was found to be a novel steroid and was considered to be an oxidised product of ergosterol.

Numerous novel steroidal compounds have been isolated from methanol extracts of cultured mycelia of *A. blazei* (Hirotani *et al*. 1999, 2000a, 2000b, 2001, 2002a, 2002b, 2002c). These compounds are described to be structurally related to ergosterol and two of these compounds, blazeispirol A, and blazeispirol C have since been found to be very cytotoxic against hepatoma cells HepG2 and Hep3B *in vitro* (Su *et al.* 2008). In addition, ergosterol has been isolated from *A. blazei* fruiting bodies and oral administration of the compound was found to retard tumour growth in Sarcoma180 bearing mice (Takaku, *et al*. 2001).

Marked antioxidant properties, discussed later in Section 2.7.4, have been attributed to the high phenolic content of *A. blazei* fruiting bodies (Huang & Mau 2006; Tsai & Mau 2007; Soares *et al*. 2009).

2.6 Bioactivity and Medicinal properties of substances from *Agaricus blazei*

2.7.1 Anti-cancer effects

There are three main modes of antitumour activity identified in compounds from *A. blazei*. These are host-mediated antitumour activity, immunomodulation, and cytotoxicity against tumour cell lines *in vitro*. Compounds from *A. blazei* demonstrating these properties, most notably polysaccharides, are discussed in the following sections.

2.7.1.1 Host-mediated anti-tumour activity

Host-mediated antitumour activity, which is identified by a significant, sometimes complete reduction in the size of solid tumours *in vivo*, has been examined in *Agaricus blazei* polysaccharides and extracts using various mouse tumour models, particularly transplanted Sarcoma 180.

Polysaccharides of various structures isolated from *A. blazei* fruiting bodies have demonstrated marked antitumour activity against transplanted Sarcoma 180 in mice.

Kawagishi *et al*. (1989) isolated a polysaccharide composed of (1→6)-β-D glucan-protein complex that resulted in 99% inhibition of Sarcoma 180 tumour growth in mice. They further examined formolysis of the complex into its respective polysaccharide and protein components, and found that the pure glucan did not exhibit anti-tumour activity indicating the protein component is necessary for the potent activity of the complex (Kawagishi *et al*. 1990). Itoh *et al*. (1994) further investigated this (1→6)-β-D glucan-protein complex (designated FIII-2-b) by examining its antitumour effect against Meth A fibrosarcoma in BALB/c mice, both alone, and in combination with 5-fluorouracil (5-FU), a pyrimidine analogue used widely for a large spectrum of human cancers. They found that FIII-2-b alone demonstrated a moderate reduction in tumour size, but it was not as significant as 5-FU alone. When both FIII-2-b and 5-FU were administered together however, the antitumour effect observed was greater than either of the two compounds alone, indicating its possible use in combination cancer therapy. There are several reports of synergenistic effects of *A. blazei* polysaccharides combined with other anticancer compounds (Ebina *et al*. 2004; Liu *et al*. 2006; Gonzaga *et al*. 2009).

Mizuno *et al*. (1990a) isolated four water soluble polysaccharides from *A. blazei* fruiting bodies that showed 93-97% inhibition using Sarcoma 180/ICR-JCL mice. These polysaccharides consisted of several structures and different properties including a (1→6)- (1→3)-β-D glucan, an acidic (1→6)- (1→4)-α-D glucan, an acidic (1→6)-(1→3)-α-D glucan, and an acidic RNA-protein complex. The latter is of particular interest as it is rare to observe host-mediated antitumour activity in mushroom nucleic acids (Mizuno, T. 1995). The same research group also examined the effect of water insoluble polysaccharides obtained with successive extraction of fruiting bodies with 1% ammonium oxalate, 5% sodium hydroxide (NaOH), 20% NaOH, and 5% lithium chloride-dimethylacetamide (Mizuno *et al*. 1990b). Heteroglycan-protein complexes extracted with ammonium oxalate had weak antitumour properties, while remarkable antitumour activity was reported in a (1→6)-β-D glucan-protein and a xyloglucan-protein complex isolated with 5% NaOH. Antitumour activity was also identified in a glucoxylan isolated from the 20% NaOH fraction, while no activity was detected in substances fractionated with 5% lithium chloride-dimethylacetamide.

Two polysaccharides have been isolated from *A. blazei* fruiting bodies by extraction with NaOH (Ohno *et al*. 2001). Their primary structures consisted of (1→6)-β-D glucan and both showed antitumour activity against the solid form of Sarcoma 180 in mice. In addition they carried out an examination of the glucosidic linkages and found that a high degree of branched 1,3-β-1,3-β-glucan segments form the active centre of antitumour activity.

Fujimiya *et al*. (1998a) examined water soluble and insoluble, ammonium oxalate and water-ethanol insoluble extracts from fruiting bodies of *A. blazei* on Meth A tumour cell growth in mice. Intra-tumoral administration of the extracts resulted in antitumour activity in all fractions, with the ammonium oxalate extract demonstrating the highest activity. Oral administration of these fractions however resulted in no tumour inhibition. They observed, however, that acid treatment of the ammonium oxalate fraction, while retaining its antitumour activity via intra-tumoral administration, also showed significant inhibition by oral administration prior to, but not at the time of, tumour inoculation. The authors hypothesized that the acid treatment increased absorption from the digestive system, leading to increased levels of the active component in the blood stream that could then travel to the tumour site. In a subsequent study, Fujimiya *et al*. (1998b) also discovered that the acid treated fraction also inhibited Meth A tumour cell growth *in vitro*. Fractionation of the ammonium oxalate extract found the most active component was an (1→4)-α-D glucan with (1→6)-β branching. Interestingly, acid treatment of the fruiting bodies themselves prior to extraction, led to the isolation of extracts exhibiting nearly identical antitumour activity via intra-tumoral administration, as those fractions acid treated after extraction (Fujimiya *et al*. 2000). Both extracts also exhibited antitumour activity via oral administration.

A polysaccharide isolated from *A. blazei* liquid cultured mycelium that exhibited a remarkable antitumour activity against Sarcoma 180, was found to have a completely different structure to the common (1→6)-β-D glucan structure of antitumour polysaccharides isolated from *A. blazei* fruiting bodies (Mizuno *et al.* 1999a). The polysaccharide was primarily a glucomannan with a main chain of β-1,2-linked D-mannopyranosyl residues, and β-D-glucopyranosyl residues as a side chain and

demonstrated an inhibition ratio of 89% in implanted tumours. Another polysaccharide complex with a broad spectrum of antitumour activity has been isolated from *A. blazei* mycelium (Ito *et al*. 1997). The polysaccharide-protein complex, designated ATOM , showed marked antitumour activity after 10 days treatment against Sarcoma 180 at doses of 10 and 20 mg^{-1} kg^{-1} day^{-1}, while doses of the complex at 50 and 100 mg^{-1} kg^{-1} day^{-1} were active against Ehrlich ascites carcinoma, Shionogi carcinoma 42, and Meth A fibrosarcoma.

There are a few reports of antitumour polysaccharides being obtained from the culture filtrate of *Agaricus blazei*, and again its structure was found to be markedly different to those isolated from mushroom fruiting bodies. Mizuno T, (1995) identified a mannan-protein complex from concentrated culture filtrate *A. blazei* that showed high antitumour activity. Fan *et al*. (2003), identified exopolysaccharides from *A. blazei* culture fluid that significantly inhibited the growth of Sarcoma 180 in mice.

2.7.1.2 Immunomodulation

The second mode of antitumour action observed in *A. blazei* compounds and extracts is immunomodulation, or stimulation of the immunological functions of the host. Compounds that demonstrate this mechanism of action (a cell-mediated immune response) are also referred to as immunopotentiators, or biological response modifiers (BRM s) (Ooi & Liu, 1999). Some compounds from mushrooms have the ability to induce gene expression and production of cytokines including TNF-α, interleukin-1 (IL-1), interleukin-8 (IL-8), and interleukin-6 (IL-6). These cytokines, produced by monocytes, macrophages and other cell types, stimulate cytotoxic T cells against tumours, and induce production of antibodies by B lymphocytes among other biological effects associated with stimulation of the immune system (Daba & Ezeronye 2003). While this activity is regarded as a main factor responsible for the antitumour activity observed in *Agaricus blazei* polysaccharides discussed in the previous section, studies have also been reported examining immunomodulatory activities without undertaking antitumour based investigations.

Various extracts from *A. blazei* have been reported to stimulate both the *in vitro* and *in vivo* release of cytokines. Water extracts from *A. blazei* fruiting bodies have been reported to induce TNF-α, IL-8, and nitric oxide secretion by macrophages derived from rat bone marrow *in vitro* (Sorimarchi *et al*. 2001b), stimulate T-cells and macrophages to release IL-1β and IL-6 resulting in augmentation of antibody production in mice (Nakajima *et al*. 2002), and increase serum immunoglobulin G levels, T-cell numbers, TNF-α secretion and phagocytic activity in Balb/cByJ mice (Chan *et al*. 2007). Furthermore, Yuminamochi *et al*. (2007) demonstrated that oral administration of *A. blazei* extracts augmented natural killer cell activation in mice through Il-12-interferon-gamma production, while increased cytokine production has been observed in keratinocytes treated with low molecular weight products from *A. blazei* (Sugita *et al*. 2008). Levels of IL-1α, granulocyte macrophage-colony stimulating factor, and TNF-α increased in a dose dependant manner, while not affecting the proliferation of keratinocytes.

Solvent extracts of *A. blazei* fruiting bodies have also demonstrated immunomodulatory activity (Kaneno *et al*. 2004). Ehrlich tumour bearing mice treated with the hexane extract for 10 days showed restored natural killer cell activity, while 30 days of treatment with dichloromethane or methanol extracts resulted in higher specific antibody production.

In one of the few accounts of bioactivity in *A. blazei* exopolysaccharides, Zhang *et al*. (2007), reported that phagocytic function of macrophages was increased in Kunming mice administered *A. blazei* exo-polysaccharide at concentrations of 5-30 mg kg^{-1}.

2.7.1.3 *In vitro* Cytotoxic activity

The third main mode of antitumour activity observed for *Agaricus blazei* is cytotoxicity against tumour cell lines *in vitro*. Various polysaccharides, particularly those extracted from *A. blazei* fruiting bodies, have been shown to inhibit the growth of tumour cells using various cell proliferation assays.

A majority of the polysaccharides found to have cytotoxic activity have exhibited this activity against leukemic cell lines. The most commonly employed method for the determination of cytotoxic activity is the colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Various polysaccharides and polysaccharide-protein complexes have been found to be active against leukemic cell lines *in vitro* using this method (Shen *et al*. 2001; Chu & Chen, 2006; Chen *et al*. 2007). Ito & Itoh (2000) reported that a (1→6)-β-D-glucan-protein complex had antiproliferative action against human lymphoid leukaemia Molt4B cells at concentrations of 400-2000 μg mL^{-1}, using ^{3}H-thymidine and ^{3}H-uridine incorporation assays. Some *A. blazei* fruiting body extracts have also shown activity against leukaemia cells (Jin *et al*. 2007; Cai *et al*. 2008; Kim *et al*. 2009). Polysaccharides from *A. blazei* fruiting bodies have also been shown to have activity against other tumour cell lines such as liver cancer cells (Liu *et al*. 2006), human thyroid carcinoma (Shimizu *et al*. 2002), while a polysaccharide-protein complex was found to inhibit the growth of Meth A fibrosarcoma cells (Fujimiya *et al*. 1999).

There have been a few accounts of cytotoxic activity in polysaccharides from liquid cultured mycelium, and homogenised liquid cultures of *Agaricus blazei*. Jiang & Gu (2005) extracted soluble polysaccharides from *A. blazei* mycelia with hot water. One of these extracts consisted mainly of mannose, and another two consisted mainly of glucose and mannose. They were tested against rat colon carcinoma cells (CT-26) and human liver carcinoma cells (Bel-7402) with varied results for each of the three polysaccharides. The mannose polysaccharide was found to be devoid of cytotoxic activity in Bel-7402 cells, while the two glucose-mannose compounds significantly inhibited the growth of these cells. While all three polysaccharides inhibited CT-26 cells, they did so with varying degrees, with the glucose-mannose polysaccharides showing the greatest level of inhibition of cell proliferation.

Yu *et al*. (2008) reported that the broth fractions from *Agaricus blazei,* (a mixture of both mycelium and culture filtrate) consisted of approximately 50% polysaccharide (consisting of 1.2% (1,3)-β-D-glucan). It demonstrated *in vitro* cytotoxic activity against both androgen-dependant (LNCaP) and androgen-independent (DU145 and

PC3) human prostate cancer cell lines at concentrations of 400 μg mL^{-1} and 800 μg mL^{-1}.

Steroidal compounds extracted from *A. blazei* have also been shown to inhibit the growth of tumour cells *in vitro*. As previously mentioned, Kawagishi *et al*. (1988) isolated six steroids from an acetone extract of the fruiting bodies of *A. blazei*, three of which demonstrated cytotoxic activity against human cervical cancer cells (HeLa) *in vitro*. Cells were incubated in the presence of the test compounds for 72 hours, after which the number of viable cells was determined by staining using the Giemsa method. The minimum concentrations that gave complete growth inhibition of HeLa cells were between 8-63 μg mL^{-1}. The steroids were also found to be active against hepatoma cells. Two steroidal compounds isolated from hexane/ethyl acetate extracts of cultured mycelia of *A. blazei*, blazeispirol A, and blazeispirol C, have been reported to be very cytotoxic to hepatoma cells HepG2 and Hep3B *in vitro* (Su *et al.* 2008). Blazein , a novel steroid isolated from *A. blazei* fruiting bodies inhibited the growth of human lung cancer cells (JU99) and stomach cancer cells (KATOIII) *in vitro* (Itoh *et al*. 2008).

The main mode of action for the cytotoxic activity observed for various *A. blazei* polysaccharides, steroids, and extracts has been identified as via the induction of apoptosis (Fujimiya *et al*. 1999; Chu & Chen, 2006; Liu *et al*. 2006; Chen *et al*. 2007; Jin *et al*. 2007; Cai *et al*. 2008; Yu *et al*. 2008 ; Itoh *et al*. 2008; Kim *et al*. 2009).

2.7.2 Anti-genotoxic activity

Genotoxic agents are those that have a deleterious action on cell genetic material, and are often implicated in carcinogenesis (Derelanko, M.J. 1995). They may be mutagenic in that they cause mutations in DNA or other genetic material. These mutations may induce cancer and such agents are termed carcinogens. They also may demonstrate clastogenic activity in which they cause or induce disruption to, or cause breakages in chromosomes (Putman *et al*. 1995).

Methods for the determination of anti-mutagenic or anticlastogenic activity may involve examining the effect of test substances on their ability to protect against DNA damage

by known carcinogens, often described as a chemoprotective effect, while other methods commonly used are the Ames test, comet assays, and in particular *in vitro* and *in vivo* micronucleus tests, due to the strong correlation between micronuclei formation and chromosome breakage (Putman *et al*. 1995). Chemoprotective effects and anticlastogenic activity have been identified in numerous polysaccharides and extracts from *A. blazei* using various methods.

The chemoprotective effects of *A. blazei* polysaccharides and aqueous extracts have been identified against known DNA damaging agents such as methyl methanesulfonate (Menoli *et al*. 2001; Martins de Oliveira *et al*. 2002; Bellini *et al*. 2003; Guterrez *et al*. 2004), ethylmethane sulfonate (Machado *et al*. 2005), cyclophosphamide (Delmanto *et al*. 2001; Sun *et al*. 2006; Matuo *et al*. 2007), diethylnitrosamine (Barbisan *et al*. 2002, 2003), benzo[a]pyrene (Angeli *et al*. 2009), and aminoanthracene (Matuo *et al*. 2007) using micronucleus tests, and comet assays. Different degrees of antimutagenic activity have been observed for the same test compound when using different methods for assessment of the activity (Menoli *et al*. 2001; Delmanto *et al*. 2001; Guterrez *et al*. 2004; Angeli *et al*. 2009).

There have been reports of similar mutagenic activities in *A. blazei* extracts from different lineages (Bellini *et al.* 2003) however this is not always the case, with some accounts to the contrary. For example, Guterrez *et al*. (2004) examined extracts from three lineages of *A. blazei* and demonstrated varying degrees of antimutagenic activity, with extracts from some lineages being found to be devoid of the activity observed in other lineages. Furthermore, Delmanto *et al*. (2001) found that while extracts of a mixture of lineages of *A. blazei* showed marked antimutagenic activity extracts from single lineages did not. Overall, the numerous reports of antimutagenic activity in polysaccharides and extracts from *A. blazei* might contribute to an anticarcinogenic effect.

2.7.3 Anti-oxidant activity

Hydroxyl radicals and other reactive oxygen species such as superoxide anions can induce carcinogenesis and other diseases by acting as initiating or promoting agents,

and enhance the degenerative processes of ageing (Lee *et al*. 2001; Soares *et al*. 2009). Anti-oxidant compounds that eliminate, or reduce the effects of these compounds can be beneficial in preventing these diseases. Different antioxidant compounds may act through different mechanisms *in vivo* (Soares *et al*. 2009) therefore different methods for the evaluation of antioxidant potential of compounds are often employed. Some of these include examining reducing power, free radical scavenging ability, and ferrous ion chelating ability.

Ker *et al*. (2005) examined the radical scavenging ability and ferrous ion chelating power of five polysaccharide fractions extracted from *A. blazei* mycelia. Two of the polysaccharide fractions demonstrated pronounced radical scavenging activity, while three other polysaccharide fractions had ferrous ion chelating powers three times the level of the positive control. Ferrous ion chelating ability was observed in polysaccharides with lower molecular weights 600-900 kDa, while free radical scavenging ability was associated with the solubility of the polysaccharides.

Methanol extracts from *A. blazei* fruiting body extracts, were examined for their antioxidant activity by Huang & Mau (2006). They investigated both γ- irradiated and non-irradiated fruiting bodies in their investigation, as γ- irradiation is often used as a method for the prevention of contamination in mushroom fruiting bodies. They reported that reducing powers of both irradiated and non-irradiated fruiting body extract were comparable. All extracts showed excellent scavenging abilities against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and hydroxyl radicals. Total phenols were the major naturally occurring antioxidant components in *Agaricus blazei* methanol extracts, and they concluded that 20 kGy of irradiation, did not affect activity of antioxidant components in *A. blazei* methanol extracts of fruiting bodies.

Soares *et al*. (2009) also examined *A. blazei* methanol extracts for antioxidant properties, and compared the relative activity, and phenolic content, of young fruiting bodies to that of the mature mushroom fruiting bodies. They identified strong antioxidant properties in extracts from fruiting bodies of both stages of maturity in four complementary assays: radical scavenging ability, reducing power, chelating ability for ferrous ions, and inhibition of lipid peroxidation. The antioxidant activity and total

phenolic content was comparable for both young and mature fruiting bodies, except the latter demonstrated a greater ability in the chelation of ferrous ions, indicating that compounds other or factors may be responsible for this property.

Tsai & Mau, (2007) tested ethanol and hot water extracts of *Agaricus blazei* fruiting bodies for antioxidant activity by examining radical scavenging ability, reducing power, and ferrous ion chelating ability. Both extracts showed antioxidative properties although their activity varied depending on the method used. The ethanol extracts were most effective in scavenging DPPH radicals, whereas the hot water extracts had greater scavenging ability on hydroxyl radicals, increased reducing power and a greater ability to chelate ferrous ions. Their results were similar to those obtained by Huang & Mau (2006) and Soares *et al*. (2009). They attributed the antioxidant activity to the high proportion of total phenols in the extracts. A correlation has also been observed between total phenolic content and antioxidant activity in other mushroom species (Cheung *et al*. 2003; Turkoglu *et al*. 2007).

2.7.4 Anti-viral activity

The ability for a virus to propagate depends on the metabolic activity of host cells (Eo *et al*. 1999). Potential antiviral agents must be specific for the inhibition of viral replication without affecting the host cell (Oxford *et al*. 1999). There have been limited reports of specific antiviral activity of extracts from both liquid cultured mycelium and the fruiting bodies of *Agaricus blazei*.

Water extracts of the cultured mycelia and fruiting bodies of *A. blazei* have been fractionated with concentrations of ethanol ranging from 0-50%, and tested for *in vitro* antiviral activity against viruses that have cytopathic effects on Vero cells, namely western equine encephalitis virus (WEE), herpes simplex virus (HSV), and polio virus (Sorimarchi *et al*. 2001a). At a test concentration of 100 μg mL^{-1}, all mycelial fractions demonstrated strong antiviral activity against WEE virus, particularly fractions generated from ethanol concentrations of 44% and 50%, which completely inhibited CPE in VERO cells infected with the virus. Slight antiviral activity against HSV was also observed in these fractions, although none of the mycelial fractions displayed

activity against polio virus. Ethanol extracts from *A. blazei* fruiting bodies did not exhibit any antiviral activity against WEE and HSV viruses at the test concentration of 100 μg mL^{-1}. Slight inhibition of CPE was observed against the polio virus after one week of culture, although this effect had disappeared after 2 weeks.

Faccin *et al*. (2007) further investigated *A. blazei* for its ability to prevent polio virus infection *in vitro*. They examined aqueous extracts, ethanol extracts, and an isolated polysaccharide for antiviral activity against polio virus type 1 in Hep-2 cells. A concentration dependant reduction in the number of plaques was observed for all three test solutions, with the ethanol extract being the most effective, showing an 88% reduction in the number of plaques compared to that of the control. The percentage reduction in plaques for the aqueous extract and polysaccharide were 50% and 67% respectively. All test substances were more effective when added during poliovirus infection, although they had little effect on viral adsorption and were not virucidal, suggesting that they act at the initial stages of poliovirus replication.

A study described by Grinde *et al*. (2006) investigated a possible role for *A. blazei* extract in the treatment of five chronic Hepatitis C (HCV) patients who had failed to respond to regular treatment with interferon. The patients were administered 20 mL *A. blazei* extract (Gold label from ACE Co., Ltd., Gifu-ken, Japan) three times a day for 1 week as recommended by the manufacturer. HCV RNA was quantified from samples of the patients blood prior to, after 4 days, and after 7 days treatment with the extract. The authors found that although the average titre of the virus was slightly lower after one week treatment with *A. blazei* extract, the difference was not clearly significant and the extract was deemed unsuitable for the treatment of chronic Hepatitis C.

2.7.5 Anti-bacterial activity

There appear to be very few reports of antibacterial activity in extracts or compounds from *Agaricus blazei*, however there are a number of reports of other species of mushroom that have shown antibacterial properties. The majority of these reports focus on solvent extracts from mushroom fruiting bodies that have been found to be active against a range of Gram positive and Gram negative bacteria (Park *et al.* 1995a, 1995b;

Beltran-Garcia *et al*., 1997; Hirasawa *et al*., 1999; Hatvani, N. 2001; Rosa *et al*. 2003; Eggert C., 1997).

Two previous reports of the antibacterial activity of *A. blazei* involve extracts of the mushroom fruiting bodies. Bernardshaw *et al*. (2005) tested a commercially available aqueous extract of *A. blazei* from ACE Co., Ltd (Gifu-ken, Japan), against *Streptococcus pneumoniae* infections in mice. The aqueous extract was administered orally, either before or with challenge to NIH/OlaHsd mice that had been infected intraperitoneally with *S. pneumoniae* serotype 6B. The extract was reported to protect mice against *S. pneumoniae* infection, and was most effective when given 24 hours prior to inoculation with the bacterium although a protective effect was also observed when administered with challenge. The authors proposed that the antibiotic effect involved the native immune system, as the antibiotic effect against *S. pneumoniae* was not observed *in vitro*, and increased levels of cytokines MIP-2 and TNF-α were detected in the serum of mice receiving the *A. blazei* extract.

Osaki *et al*. (1994) have previously identified a bactericidal compound from a hexane extract of *Agaricus blazei* fruiting bodies that was active against *Salmonella typhimurium* TA100 *in vitro*. The compound responsible for the antibacterial activity was identified as 13-hydroxy *cis*-9, *trans*-11-octadecadienoic acid.

2.7.6 Adjuvant activity

There have been several reports of the effect of *A. blazei* extracts on the ability to enhance immune responses when administered in conjunction with vaccines. Chen *et al*. (2004) investigated whether co-immunization of *A. blazei* extract with hepatitis B virus (HBV) core DNA vaccine could increase the immune response in mice. A significant increase in not only the hepatitis B virus core antigen (HBcAg) specific antibody response, but also T cell proliferation, was observed in mice which received HBcAg DNA vaccine, compared to control mice which received HBcAg alone. Their results suggested that *A. blazei* extract may represent an adjuvant for improving the efficacy of DNA vaccines *in vivo*.

This effect was further examined in 2006 with a report from the same research group (Chen & Shao, 2006). Extract from *A. blazei* fruiting bodies co-administered with foot-and-mouth disease virus (FMDV) DNA vaccine significantly enhanced the anti-FMDV antibody titre, compared to control mice receiving FMDV vaccine only. These results also supported the study by Nakajima *et al*. (2002) in which enhancement of antibody production by *A. blazei* was reported through the increased production of IL-1β and IL-6 from activated macrophages and T cells. In addition to the increased antibody production observed by Chen & Shao (2006), increased T cell proliferation was observed in mice receiving both the FMDV vaccine and *A. blazei* fruiting body extract, indicating once again that *A. blazei* extract possess adjuvant activity for this DNA vaccine.

2.7.7 Anti-anaphylaxis activity

Pathogenesis of allergic and anaphylactic reactions commonly involves the release of histidine from mast cells. Choi *et al*. (2006) examined the effect of a water extract of *Agaricus blazei* (ABWE) on mast cell-mediated anaphylactic reactions and histidine release in induced anaphylactic reactions in mice. The study identified that ABWE significantly inhibited systemic anaphylaxis-like reactions, ear swelling response, and passive cutaneous anaphylaxis reactions. In addition, ABWE was also inhibited induced histidine release from rat peritoneal mast cells (RPMC). These results indicate that *A. blazei* may be beneficial in the treatment of mast cell-mediated anaphylactic reactions.

2.7.8 Anti-hypertensive activity

High blood pressure (hypertension) and elevation of cholesterol in plasma are main risk factors in the development of heart disease. Antihypertensive activity has been examined in gamma-aminobutyric acid (GABA) enriched *A. blazei* extracts from mushroom fruiting bodies (AG-GABA), and liquid cultured mycelium (AG-M) (Watanabe *et al*. 2002). Spontaneously hypertensive rats were fed AG-GABA and AG-M for up to 28 days, after which the blood pressure in these rats was determined to be significantly lower compared to the control group. Blood serum levels of total cholesterol and triglycerides were also reduced. AG-GABA was found to have a higher

anti-hypertensive effect than AG-M. The effect of AG-GABA on blood pressure was then further examined in humans suffering mild hypertension (Watanabe *et al*. 2003). Subjects were given capsules of AG-GABA daily for four weeks after which both systolic and diastolic blood pressure were found to decrease compared to pre-test values and placebo group. No significant difference however, was observed in levels on total cholesterol or glycerides in human subjects.

2.7.9 Anti-diabetic activity

Kim *et al*. (2005) identified that β-glucans and oligosaccharides from *Agaricus blazei* have anti-diabetic activity. β-glucans were obtained with repeated hot water extraction and had molecular weights of 30-50kDa. Oligosaccharides, mainly di- and tri-saccharides, were then derived by hydrolysing the β-glucans obtained with an endo-β-(1→6)-glucanase. Though both β-glucans and oligosaccharides showed anti-hyperglycemic, anti-hypertriglyceridemic, anti-cholesterolemic, and anti-arteriosclerotic activity, indicating anti-diabetic activity in diabetic rats, the oligosaccharides displayed twice the activity of β-glucans.

2.7.10 Anti-thrombotic activity

Omega -6 polyunsaturated fatty acid (ω-6APFA) from *Agaricus blazei*, has been shown to exhibit an anti-thrombotic effect in rats (Zhang *et al*. 2006). Treatment of rats with 0.14 and 0.28 g ω-6APFA kg^{-1} day^{-1} significantly inhibited thrombosis formation, and prolonged blood clotting time compared to the control group, indicating administration of the fatty acid from *A. blazei* can promote blood flow and lower blood viscosity.

2.8 Other uses of *Agaricus blazei*

Agaricus blazei has also been used in the production of alcoholic beverages. Traditionally, *Saccaromyces cerevisiae* (yeast) is the main microorganism used in wine brewing, due to its potent ability to produce alcohol dehydrogenase (ADH) (Ayres *et al*. 1980). It has been discovered that some genera of mushrooms can also produce alcohol

dehydrogenase, including *Agaricus blazei*. Okamura *et al*. (2001) and Okamura-Matsui *et al*. (2003) have reported on the production of wine using the cell free extract of *A. blazei* liquid cultured mycelium, as a substitute for *Saccaromyces cerevisiae*. The cell-free extract had high ADH activity, and using both aerobic and anaerobic conditions the wine produced using *A. blazei* was 8% (1.7M), with a β-glucan content of 0.68%. Another novel use for *A. blazei* includes the use of dietary fibre from fruiting bodies for the production of bread, (Zhang *et al*. 2007).

Yoshimoto, H. (2006) investigated the use of spent compost from *Agaricus blazei* fruiting body cultivation for use as an organic fertiliser for the growth of eggplant, carrot, strawberry and rice. Use of the spent mushroom compost improved the chemical properties of the soil, and had a growth promoting effect, including increasing the content of vitamins, minerals and amino acids of the crops examined.

2.9 Summary

This review summarised the main areas of research carried out on *Agaricus blazei* and its extracts around the world since its discovery, particularly the work performed in Japan and China.

The majority of research has focussed on extracts and compounds from *A. blazei* fruiting bodies, with far less information available regarding the bioactive properties of the liquid cultured mycelium or liquid culture filtrate. More recently, however, a body of information is emerging that describes the bioactive properties of these latter two sources . Liquid cultivation of fungi has the advantage over fruiting body cultivation in that the biomass can be produced in less time and requires less space compared to the vast climate controlled growing houses required for *A. blazei* fruiting body cultivation. This review also highlighted the fact that nutrients in the culture media can profoundly influence the amount of biomass and bioactive compound production. Manipulation of the liquid culture media to optimise the yield of a particular bioactive compound can be achieved with less difficulty than in most methods for the cultivation of fruiting bodies.

The review of the medicinal properties of extracts of *Agaricus blazei* has highlighted the emphasis that has been placed on polysaccharides and polysaccharide-protein complexes from mushroom fruiting bodies. These compounds have demonstrated antitumour or immunomodulatory activity. More recently, the antimutagenic and antioxidant properties of *A. blazei* extracts have been investigated, and the published results would suggest that claims of these properties have a good scientific basis. There is scope for more research into the bioactivity of steroidal compounds and other small molecules from *A. blazei*. Moreover, there have been only a limited number of reports detailing the antibacterial and antiviral properties of *A. blazei*. These important medicinal properties warrant further investigation for the purpose of identifying potential new anti-infective drugs from natural sources.

Chapter Three

Materials and Methods

Freeze-drying (lyophilization) was carried out using a Dynavac freeze drying unit (Dynavac, Hingham, MA, USA). All centrifugation procedures were carried out using a Sorvall RC5C centrifuge (Kendro Laboratory Products, Asheville, NC, USA), unless otherwise stated.

3.1 Chemicals and reagents

All reagents used were of analytical reagent quality unless otherwise specified, and solutions were prepared using de-ionised water (dH_2O). All chemicals used were of analytical reagent (AR) grade and purchased from Sigma Aldrich (St. Louis, MO. U.S.A.) unless otherwise specified.

3.2 Fungi and fungal growth methods

3.2.1 Fungi

Agaricus blazei (ATCC 76739) was obtained from the American Type Culture Collection (ATCC), Manassas, USA. Stock cultures were stored on yeast malt agar (YMA-Difco) slants under sterile mineral oil at 4°C. Agar plugs containing *A. blazei* mycelium were stored at 80°C in vials containing 10 % (v/v) glycerol in water, and recovered at regular intervals for use in experimental procedures.

Agaricus bisporus 885 grain spawn was donated by Chiquita Mushrooms, Victoria, Australia. Samples of grain spawn with healthy growth of *A. bisporus* 885 mycelium were inoculated onto malt extract agar (MEA-Oxoid) plates and incubated at 25°C for 15-20 days until healthy mycelial growth appeared on the surface of the agar. Stock cultures were prepared by subculturing of fungal mycelial plugs on MEA slants. Stock cultures were stored on the MEA slants under sterile mineral oil at 4°C.

Lentinus edodes (ATCC 48857) was obtained from the ATCC (American Type Culture Collection). Stock cultures were stored on MEA slants under mineral oil at 4°C.

Pycnoporous coccineus 1096 was obtained from CSIRO Forest Products, Melbourne. Stock cultures were stored on MEA slants under sterile mineral oil at 4°C.

3.2.2 Media for fungal growth

Culture media and media supplements were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK) unless otherwise specified. All culture media was sterilised by autoclaving at 121°C for 15 minutes unless otherwise stated.

3.2.2.1 Media

Yeast malt agar (YMA) was obtained from Difco (BD Biosciences, NJ, U.S.A.) and contained 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 5 g L^{-1} peptone, 10 g L^{-1} dextrose, and 20 g L^{-1} agar. Yeast malt broth (YMB-Difco) was also used for liquid culture of fungi and consisted of the same ingredients as YMA excluding the agar. The natural pH of YMA and YMB was 6.2 ± 0.2.

Malt extract agar (MEA) contained the following ingredients: 30 g L^{-1} malt extract, 5 g L^{-1} mycological peptone, and 15 g L^{-1} agar. Malt extract broth (MEB) used for liquid cultivation of fungi was composed of the same ingredients as MEA without agar. Both media were sterilised at 115°C for 10 minutes, and had a natural the pH of 5.4 ± 0.2.

Potato dextrose agar (PDA) consisted of 4 g L^{-1} potato extract, 20 g L^{-1} glucose, and 15 g L^{-1} agar with a pH of 5.6 ± 0.2. Potato dextrose broth (PDB-Difco) was also used for liquid cultivation of fungi, and consisted of 4 g L^{-1} potato starch and 20 g L^{-1} glucose. The natural pH of the medium was 5.1 ± 0.2. PMP Media (Kim S.W., *et al*. (2002)) was also employed and consisted of 24 g L^{-1} PDB, 10 g L^{-1} malt extract, and 1 g L^{-1} peptone. The initial pH of the medium was 5.1 ± 0.15.

Fresh potato dextrose agar (F-PDA) was prepared as follows: 300 g of peeled, thinly sliced potatoes were boiled in 1 L of water for 1 h. The potato broth was then allowed to cool and passed through a sieve, adding a small amount of mashed potato solid to the filtrate. The volume of the broth was made up to 1 L to replace water lost to evaporation, followed by the addition of 20 g bacteriological agar (Agar No. 1) and 8 g dextrose. The medium was then sterilised at 121°C for 45 min.

Complete Mushroom Medium (CMM) as described by Kim, S.W., *et al* (2002) consisted of 20 g L^{-1} glucose, 2 g L^{-1} peptone, 2 g L^{-1} yeast extract, 0.5 g L^{-1} $MgSO_4.7H_20$, 1 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4. The initial pH of the medium was 6.9 ± 0.2.

Glucose and Sucrose Liquid Media, described by Eguchi, *et al*. (1994) for *A. blazei* growth, consisted of 10 g L^{-1} glucose or sucrose, 10 g L^{-1} malt extract, 4 g L^{-1} yeast extract, 0.3 g L^{-1} casamino acid, and 0.03 g L^{-1} inositol. The initial pH of both media was adjusted to pH 6.0.

Sabouraud dextrose agar (SDA) contained the following ingredients: 10 g L^{-1} mycological peptone, 40 g L^{-1} glucose, and 15 g L^{-1} agar. The natural pH of the medium was 5.6 ± 0.2. Sabouraud maltose agar (SMA) was also used. The ingredients and natural pH of which were the same as that used to prepare SDA, with the exception that 40 g L^{-1} maltose was substituted for glucose.

Czapek dox agar (CDA) contained the following ingredients: 2 g L^{-1} sodium nitrate, 0.5 g L^{-1} potassium chloride, 0.5 g L^{-1} magnesium glycerophosphate, 0.01 g L^{-1} ferrous sulphate, 0.35 g L^{-1} potassium sulphate, 30 g L^{-1} sucrose, and 12 g L^{-1} agar. The natural pH of the medium was 6.8 ± 0.2.

Corn meal agar (CMA) consisted of 2 g L^{-1} corn meal extract and 15 g L^{-1} agar, which exhibited an initial pH of 6.0 ± 0.2.

Basal medium (BM) was prepared with the following ingredients in w/v quantities; 1% glucose (Sigma), 0.3% yeast extract, 0.3% peptone, 0.05% $NaNO_3$, 0.05% NaH_2PO_4 (BDH, Merck Chemicals Ltd., Dorset, UK), 0.02% $MgSO_4$, 0.01% KCl. The initial pH of the liquid medium was adjusted to 6.0 ± 0.2 prior to autoclaving.

3.2.2.2 Preparation of media

Agar plates were prepared by aseptically pouring 15-20 mL of molten (50°C), sterilised medium into 90 mm diameter sterile petri dishes. In cases were the preparation of agar plates with a defined volume was required, a sterile syringe was used to transfer the molten agar to the Petri dishes. For experimental procedures that required agar plates at different pH values, the pH of the medium was adjusted by the addition of 1 M or 6 M HCl or NaOH before sterilisation, except for extreme pH values of 3 and 9 where the pH was adjusted after autoclaving. This ensured maximum solidity of the agar at these pH values.

Agar slants were prepared by transferring 12 mL of dissolved medium into McCartney bottles. After sterilisation for the appropriate time, the lids were tightened and the bottles laid at an angle of approximately 30° and allowed to solidify.

Liquid medium was prepared in conical or baffled shake flasks by dissolving the appropriate amount of powdered medium in distilled water. The opening of the flask was plugged with cotton wool and the flask sterilised for the appropriate time.

3.2.3 Inoculation and cultivation of fungal cultures

All fungal inoculation procedures were performed in a Class 1 ultraviolet biological safety cabinet (Email Westinghouse Pty Ltd.) with strict adherence to aseptic technique. Liquid cultivation of fungal species was carried out in a Bioline 4700 environmental shaker (Quantum Scientific, Queensland, AUS).

3.2.3.1 Cultivation on solid media

Fungal cultures were routinely grown on YMA or MEA agar plates generated by mycelial inocula from slant stock cultures. Inocula from slant cultures were prepared by

taking a 1 cm^2 section of mycelium from the growing edge of fungal culture on the slant. The section was then transferred to the surface of an agar plate with the mycelium face down onto the surface of the agar, followed by incubation at 25 °C for 15-20 days.

Plug inoculums inocula were then prepared from agar plates containing viable mycelium. Plugs of the sub-cultured fungus were cut from the actively growing outer edge of the culture using a sterile 5 mm diameter cork borer. Agar plates (or slants) were inoculated by centrally placing the mycelial plugs onto the surface of a freshly prepared agar plate with the mycelial facing down. Agar plates were transferred to humidified plastic trays, and incubated at the appropriate temperature for the fungal species under investigation.

Due to the fact that routine subculturing of fungi may compromise the genetic stability of the species, fungal samples that had been consecutively subcultured using the plug inoculum technique only 2-4 times were used to ensure the viability and genetic integrity of the mycelium. Older cultures were not used for experimental work and destroyed by autoclaving at 121°C for 30 minutes.

3.2.3.2 Cultivation in liquid media

The preparation of homogenised *A. blazei* mycelium for inoculation of liquid culture flasks was based on the method described by Zweck *et al.* (1978). A volume of 50 mL of YMB was prepared in 250 mL baffled conical flasks. Four plugs of *A. blazei* mycelium were transferred to each flask, followed by incubation at 30°C for 20 d, with shaking at 100 rpm to allow for mycelial growth.

The contents of the culture flask was then homogenised using an Ystral GmbH homogeniser (Ystral GmbH, Ballrechten-Dottingen, Germany) with a 2 mm sterile blade for 30 s. Volumes of the prepared homogenised mycelium were then used to inoculate culture flasks for experimental procedures.

The desired amount of medium was prepared in baffled shake flasks stoppered with cotton wool (Section 3.2.2.2). An aliquot of homogenised mycelium was transferred to each flask, typically 2 mL for 100 mL of medium, 4 mL for 200-250 mL medium, and 10 mL for 1 L of medium. The flasks were then incubated at an appropriate temperature and duration, depending on the experiment, with shaking at 100 rpm.

3.2.4 Radial growth measurement of fungal cultures

Quantification of radial growth was carried out according to the method described by Lonergan *et al*. (1993), and involved measurement of the radius of the mycelial culture in mm, minus the plug radius in mm. Four perpendicular measurements were made for each culture, and the average radius recorded.

3.2.4.1 Effect of pH and temperature on fungal growth

PDA plates (20 mL) were prepared at pH 3, 4, 5, 6, 7, 8, and 9, in 90 mm diameter Petri dishes according to the method described in Section 3.2.2.2. PDA was used for this experiment as its translucent nature enabled clearer visual inspection of mycelial growth, therefore permitting more precise measurement of the radius of the fungal cultures.

Mycelial plugs from a working culture of the fungus under investigation were used to centrally inoculate each agar plate (Section 3.2.3.1), and triplicate inoculated plates of each pH value were incubated at temperatures of 10°, 15°, 20°, 25°, 30°, 37°, and 45°C. Growth of *A. blazei* mycelium was also measured at temperatures of 26°, 27°, 28°, and 29°C. The radial growth of each culture was measured at 24-hour intervals for 14 days using the procedure described in Section 3.2.4.

3.2.4.2 Effect of culture media on fungal growth

Triplicate agar plates (20 mL) of the following eight solid media were prepared: YMA, MEA, PDA, F-PDA, SMA, SDA, CMA, CDA. Plugs of *A. blazei* mycelium, taken from a working stock culture, were used to centrally inoculate each agar plate and all plates were incubated at 30°C. The radial growth of *A. blazei* on each medium was measured at 24-hour intervals using the procedure described in Section 3.2.4.

3.2.4.3 Comparison of storage methods for *Agaricus blazei* cultures

Plugs of *A. blazei* mycelium were inoculated onto YMA and MEA plates, and incubated at 25°C for 15-20 days to allow for mycelial growth. Triplicate culture plates of each medium were then stored at 4-6°C. Vials containing 3 mL of a sterile 10 % (v/v) glycerol solution were each inoculated with 3 plugs of *A. blazei* mycelium, and the vials placed in a 80°C freezer for storage.

At intervals of 1, 2, and 4 weeks, freshly prepared YMA and MEA plates were inoculated with mycelial plugs in triplicate from cultures stored according to one of the two methods. The plates were incubated at 25°C and the radial growth of the inoculated cultures was measured at 24 h intervals over 16 days. The appearance of the resultant mycelial growth and evidence of sectoring was also noted.

Mycelial plugs stored at -80°C in 10% glycerol were also inoculated onto YMA and MEA plates at periods of 2, 6, 12, 18 and 24 months to investigate long term effects using this storage method.

3.2.5 Determination of fungal biomass in liquid culture

Liquid culture flasks were filtered using a Buchner Funnel and a double layer of Whatman No. 1 filter paper (Proscience; Radiometer Pacific Pty. Ltd. Blackburn, VIC, AUS). The wet mycelial samples were washed twice with dH_2O, transferred to a pre-weighed 50 mL centrifuge tube (Falcon Becton Dickinson, NJ, U.S.A.), and frozen at -

20°C. The frozen mycelial samples were then freeze-dried and re-weighed to determine the yield of dry biomass, expressed as g L^{-1} unless otherwise stated.

3.2.5.1 Effect of temperature on biomass production

Volumes of 250 mL of sterilised YMB were prepared in 250 mL baffled flasks. Each flask was inoculated with 2 mL homogenised *A. blazei* mycelium and triplicate flasks were incubated at 20°, 25°, 30°, and 37°C for 15 days. The yield of dry biomass for each triplicate culture flasks was determined (Section 3.2.5), and the average biomass was calculated for each temperature.

3.2.5.2 Effect of pH on biomass production

Volumes of 250 mL of sterilised YMB were prepared in 250 mL baffled flasks. Triplicate flasks were adjusted to pH values of 3, 4, 5, 6, 7, 8, and 9, and inoculated with 2 mL homogenised *A. blazei* mycelium. All flasks were incubated at 30°C for 15 days. The biomass of the triplicate culture flasks was determined (Section 3.2.5), and the average biomass was calculated for each pH.

3.2.5.3 Effect of liquid media on biomass production over time

Volumes of 250 mL of sterilised YMB were prepared in 500 mL baffled flasks. Each flask was inoculated with 4 mL homogenised mycelium and incubated at 30°C with shaking at 100 rpm. At 3-day intervals, for a period of 30 days, triplicate flasks were removed and their biomass determined (Section 3.2.5). The procedure was repeated using MEB and PDB.

3.2.5.4 Effect of carbon source on biomass and exo-polysaccharide production

One hundred mL of sterilised basal media were prepared in 250 mL baffled flasks. To examine the effect of different carbon sources on the growth and exo-polysaccharide production by *A. blazei* mycelium, flasks were prepared substituting the 1% glucose in the basal media for 1% sucrose, 1% mannose, and 1% arabinose. Triplicate flasks of

each medium were inoculated with 2 mL homogenised mycelium and incubated at 30°C for 15 days with shaking at 100 rpm.

Following incubation the flasks were filtered and their biomass determined (Section 3.2.5), reserving the culture fluid for the extraction of exo-polysaccharides. Exo-polysaccharides were extracted using the method described in Section 3.3.3, using two volumes of ethanol for the precipitation of polysaccharides. The yield of exo-polysaccharide extract (g L^{-1} medium) for each carbon source was determined.

3.3 Preparation of fungal extracts

3.3.1 Soxhlet extraction of liquid cultured mycelium

Freeze-dried biomass obtained from filtration of fungal liquid cultures (Section 3.2.5.1), was ground to a fine powder using a mortar and pestle. Approximately 5-6 g of the mycelial powder were transferred to a 25 mm x 80 mm Whatman cellulose extraction thimble (Proscience), and placed inside a Soxhlet apparatus. Extractions were carried out at a temperature 10-20°C above the boiling temperature of the solvent being used.

Initially, samples were extracted sequentially with hexane, dichloromethane, diethyl ether, chloroform, ethyl acetate, and methanol. Preliminary investigation indicated the yield for both the diethyl ether and chloroform extracts was extremely low (0-5 μg), and did not permit the preparation of stock extracts at a concentration required for screening of bioactivity. Therefore, these two solvents were omitted from the extraction procedure, and the solvents hexane, dichloromethane, ethyl acetate, and methanol were used for all subsequent Soxhlet experiments. A volume of 150 mL of each solvent was used for each extraction, with the extraction proceeding for 6-7 hours.

Solvent extract solutions were transferred to pre-weighed 250 mL round bottom flasks and the solvent evaporated under vacuum using a rotary evaporator (Rotavapor R, Büchi, Switzerland). The round bottom flasks were then re-weighed and the mass of the extract determined. Each extract was redissolved in its corresponding solvent to a

concentration of 100 mg mL^{-1}, and stock extract solutions were stored at -20°C for bioactivity testing.

3.3.2 Extraction of protein components

Fifty mL of saline (0.85% (v/v) NaCl) were added to approximately one gram of freeze-dried *A. blazei* mycelium previously ground to a fine powder using a mortar and pestle. The mixture was incubated overnight at 4°C with constant stirring to allow for the solubilisation of protein. The slurry was then centrifuged at 10,000 rpm for 20 minutes to remove insoluble material, and the supernatant transferred to a clean 250 mL centrifuge tube.

Ammonium sulphate was added to the supernatant at a level of 80% saturation, and the mixture was incubated overnight at 4°C with constant stirring to allow for the precipitation of protein. The precipitated protein was collected by centrifugation at 12,000 *g* for 20 minutes, and the supernatant decanted.

The precipitated protein was then dissolved in 10 mL of dH_2O, transferred to dialysis tubing (MW cut-off 10,000 Da), and extensively dialysed against dH_2O to remove ammonium sulphate and other small molecular weight impurities. The resulting solution was transferred to a 50 mL centrifuge tube (Falcon), frozen at -20°C, and freeze-dried. The lyophilised solid comprised the crude protein extract and was stored at -20°C for use in bioactivity testing.

3.3.3 Extraction of exo-polysaccharide components

A. blazei culture filtrate was freeze dried and resuspended in dH_2O to concentrate the filtrate to 1/5 of the original volume. A volume of absolute ethanol was added to the concentrated culture fluid, and the mixture incubated overnight at 4°C with constant stirring to allow for the precipitation of polysaccharides from the solution.

Exo-polysaccharides were then collected by centrifugation of the solution at 12,000 *g* for 15 minutes. The supernatant was decanted and the crude polysaccharide extract was

washed with acetone (BDH) and diethyl ether (Fluka), and resuspended in 10 mL of dH_2O. The solution was transferred to dialysis tubing (MW cut-off 10,000 Da) and extensively dialysed against dH_2O. The resulting solution was then transferred to a 50 mL centrifuge tube (Falcon), frozen, and freeze-dried. The lyophilised solid comprised the crude exo-polysaccharide extract and was stored at 20°C for use in bioactivity testing.

3.4 Screening of mycelial solvent extracts for anti-bacterial activity

3.4.1 Bacteria

Bacterial species used in anti-bacterial screening experiments were obtained from the ATCC or the Australian Collection of Micro-organisms (ACM), Qld, Australia. The source of each species, and their culture and incubation conditions, are summarised in Table 3.1.

Bacterium	Source & ID no.	Incubation temperature °C	Media for growth
Bacillus cereus	ATCC 1178	37	NA & NB
Branhamella catarrhalis	ATCC 23246	30	BHI & HBA
Escherichia coli K12	ATCC 11105	37	NA & NB
Pseudomonas aeroginosa	ATCC 10145	37	NA & NB
Salmonella entrica	ATCC 13311	37	NA & NB
Staphylococcus aureus	ATCC 12600	37	NA & NB
Streptococcus pyogenes	ACM 178	37	BHI & HBA

Table 3.1: *Bacterial species used for antibacterial testing*

3.4.2 Media for bacterial growth

Culture media and media supplements were purchased from Oxoid Ltd. unless otherwise stated.

Nutrient agar (NA) contained the following ingredients: 1 g L^{-1} Lab-Lecco powder, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone, 5 g L^{-1} sodium chloride, and 15 g L^{-1} agar. Nutrient broth (NB) was also used for growth of bacteria and consisted of the same ingredients as NA excluding the agar. The natural pH of NA and NB was 7.4 ± 0.2.

Mueller-Hinton agar (MHA) consisted of: 300 g L^{-1} beef (dehydrated infusion), 17.5 g L^{-1} casein hydrolysate, 1.5 g L^{-1} starch, and 17 g L^{-1} agar. Mueller-Hinton broth (MHB) used for the growth of bacteria was composed of the same ingredients as MHA without the agar. The natural pH of MHA and MHB were 7.4 ± 0.2, and 7.3 ± 0.2 respectively.

Brain Heart Infusion Broth (BHI) consisted of: 12.5 g L^{-1} calf brain infusion solids, 5.0 g L^{-1} beef heart infusion solids, 10.0 g L^{-1} protease peptone, 2.0 g L^{-1} glucose, 5.0 g L^{-1} sodium chloride, and 2.5 g L^{-1} di-sodium phosphate. The natural pH of the medium was 7.4 ± 0.2.

Horse Blood agar (HBA) was prepared using blood agar base (10.0 g L^{-1} Lab-Lemco powder, 10.0 g L^{-1} peptone, 5.0 g L^{-1} sodium chloride, 15.0 g L^{-1} agar) with a natural pH of 7.3 ± 0.2. Fifteen mL aliquots of blood agar base were prepared in McCartney bottles, autoclaved, and placed in a 50°C water bath for hour. One mL of defibrinated horse blood (Oxoid Ltd.) was added to the blood agar base, and the bottle rotated gently to mix thoroughly. The agar was then poured into sterile 90 mm Petri dishes and allowed to set for 1 hour before storage at 4°C.

3.4.3 Disc-diffusion assay

Blank 6 mm sterile filter discs were impregnated with 10 µL of the 100 mg mL^{-1} stock solvent extracts (1 mg per disc). Discs impregnated with solvent alone were used as negative controls. All discs were stored in glass petri dishes at 4°C for 2-3 hours until completely dry. Bacterial broth cultures were prepared by inoculation of 3 mL NB or BHI with a loopful of bacterial stock culture, and incubating for 24 or 48 hours. Each bacterial culture was diluted with saline to match a McFarland 0.5 turbidity standard (≈1.5 x 10^7 cfu mL^{-1}).

A volume of 200 μL of the dilute bacterial culture was added to 4 mL of molten (50°C) MHA, gently mixed, and poured evenly over the surface of a 16 mL MHA plate to create a bacterial lawn. Once the agar had set, the dry filter discs were placed onto the surface of 20 mL MHA plates. Plates were incubated at 30°C or 37°C, for 24 or 48 hours depending on the bacterial species.
After incubation the plates were examined for zones of inhibition- a clear zone around the disc where no bacterial growth is visible. Visible zones greater than 6 mm in diameter were measured and recorded.

3.5 Screening of fungal extracts for cytotoxic activity

All cell culture media and media supplements were purchased from Invitrogen (Carlsbad, CA, U.S.A), unless otherwise stated.

Unless specified, incubation of cell cultures was carried out in a humidified incubator (Napco 6500; Napco, Winchester, VA, USA) in an atmosphere of 5% CO_2 and 95% air at 37°C.

All centrifugation procedures involving cell cultures were carried out at 4°C using a Hettich universal 1637 centrifuge (Hettich GmbH & Co., Tuttlingen, Germany).

3.5.1 Cells and media

The human cervical cancer cell line HeLa, was donated by the Walter & Eliza Hall Institute, Parkville, Victoria, Australia. An adherent cell lines, HeLa cells were routinely cultivated in Dulbecco s modified Eagle s medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), and 1% penicillin-streptomycin solution (p-s).

The Raji cell line (a B-cell lymphoma cell line) was donated by the Walter & Eliza Hall Institute. Raji cells, a suspension cell line, were routinely cultivated in RPMI 1640 medium supplemented with 10% FBS and 1% p-s solution.

The monkey kidney epithelial cell line, MA104, was kindly provided by the Murdoch Childrens Research Institute, Royal Children s Hospital, Parkville, VIC, AUS. An adherent cell line, cells were routinely cultivated in Minimum Essential medium (MEM) supplemented with 10% (v/v) foetal bovine serum FBS and 1% p-s solution.

3.5.2 Cell cultivation

All cell culture procedures were performed in a Class II ultraviolet biological safety cabinet (Email Westinghouse Pty Ltd.) with strict adherence to aseptic technique.

3.5.2.1 Passaging of adherent cell lines

Adherent cells were routinely cultivated as monolayers in disposable 75 cm^2 flasks (Falcon), using the appropriate medium for each cell line, and were passaged upon reaching confluence.

To passage cells, the spent medium was aspirated using a Pasteur pipette, and the cells washed twice with 3-5 mL sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4). Any residual PBS was removed with a Pasteur pipette. Two mL of trypsin-EDTA (0.05% trypsin, 0.53mM EDTA) were added to cover the monolayer, and the flask was incubated for 2-5 minutes or until the cells detached.

The trypsinized cells were diluted with growth medium for a split ratio of 1:4 (HeLa and MA104 cells), and cell solutions were then transferred to new flasks for further incubation.

3.5.2.2 Passaging of suspension cell lines

Suspension cell lines were routinely cultivated as cell suspensions in disposable 75 cm^2 flasks (Falcon), using the appropriate medium. Cell cultures were passaged upon reaching a cell density of 10^6 10^7 cells mL^{-1}. Cell solutions were counted using an

improved Neubauer haemocytometer (Weber Scientific International Ltd., Middlesex, UK).

To passage cells, the contents of the 75 cm^2 culture flask was transferred to a 50 mL sterile centrifuge tube (Falcon) and centrifuged at 1000 rpm for 3 minutes at 4°C. The spent medium was then aspirated off, and fresh media added to dilute the cell suspension to a cell concentration of approximately 10^4 cells mL^{-1}. The cell suspension was thoroughly mixed and divided between new 75 cm^2 flasks. The flasks were then incubated for 1-2 days, or until the cell density returned to approximately 10^6 cells mL^{-1}.

3.5.3 MTT cell viability assay

Cellular viability was determined using a modified method of the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT) assay described by Mossman (1983). The assay was carried out in 96 well microtitre plates (Proscience), previously seeded and incubated with cell solutions and test extracts if appropriate.

3.5.3.1 MTT assay for adherent cell lines

After aspiration of media (200 μL) from each well, cells seeded in 96-well microtitre plates were washed three times with PBS, and 20 μL of MTT (Sigma) solution (5 mg mL^{-1} in PBS) were added to each well. The plates were then incubated for three hours to allow tetrazolium crystals to form.

One hundred and fifty μL of acid-isopropanol solution (88% (v/v) isopropanol, 12% (v/v) 0.2M HCl) were added to each well and the contents of each well mixed thoroughly using a micropipette to solubilise the dark-blue tetrazolium crystals. After standing for 10-20 minutes at room temperature to ensure all the crystals had dissolved, the plates were read using a Molecular Devices Emax microplate reader, at a wavelength of 570 nm. Plates were read within an hour after the addition of the acid-isopropanol solution.

3.5.3.2 MTT assay for suspension cell lines

A total well volume of 100 μL was the maximum allowed for the determination of viable cells growing in suspension. A volume of 20 μL of MTT solution (5 mg mL^{-1} in PBS) was added to the 100 μL cell solutions in each well and the plates were then incubated for three hours.

Addition of acid-isopropanol solution and reading of the plates was carried out as described above (Section 3.5.3.1).

3.5.4 MTT assay standard curve

The optimal cell density of each cell line for cytotoxicity testing was determined by the preparation of a standard curve. Ten-fold serial dilutions of each cell line were prepared in the appropriate medium, with a final concentration range from 10^1 to 10^7 cells mL^{-1}. The cell solutions were seeded in quadruplicate wells of a 96 well plate and incubated for 48 or 72 hours.

The MTT assay (Section 3.5.3) was used to determine the optimal cell density to use for each cell line involved in cytotoxicity testing by preparation of a growth response curve. Cells at the optimum seeding density were still in a log phase of growth after 72 hours incubation (adherent cells) and 48 hours (suspension cell lines). The optimum cell concentration at this growth stage was identified by the mid-point of the linear portion of the growth curve. In addition, cell concentrations that resulted in an absorbance reading at 570 nm of between 0.8-1.2 were used for cytotoxicity testing, in order to allow for the discrimination between cell proliferation and cell death.

3.5.5 Assays for cytotoxic activity

3.5.5.1 Cytotoxicity assay for adherent cell lines

For the assessment of cytotoxicity against adherent cell lines, 200 μL of cell suspension was added to the wells of 96-well plates at an optimal density determined for each cell line (Section 3.5.4).

The cells were then incubated for 16-18 hours to allow for recovery from handling. The media was then removed by aspiration, and 100 μL of various concentrations of *A. blazei* extracts (5-500 μg mL^{-1}) were added to triplicate wells, followed by one hundred μL of fresh media. Wells containing cells and 200 μL of fresh media served as the cell-only control. Triplicate wells containing 200 μL of media only were assayed for blank readings. The cells were then incubated in the presence of the extracts for 48 hours.

The MTT cell viability assay for adherent cells (Section 3.5.3.1) was used to determine the proportion of viable cells remaining after treatment with *A. blazei* extracts at different concentrations. The proportion of viable cells remaining after treatment was calculated by dividing the absorbance at 570 nm of the test extract (A) by the absorbance at 570 nm (OD) of the cell only control (B), and expressing the ratio as a percentage, according to the formula below.

$$\% \text{ viable cells remaining} = (OD_{test} \quad OD_{blank}) / (OD_{control}-OD_{blank}) \times 100$$

The unpaired Student s t-test was used for statistical analysis. The mean values of the percentage viable cells – standard deviation for cells incubated in the presence of extracts, and cell only controls were calculated. A p-value <0.0001 was considered statistically significant.

3.5.5.2 Cytotoxicity assay for suspension cell lines

For the assessment of cytotoxicity against suspension cell lines, 50 μL of cell solution was added to wells of 96-well plates at an optimal density for the cell line (Section 3.5.4). The cells were then incubated for 2-3 hours to allow recovery from handling.

Fresh media (50 μL) was added to replicate wells to serve as a cell-only control. One hundred μL of media in replicate wells containing no cells were assayed for blank readings. Test extracts at different concentrations (50 μL) were added to triplicate wells and the cells incubated in the presence of the extracts for 48 hours.

The MTT cell viability assay for suspension cells (Section 3.5.3.2) was used to determine the proportion of viable cells remaining after treatment with *A. blazei* extracts at different concentrations.

The proportion of viable cells remaining after treatment, was calculated by dividing the absorbance at 570 nm of the test extract by the absorbance at 570 nm of the cell only control, and expressing the ratio as a percentage, according to the same formula described above (Section 3.5.5.1).

3.6 Screening of protein and exo-polysaccharide extracts for anti-viral activity

All cell culture media and media supplements were purchased from Invitrogen unless otherwise stated.

Unless specified, incubation of cell cultures was carried out in a humidified incubator (Napco 6500; Napco, Winchester, VA, USA) in an atmosphere of 5% CO_2 and 95% air at 37°C.

All centrifugation procedures involving cell cultures were carried out at 4°C using a Hettich Universal 1637 centrifuge (Hettich GmbH & Co., Tuttlingen, Germany).

3.6.1 Cells

Routine passaging of MA104 cells was carried out according to the method described in Section 3.5.2.1, and trypsinized cells were diluted with growth medium to give a split ratio of 1:4 for the cultivation of cells.

3.6.2 Virus

Simian Rotavirus SA11 was kindly donated by the Murdoch Childrens Research Institute, Royal Children s Hospital, Parkville, VIC, AUS. Virus was propagated in monolayers MA104 cells. Prior to infection, confluent cell monolayers in 75 cm^2 culture flasks were incubated overnight in serum-free MEM (SF-MEM). SA11 was pre-activated by incubation of stock virus at 37°C in the presence of 10 $\mu g\ mL^{-1}$ porcine trypsin (Sigma) for 30 minutes. The SF-MEM was removed and the monolayer was infected with 1 mL of pre-activated SA11 virus. One hour was allowed for virus adsorption with gentle rocking every 15 minutes, followed by removal of the viral inoculum, and the addition of 10 mL SF-MEM containing 5 $\mu g\ mL^{-1}$ porcine trypsin (SF-MEM-T). The flasks were incubated for 48-72 hours or until complete CPE was observed.

Virus was then harvested after three freeze (-80°C)- thaw cycles, and low speed centrifugation (20 *g* for 5 minutes), to remove large cellular debris from the lysate. One mL aliquots of the virus stock were stored in cryogenic tubes (Nalgene; Nalge Company, Rochester, NY, USA) at -80°C for use in anti-viral testing.

3.6.3 Optimisation of viral titre for anti-viral screening

The optimal viral concentration for use in anti-viral screening was determined by construction of a standard curve. This was necessary due to the fact that if the virus concentration was too low, the majority of virus infected control cells would survive over the period of the assay. As a result, comparisons made with cells treated with test extracts would not indicate a significant difference, and hence anti-viral activity could not be determined. Conversely, if the virus concentration was too high or in excess,

extracts possessing antiviral activity may not be evident, as an extract concentration higher than the maximum concentration tested (1000 μg mL^{-1}) may be required for the antiviral activity to become apparent.

MA104 cells in MEM were seeded in wells of a 96-well plate at the cell density determined as described in Section 3.5.4, and incubated for 16-18 hours to allow recovery from handling. The medium was removed and replaced by SF-MEM, followed by further incubation for 16-18 hours. Ten-fold serial dilutions of virus stock (10^{-1} 10^{-9}) and neat virus stock were prepared in SF-MEM-T.

The SF-MEM was removed from the MA104 cell monolayers, and 100 μL of each virus dilution, and neat virus, were added to eight replicate wells. One hundred μL of SF-MEM-T were also added to replicate seeded wells (mock virus adsorption), and cell free wells for blank readings.

The cells were incubated for 1 hour to allow for virus adsorption, followed by removal of the viral inoculum and the addition of 200 μL SF-MEM-T to each well. The plate was incubated for 48-72 hours, or until complete CPE was observed in the cells treated with neat virus stock.

The MTT assay for cell viability was performed on all cell conditions. The concentration of virus that resulted in an absorbance at 570nm greater than 5%, and less than 50% of the mock-infected cells, was determined to be the optimum viral concentration for use in antiviral testing.

3.6.4 Initial screening assay for anti-viral activity

MA104 cells were seeded into 96 well plates at an optimum cell density (Section 3.5.4), and incubated for 16-18 hours to allow the cells to recover from handling. The medium was removed and replaced by SF-MEM followed by further incubation for 16-18 hours.

The SF-MEM was removed and 100 μL of each test extract concentration (12.5-1000 μgmL^{-1}) in SF-MEM-T were added to eight replicate wells on the plate.

Pre-activated SA11 rotavirus stock solution was diluted to the optimum concentration (Section 3.6.3) in SF-MEM-T, and 100 µL added to replicate cell monolayers. Wells containing SF-MEM-T only were used for blank readings. Wells containing cells with virus only (no extract) were used as positive controls for antiviral testing.

The effect of the extracts, or trypsin, on the viability of MA104 cells was also determined. One hundred µL of SF-MEM-T were added to cell monolayers previously inoculated with 100 µL volumes of increasing extract concentrations. Wells containing cells only (no extract), were used as controls for cytotoxicity testing.

The plates were incubated for 72 hours and the MTT cell viability assay (Section 3.5.3.1) was performed in all wells. The cytotoxic and antiviral effects of test extract solutions were determined by comparison of the proportion of viable cells remaining after treatment compared with the relevant controls.

3.6.5 Viral attachment assay

The effect on attachment of rotavirus SA11 to MA104 cells in the presence of *A. blazei* extracts was tested using a viral attachment assay at 4°C. This temperature allows for virus attachment to MA104 cells without viral penetration.

Pre-activated SA11 rotavirus stock solution was diluted to the optimum concentration (Section 3.6.3) in SF-MEM-T, and 100 µL added to the cell monolayers in the microtitre plate wells. One hundred µL of test extracts were then added to replicate wells. Wells containing SF-MEM were used for blank readings. Wells containing cells and virus only (no extract) were used as controls for antiviral testing. Plates were incubated for 1 hour at 4°C to allow for viral attachment in the presence of the test extracts, followed by removal of the extract-virus inoculum.

The effect of the extracts, trypsin, or the assay procedure on the viability of MA104 cells was also investigated. To cell monolayers previously inoculated with 100 µL of

test extract, 100 µL of SF-MEM-T were added for mock viral infection. Wells containing cells only (no extract), were used as controls for cytotoxicity testing. Wells containing SF-MEM were used for blank readings. After incubation for 1 hour at 4°C, the extract inoculum was removed.

Two hundred µL of SF-MEM-T were added to all wells, and the plates incubated for 72 hours at 37°C, or until complete CPE was observed in the cells treated with virus only. The MTT assay was then performed as described previously (Section 3.5.3.1) to examine the effect of *A. blazei* extracts on attachment of SA11 to MA104 cells. The cytotoxic and antiviral effects of test extract solutions were determined as before.

3.6.6 Viral entry assay

The viral entry assay method was identical to the viral attachment assay (Section 3.5.5), with the exception of the incubation temperature at which the virus underwent attachment to the MA104 cells. For the viral entry assay, an incubation temperature of 37°C was used as this temperature allows for viral adsorption as well as entry of the virus into the MA104 cells.

3.6.7 Assay for virucidal activity

In order to determine the virucidal effect of *A. blazei* extracts on SA11, volumes of test extracts over the concentration range examined (12.5-600 µg mL^{-1}) were incubated with an equal volume of pre-activated SA11 at the appropriate dilution (Section 3.6.3) in SF-MEM-T. Equal volumes of extract and media were used as mock vial infections to examine the cytotoxic effects on the MA104 cells. The extract-virus solutions, or mock-infected extracts, were incubated for 1 hour.

After incubation, 100 µL volumes of the extract-virus, and extract only solutions were added to wells of a 96-well plate previously seeded with MA104 cells that had been incubated for 16-18 hours in SF-MEM. Media alone, and virus alone (previously incubated as for test solutions) were also analysed as controls.

Cells were incubated for 1 hour to allow for virus adsorption, followed by removal of the viral, or mock inoculum, and the addition of 200 µL SF-MEM-T to all wells. Plates were incubated for 72 hours, or until complete CPE was observed in the cells treated with the virus only. The MTT assay was then performed as described previously (Section 3.5.3.1), to determine the virucidal effect of *A. blazei* extracts on SA11. The cytotoxic and antiviral effects of test extract solutions were determined as before.

3.7 Characterisation of bioactive components

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO., U.S.A), and were of analytical grade unless otherwise specified.

3.7.1 SDS-PAGE of protein extracts

The buffers and reagents used for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein extracts, and their composition, are described in Table 3.2.

Buffer	Composition
SDS-PAGE resolving gel	10% (w/v) acrylamide (Bio-Rad) 0.4 % (w/v) methylene bisacrylamide (Bio-Rad) 0.1 % (w/v) SDS in 375 mM Tris-HCl [pH 8.8]
SDS-PAGE stacking gel	5.0% (w/v) acrylamide 0.2 % (w/v) methylene bisacrylamide 125 mM Tris-HCl [pH 6.8]
SDS-PAGE running buffer	0.3% (w/v) Trisma base 1.44% (w/v) glycine 0.1% (w/v) SDS
SDS-PAGE sample buffer	2% (w/v) SDS 2% (v/v) 2-mercaptoethanol 62.5 mM Tris-HCl [pH6.8] 10 % (v/v) glycerol 0.005% (w/v) bromophenol blue
Coomassie protein staining solution	0.03 % (w/v) Coomassie brilliant blue R-250 50% (v/v) methanol 8.75% (v/v) acetic acid
De-staining solution 1	50 % (v/v) methanol 8.75 % (v/v) acetic acid.
De-staining solution 2	10 % (v/v) methanol 7.5 % (v/v) acetic acid

Table 3.2 *Buffers and reagents for SDS-PAGE*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the method described by Laemmli (1970), using the BioRad Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA).

Gels comprised a lower resolving gel, and an upper stacking gel. The composition of the lower resolving gel is outlined in Table 3.2. Polymerisation was initiated by the addition of 0.1% (w/v) ammonium persulphate and 0.04% (v/v) TEMED (Bio-Rad). The gel was cast between secured glass plates, assembled according to the manufacturer s instructions. The gel solution was then overlaid with isopropanol in order to produce a level interface and allow polymerisation. The isopropanol overlay was removed following polymerisation and the gel rinsed four times with dH_2O.

The upper stacking gel was prepared as outlined in Table 3.2. Ammonium persulphate [0.1% (w/v)] and TEMED [0.1% (v/v)] were added and the solution applied above the resolving gel. Appropriate combs were inserted into the stacking gel, and the gel was allowed time to polymerise. After placing the gel apparatus in the Mini-Protean® Electrophoresis Cell, SDS-PAGE running buffer was added to the inner and outer reservoirs.

The samples were denatured by boiling for 5 minutes in an equal volume of SDS-PAGE sample buffer, loaded into the wells and electrophoresed at a constant voltage of 150 V for 1 hour.

After electrophoresis, the gel was stained overnight by immersing in Coomassie brilliant blue staining solution with gentle rocking using a Bioline platform rocker (Edwards Instruments Co., Sydney, Australia). Gels were initially destained in destain solution 1 for 30-40 minutes, followed by 1-2 hours in destain solution 2.

The molecular weight of proteins was determined by comparison of electrophoretic mobility with Benchmark™ Protein Ladder molecular weight marker proteins (Invitrogen, Carlsbad, CA, U.S.A).

3.7.2 Ion-exchange chromatography

Crude exo-polysaccharide extracts were fractionated using the anion exchanger DEAE-Sepharose CL-6B (Sigma). A 2-3 mL volume of exo-polysaccharide extract (10 mg

mL^{-1} in dH_2O) was applied to the packed column (1.8 cm x 9 cm), previously equilibrated with dH_2O.

Thirty mL of each eluant was passed through the column and 2mL fractions were collected. After elution of the unadsorbed compounds with dH_2O (Fraction 1), bound compounds (Fraction 2-4) were eluted by raising the NaCl concentration in the elution buffer (0.1 M, 0.2 M, and 0.5 M NaCl).

Fractions were assayed for polysaccharide content using the Phenol-sulphuric acid method (Section 3.6.3). An elution profile displaying the protein or polysaccharide content in each fraction was constructed.

For each eluant, the fractions containing the compounds of interest were pooled and extensively dialysed against dH_2O to remove the NaCl. The remaining dialysate was then freeze-dried, and each fraction stored at 20°C for use in bioactivity testing.

3.7.3 Phenol sulphuric acid method for exo-polysaccharide quantitation

A modified method of the Phenol-sulphuric acid assay described by Dubois *et al.* (1956) was used for the quantitation of polysaccharide components in *A. blazei* extracts.

Standard solutions were prepared from a stock glucose solution (1mg mL^{-1} in dH_2O) to give a glucose concentration range of 20-100 μg mL^{-1}. Test sample solutions were prepared at a concentration of 1 mg mL^{-1}, for polysaccharide determination.

One mL of 5% aqueous phenol was added to 1 mL of test solution or standard in a 10 mL test tube, and mixed thoroughly by vortexing. Five mL of concentrated sulphuric acid was added as a stream directly to the solution surface and the samples were allowed to stand for 20 minutes at room temperature to allow colour to develop.

The absorbance of all solutions at 490 nm was then measured on a Carey 300 Bio UV-Visible Spectrophotometer (Varian, Palo Alto, CA, U.S.A.). The polysaccharide concentration of samples was determined by comparison with the glucose standard

curve constructed. In cases where samples were found to exhibit higher absorbance readings than the most concentrated glucose standard (100 μg $mL^{-1)}$, the sample was diluted accordingly in dH_2O and re-analysed.

3.7.4 Protein quantitation assay

A modified method of the Bradford protein assay (Bradford, 1976) was used for the quantitation of the amount of protein in the exo-polysaccharide and protein extracts from *A. blazei*.

Protein standard solutions in the range of 2-40 μg mL^{-1} were prepared by the dilution of a stock bovine serum albumin (BSA) solution (2 mg mL^{-1} in saline) with dH_2O. Using 96-well microtitre plates, 100 μL of test extract solution (1 mg mL^{-1} in dH_2O) were added to triplicate wells. Triplicate wells of 100 μL of each standard solution were also analysed for the construction of a standard curve, in addition to triplicate wells of 100 μL dH_2O for blank readings.

Bradford protein reagent was prepared by dissolving 100 mg of Coomassie brilliant blue G-250 in 50 mL of 95% ethanol. One hundred mL of 85% phosphoric acid were added, and the solution diluted to 1 L with dH_2O. The solution was then filtered through Whatman No. 1 filter paper, and stored in a brown bottle at 4°C.

One hundred and fifty μL of Bradford protein reagent were added to standards and samples in each well, and the plate allowed to stand at room temperature for 20 minutes. The plates were read on a Molecular Devices Emax microplate reader, using a test wavelength of 595 nm, and plates were read within an hour after the addition of the Bradford protein reagent.

The protein concentration of test samples was determined by comparison of the absorbance obtained, with the absorbance of the protein standards using the standard curve constructed. In cases where the samples gave higher absorbance readings than the most concentrated protein standard (40 μg mL^{-1}), the sample was diluted accordingly in dH_2O and re-analysed.

3.7.5 Glucose quantitation assay

The glucose content of protein and exo-polysaccharide extracts was determined using a Glucose Assay Kit (Megazyme, Wicklow, Ireland). Triplicate glucose standard, blank, and sample tubes were prepared for analysis according to the manufacturers instructions. Each tube was thoroughly mixed by vortexing, and incubated at 40°C for 20 minutes.

The absorbance (O.D.) of all solutions at 510 nm was measured against the reagent blank, using a Carey 300 Bio UV-Visible Spectrophotometer (Varian, Palo Alto, CA, U.S.A.). The amount of glucose (μg mL^{-1}) in each sample was calculated by reference to the glucose standard according to the following equation:

Amount of Glucose, μg 0.1 mL^{-1} = [O.D. (sample) /O.D. (standard)] x 100

3.7.6 TLC Monosaccharide determination

The determination of the monosaccharide composition of exo-polysaccharides (EPS) was carried out by acid hydrolysis and separation on silica gel plates (Robyt & White (1987)). 100 μL EPS solutions (1 mg mL^{-1}) were acid hydrolysed by adding 10 μL 1M HCl in a screw-top 1.8 mL centrifuge tube and placed in a 100 °C oven for 60 minutes. 10 μL of hydrolysed EPS was spotted on silica gel 60 plates (Merck) in addition to glucose and mannose standard solutions. The prepared TLC plate was placed in a chromatographic chamber containing acetonitrile-water (85:15 v/v), and the plate developed with four ascents of solvent, drying the plate after each ascent. The TLC plates were sprayed with sulphuric acid-methanol (1:3 v/v) followed by heating at 110-120°C. Carbohydrates give a black-brown spot and monosaccharide s present in test samples were determined by comparing Rf values with the glucose and mannose standards.

3.8 Statistical analysis

Comparison between means was carried out using an unpaired student s t-test. All statistical analysis was done using the statistical package Minitab Version 15.1 (2007). The statistical differences were considered significant at $p < 0.001$.

Chapter Four

Growth and preservation of *Agaricus blazei* on solid media

4.1 Introduction

Different fungal species require particular culture conditions to assure a healthy growth of mycelium. Of the many growth conditions that will influence fungal growth rates, the most important to consider include temperature, pH, humidity, and carbon and nitrogen sources (Brancato & Golding, 1953; Lomberh *et al*., 2000).

Whilst the conditions for optimal mycelial growth will vary with fungal species or strain, the environment in which a particular species exists, and to which it has adapted, will also influence its optimal growth parameters (Khan *et al*., 1991, Gibriel *et al*., 1996; Stott *et al*., 1996). Furthermore, the optimal growth conditions for any given species may differ, depending on whether the fungus is grown on solid or in liquid medium (Gibriel *et al.* 1996).

Determination of the optimal growth conditions for a particular fungus on solid media is important for several reasons. Working cultures of fungal species are generally maintained on solid media, and are often used to further inoculate liquid cultures. Common methods used for the storage of fungal cultures also require that a healthy growth of fungi is obtained on agar, and hence the examination of appropriate storage methods for fungal mycelium is very important to ensure maintenance of genetic stability, and viability of pure mycelium (Oei, 1996).

This chapter reports the conditions of optimal growth for *Agaricus blazei* mycelium on solid agar, including the optimal pH and temperature conditions, optimal media for growth, and suitable preservation methods.

4.2 Results and Discussion

4.2.1 Examination of growth conditions

The growth of *Agaricus blazei* mycelium was investigated using eight different media formulations routinely employed for fungal growth. The extent of growth was also measured as a function of pH for each of the media investigated. The optimal growth conditions were then employed for the routine growth of this fungus for all further studies.

4.2.1.1 Effect of pH and temperature on radial growth of fungal mycelium

The effect of pH and temperature on the growth of *Agaricus blazei* was examined and quantified by measurement of radial growth according to the method described in Section 3.2.4.1. The results were compared to those of other fungal species on solid agar, under identical conditions. Plugs of *Agaricus blazei*, *Agaricus bisporus*, *Lentinus edodes*, and *Pycnoporous coccineus* mycelium, grown on PDA, were inoculated onto PDA plates that had been adjusted to pH values ranging from 3-9. PDA was employed for the study due to its translucent nature, which allows the edge of the growing culture for radial growth measurement to be clearly identified. Plates were prepared for each organism and pH value, and were incubated at 10°, 15°, 20°, 25°, 30°, 37°, and 45°C for 7 days, except for *P. coccineus*, which was incubated for a maximum of 4 days due to rapid mycelial growth occurring during the d-day period. Radial growth measurements were made every 24 hours over the total incubation time.

Literature sources indicate that a range of temperatures between 24 - 30°C have been used for the growth of *A. blazei* mycelium on solid agar or in liquid media (Eguchi *et al*., 1994; Mizuno, T., 1995; Ito *et al*. 1997; Mizuno, M. *et al*., 1999; ATCC). To determine the optimum temperature for mycelial growth on solid agar for the strain of *A. blazei* used in this study, triplicate pH plates inoculated with *A. blazei* were also incubated at 26°, 27°, 28°, and 29°C.

The growth profiles of the *A. blazei* over the range of temperatures and pH values examined are shown in Figures 4.1 (a) and (b).

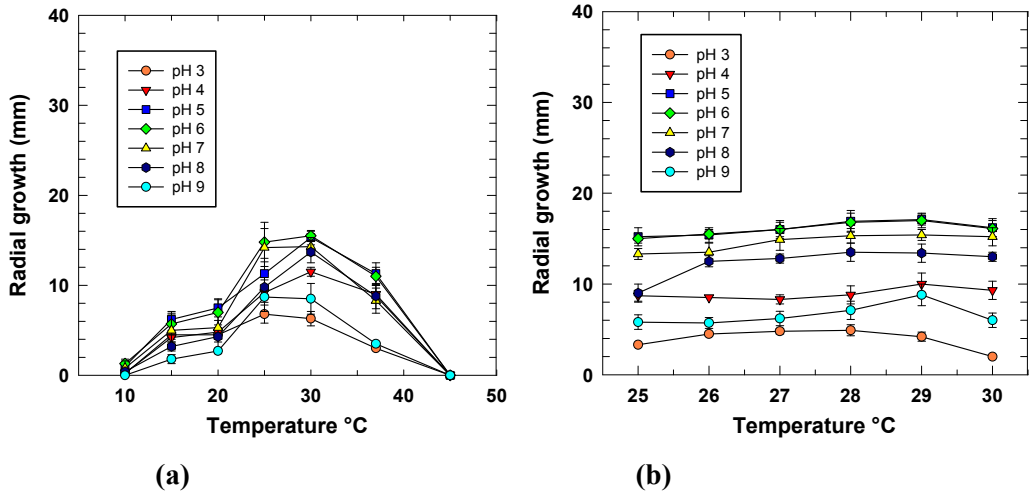


Figure 4.1 *(a) The effect of pH and temperature (10℃- 45℃) on the growth of* Agaricus blazei *mycelium (b) The effect of pH and temperature (25℃- 30℃) on the growth of* Agaricus blazei *mycelium*

Examination of the growth profile in Figure 4.1(a) indicates that *A. blazei* mycelium grows well on PDA over a pH range of 4-8, with an optimum pH range of 5-6. The optimal temperature for growth of *A. blazei* mycelium was found to be 30°C, although growth was only slightly reduced at 25°C. There was little or no growth at 10°C and 45°C, and moderate growth was also observed at 37 °C, a temperature often considered high for growth of mushroom mycelium.

Further investigation of the optimal temperature for growth of *A. blazei* on solid media was carried out by performing an additional radial growth experiment over the temperature range 25°C - 30°C. The results presented in Figure 4.1(b) indicate that the optimum pH range for growth was again pH 5 - 6, confirming the results obtained in the previous experiment. At this optimum pH range the optimum temperature for *A. blazei* growth was between 28°C- 29°C. Eguchi *et al.* (1994) reported an optimal growth temperature and pH of 26°C and 6.0, respectively, for the growth of the CJ-01 strain of *A. blazei* on solid media. The optimal growth pH range of pH 5-6 observed in this study (using the ATCC 76739 strain) was in accordance with these results however, the optimal temperature observed (28°C- 29°C) was slightly higher, and 4° higher than the growth temperature of 24°C recommended by the ATCC. These differences in growth

characteristics are not surprising, as it has been established that the specific strain of *Agaricus blazei* used in a particular study will exhibit its own unique growth characteristics. *Agaricus blazei* obtained from different global sources has been shown to exhibit different growth characteristics, and in fact a taxonomic investigations of various *Agaricus blazei* strains, highlighting these differences in growth characteristics) was reported in 2002 (Wasser *et al.,* 2002). Other researchers have highlighted that different growth characteristics can be observed for a fungal species that has been subjected to a range of environmental conditions (Stott *et al*. 1996).

In addition to studying a dissimilar strain of *Agaricus blazei* to that employed in this study, Eguchi *et al*. (1994) employed a sucrose-based medium for their experimental work. It contained 1% sucrose, 1% malt extract, 0.4% yeast extract, and agar, with an initial pH of 5.5. The PDA growth medium used in this experiment contained 2% glucose as the main carbon source, 0.4% potato extract (which contains many undefined complex carbohydrates) and agar at an initial pH of 5.1. In addition to the media used in this study containing a different carbon source, no additional nitrogen additive such as yeast extract was present in the PDA, which may have influenced the physiology of *A. blazei* mycelium and therefore the specific requirements for optimal growth. These differences may account for the fact that Eguchi *et al*. (1994) recorded a maximum radial growth of 40 mm after 15 days, more than twice the growth rate observed in this experiment, with a 16 mm growth of *A. blazei* over 7 days. This suggests that the sucrose-based medium employed by Eguchi *et al.* (1994) may provide a more optimal growth medium for the *A. blazei* than PDA.

The pH-temperature growth profiles of three other fungal species, *Agaricus bisporus*, *Lentinus edodes*, and *Pycnoporus coccineus,* were measured and compared to the growth profile obtained for *A. blazei*. The growth profiles of all four fungi are shown in Figure 4.2 (a)-(d).

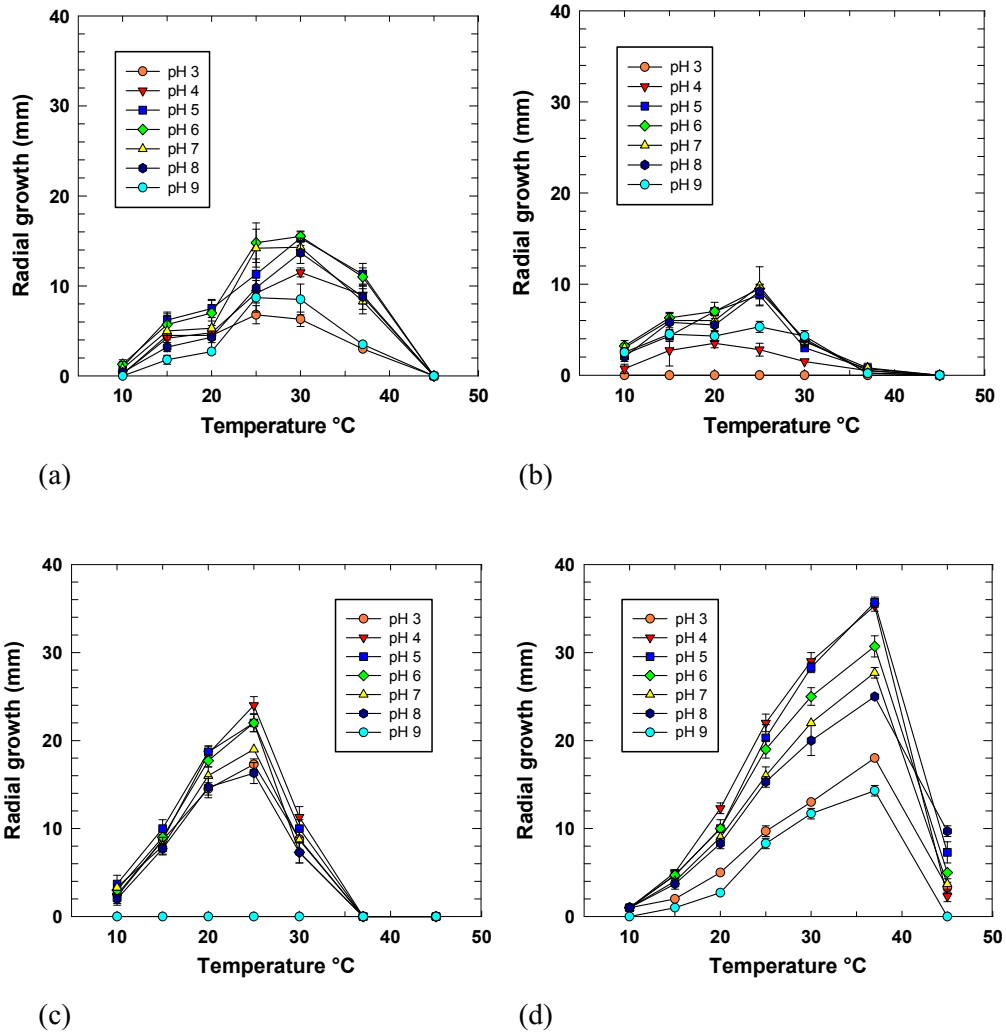


Figure 4.2 *The effect of pH and temperature on the growth of (a)* A. blazei; (b) A. bisporus; (c) L. edodes; (d) P. coccineus

In contrast to *A. blazei*, *A. bisporus* displayed a clear optimal growth temperature of 25 °C, with a moderately reduced growth at 30°C and little or no growth at or above 37°C. This suggests that *A. bisporus* is less tolerant of high temperatures than *A. blazei*. This latter species heat tolerance could possibly be attributed to its origins in the mountain regions of Brazil, an area that endures high temperatures and intense sunlight (reference needed here). The optimum pH range for growth of *A. bisporus* was pH 5-8, a slightly broader pH range for growth than that observed for *A. blazei*. *A. bisporus* displayed a slower growth rate with maximum radial growth of only 9.8 ± 2.1 mm after 7 days at 25°C compared to *A. blazei*, which recorded a radial growth of 16 mm after 7 days at its optimal temperature range of 29-30°C. The mycelial growth of a mushroom species

very closely related to *A. bisporus, Agaricus brunnescens,* has been studied previously by Khan *et al*. (1991). Similar results were reported for this mushroom species with an optimal growth temperature of 25°C on solid agar, and a reported maximum growth temperature of 30°C, above which little growth is observed.

Lentinus edodes, or the shiitake mushroom, is another fungus with reported therapeutic properties, mainly cultivated in China and Japan. It can be seen from the growth profile of *L. edodes* given in Figure 4.5 (c) that the fungus has a very defined shape with clear maxima for growth. A temperature of 25°C was found to be the clear optimum temperature for the growth of *L. edodes* mycelium, with minimal or no growth of *L. edodes* mycelium recorded at temperatures of 10°C and 37°C. Moderate growth was observed at temperatures of 15°C and 30°C.

Compared to both *A. blazei* and *A. bisporus*, *L. edodes* clearly preferred an acidic environment with an optimal pH for growth of pH 4, while good growth was observed over the pH range 4-6. While the two *Agaricus* species previously studied grew only minimally or not at all at pH 3, *L. edodes* mycelium maintained a moderate growth at this level of acidity.

Furlan *et al.* (1997) studied the growth of seven strains of mushroom, including *L. edodes*, and found that this was the only strain of mushroom whose maximal growth was not affected (by acid environments?) at pH 4. The temperature range for optimum growth of *L. edodes* reported in this study was 25-30°C.

Lentinus edodes grew at a faster rate on PDA than *A. blazei* and *A. bisporus*, with radial growth of 24.0 ± 1.0 mm measured after 7 days of incubation under its measured optimum temperature and pH conditions. While this may be attributed to the presence of several of the constituents in the culture medium, it should also be noted that different fungal species naturally have different principal growth rates (Furlan *et al.*, 1997).

An Australian isolate of the white-rot fungus, *Pycnoporus coccineus,* exhibited a higher tolerance to elevated temperatures with an optimal growth temperature of 37°C, and had

an optimal pH for growth in the range pH 4-5 (Figure 4.5 (d)). It is a rapidly growing fungus with optimal radial growth of 35.7 ± 0.6 mm after 4 days at 37 °C. These results confirm the results obtained by Lonergan *et al*. (1993), who concluded that the optimal temperature and pH for *P. coccineus* growth were 37°C and pH 4-5, respectively.

The growth profiles of the four fungi studied highlight the wide variation in pH and temperature tolerance for the different species, which is not unexpected since they come from different taxonomic groups. While many fungi prefer an acidic environment for optimal growth, which aids the suppression of bacterial contamination, the fungi studied also showed a tolerance for alkaline conditions.

This experiment permitted the determination of the optimal temperature and pH for growth by observation of the largest colony diameters. This method has been reported by Brancato & Golding (1953) as a reliable parameter for the determination of optimal growth conditions required for mycelial growth. The range of results obtained for each fungal species studied highlights the importance of the investigation of growth parameters, such as pH and temperature, when studying a particular fungal species.

4.2.1.2 Effect of culture media on radial growth of *A. blazei* mycelium

The effect of different growth media formulations on the resulting radial growth of *A. blazei* was examined using eight defined media according to the method described in Section 3.2.4.2. Plugs of *A. blazei* mycelium were inoculated onto plates of YMA, MEA, PDA, F-PDA, SMA, SDA, CMA, and CDA. Triplicate plates of each medium were incubated at 30°C, and the radial growth was measured every 24 hours over 15 days. The growth of *A. blazei* mycelium on the different solid media compositions is shown in Figure 4.3. The results shown represent the average of triplicate studies for each media, and standard deviations are shown in the error bars.

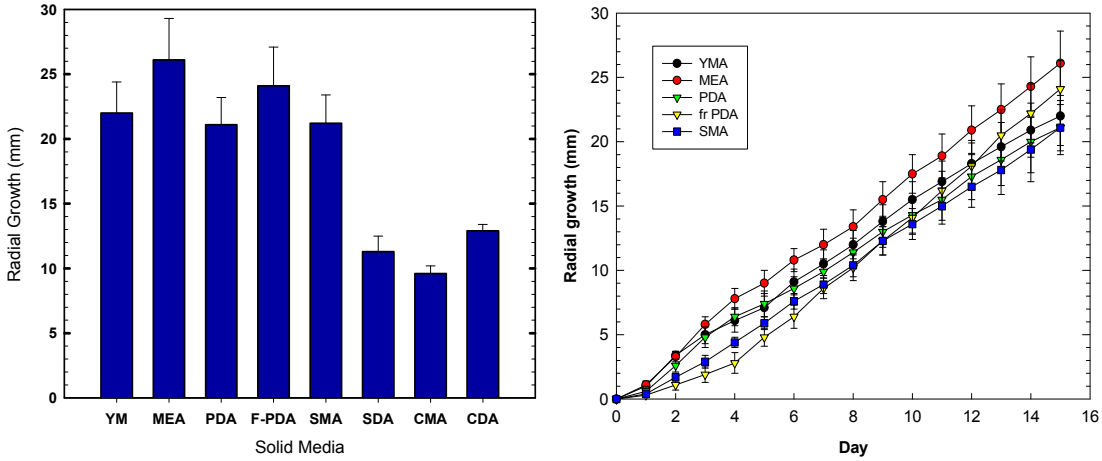


Figure 4.3 *The effect of eight media formulations on (a) the overall growth of* A. blazei *mycelium after 15 days; (b) the daily growth of A. blazei mycelium over 15 days.*

The results shown in Figure 4.3(a) indicate that *A. blazei* grows well on five of the eight media examined. The most growth was obtained using MEA, with 26.1 ± 3.2 mm growth recorded after 15 days of incubation. Growth of cultures on YMA, PDA, fresh PDA and SMA were also good, with between 20-24 mm of radial mycelial growth after 15 days incubation. Cultures grown on SDA, CMA and CDA showed very poor growth, with prevalent sectoring and irregular growth of mycelium also being observed on these three media.

A lag phase of 48 hours was observed for mycelial growth on YMA, MEA, PDA and SMA, while a 4-day lag phase was observed for growth on fresh PDA. Following this lag phase, a phase of exponential growth, or log phase growth, was observed for all five media, with no evidence of a reduction in the growth rate after 15 days of incubation.

The appearance of *A. blazei* mycelium was also different, depending on the media formulation being investigated. Photographs depicting the appearance of *A. blazei* mycelium grown on different agar media for 15 days are shown in Figure 4.4.

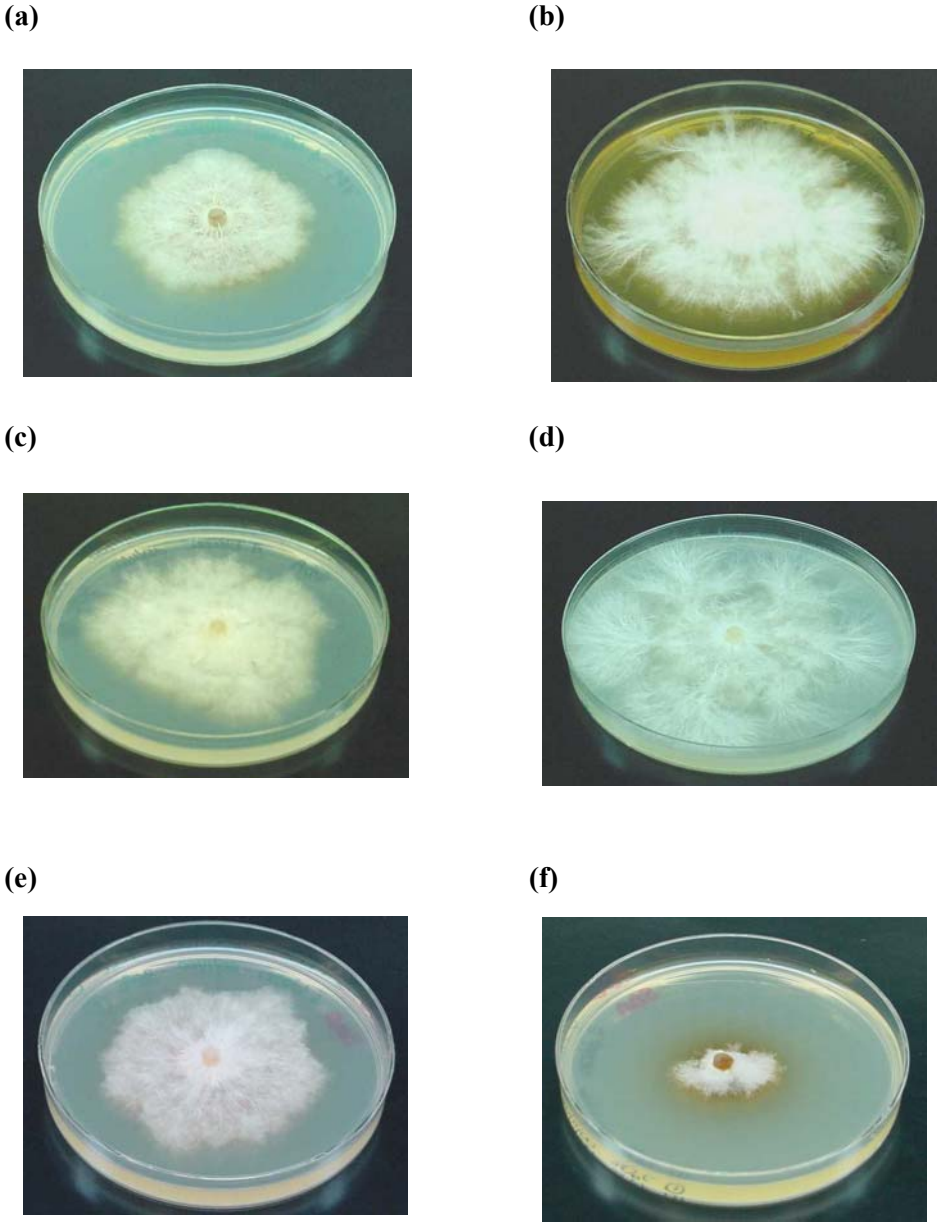


Figure 4.4 *Growth of* A. blazei *mycelium on (a) YMA (b) MEA (c) PDA (d) F-PDA (e) SMA and (f) SDA.*

Despite the longer lag phase, the fastest log growth phase was observed on fresh PDA, a medium containing glucose as a carbon source in addition to complex carbohydrates. Growth on disaccharides, oligosaccharides and polysaccharides requires the synthesis of digestive enzymes. These enzymes are frequently but not always exo-cellular, and a period of induction is required before growth will occur (Griffin, 1994), signified by the longer lag period for fresh PDA. The extensive growth may also be due to the complex carbohydrates that provide additional carbon sources for utilisation by *A. blazei*. In

addition, healthy growth of mycelium on commercially available PDA was observed. It also contains potato extract and glucose as carbon sources. Growth of *A. blazei* mycelium on F-PDA appeared as branched, white mycelial fibre networks extending with a sparse cover of aerial mycelium. On PDA, growth of *A. blazei* mycelium consisted of mainly aerial mycelium, with less branching.

As previously noted, the greatest radial growth of *A. blazei* was observed on MEA, and growth on this medium appeared as a white mycelial mat of typically aerial mycelium. There was also distinct branching of mycelium around the growing edge of the culture.

Rapid healthy mycelial growth was observed on YMA, which contains glucose, yeast and malt extract, and peptone, and SMA, which contains both maltose and peptone. Media containing base constituents of glucose/sucrose, yeast extract/peptone and malt extract are regularly used and recommended for the growth of *A. blazei* mycelium (Mizuno *et al*., 1999; ATCC). Horizontal mycelial growth, as opposed to aerial mycelium, was observed on YMA and SMA, The growth was denser on both these media compared to that observed on MEA.

It is interesting to note the difference in the growth of *A. blazei* mycelium on SMA and SDA (Figures 4.4 (e) and (f)). While rapid healthy growth was observed on SMA, growth on SDA was very poor despite these two media formulations being identical except for the composition of the carbon source. Either combination would be expected to be suitable for the growth of *A. blazei*, however, only SMA supported mycelial growth. This result indicates that *A. blazei* may have a preference for carbon sources such as maltose, as opposed to the popular glucose-based media for fungal growth. This is supported by the fact that optimum growth was obtained on MEA. MEA contains malt extract as a carbon source, which contains a high concentration of carbohydrates, particularly maltose and 60% reducing sugars.

Growth on CMA and CDA was seen to be very poor, indicating that corn meal, and sucrose and a range of salts, respectively, do not provide the nutrients required for

healthy growth of *A. blazei* mycelium. In addition the pH of CDA is 6.8 ± 0.2, a pH level higher than the optimum range of pH 5-6 for growth of *A. blazei*.

As previously mentioned, YMA, with a natural pH of 6.2 – 0.2, and other similar media containing both yeast and malt extracts, peptone, and glucose, is commonly used as a growth medium for growth of *A. blazei* mycelium. However some researchers employ other carbon sources. For growth experiments using *A. blazei* on solid media, Eguchi *et al.* (1994) employed a media consisting of the constituents mentioned above, but replaced glucose with sucrose as the carbon source, which had been determined as the best carbon source for growth of *A. blazei,* and it is often substituted for glucose in solid and liquid medium for *A. blazei* growth (Mizuno *et al*., 1999; Ito *et al*., 1997).

Other *Agaricus* species have been examined for their growth characteristics on different media. Khan *et al.* (1991) examined the growth characteristics of five strains of *Agaricus brunnescens* Peck, and found that among the media tested, including PDA and WEA (wheat extract agar), the best growth of each strain was obtained on MEA. Bilay *et al.* (2000) examined the growth of five species of *Agaricus* mushroom mycelium on four different agar media: MEA, WA (wort agar), PDA and YMA. No particular agar medium provided a more optimal growth for any of the five species investigated, however some species, including *Agaricus bisporus*, and *Agaricus arvensis*, responded favourably to an initial increase of the pH medium to pH 6. The pH adjustment resulted in more growth than the same media at its natural pH of 5.4.

4.2.2 Preservation of *Agaricus blazei* mycelium

Fungal strain conservation by means of utilising an appropriate storage method is of extreme importance, and necessary to maintain the vigour and genetic stability of the pure mycelium (Chang & Miles, 1989). Cells in fungi can degenerate due to the lack of oxygen, lack of nutrients, infections, substrate pH changes and the accumulation of undesirable metabolites (Oei, 1996).

Cultures of edible mushrooms can be preserved as either spores, or vegetative mycelia. Due to the routine fertility checks required when spore cultures are stored, vegetative mycelia of known origin are often used for storage (Chang & Miles, 1989). There are many methods available for the storage of fungal mycelium, depending on whether short-term (up to six months) or longer-term storage is required. The most common short-term storage method for fungal mycelium is periodic agar transfer, which involves subculturing mycelial plugs on solid agar every three to twelve months (Chang & Miles, 1989), or storage of mycelium on agar slants under mineral oil (Wernham, 1946). Storage of mycelium in liquid nitrogen is the most widely used method for long-term storage of fungal species (Smith, 1982), provided the appropriate facilities are available.

The effectiveness of using two different preservation methods on the viability of *Agaricus blazei* mycelium over both a short and long term period was investigated. Periodic subculturing of *A. blazei* mycelium after storage at 4°C on agar was examined for its suitability for short-term storage over a period of 1 to 8 weeks.

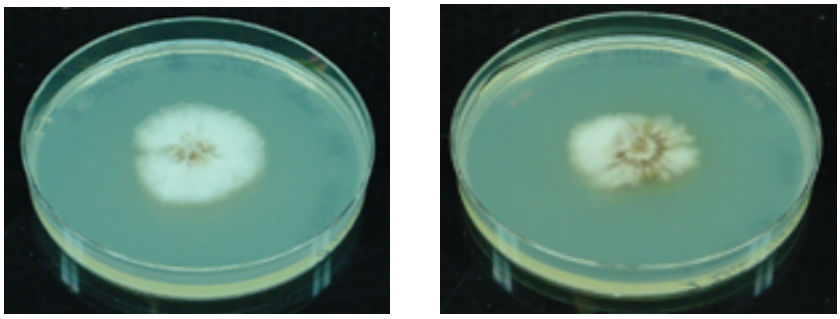
In addition, the storage method described by Ito & Yokoyama (1983) was also examined. It involved the freezing of mycelial plugs in a 10% (v/v) glycerol solution, and was reported to successfully maintain nearly eight hundred strains of various species of fungi for at least a year (Ando, 1997). This method was examined for its suitability for storage of *A. blazei* mycelium over both short-term (up to two months) and long-term (up to 3 years) periods.

4.2.2.1 Short term preservation on agar at 4°C

While carrying out the experiments described in Section 4.2.1.2, observations indicated that, although *A. blazei* mycelium grew well on MEA, F-PDA, YMA, PDA, and SMA, growth on YMA and MEA was less susceptible to viability loss after repeat subculturing and resuscitation from storage at 4°C. Therefore these two media formulations were used to examine storage at this temperature.

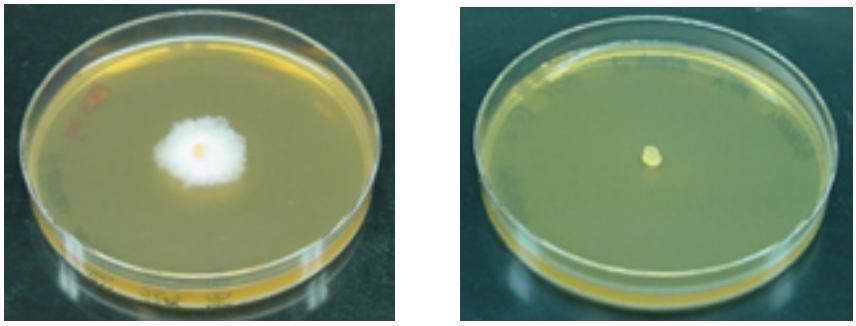
Agaricus blazei mycelium was grown on YMA and MEA for 14 days at 30°C. The plates were then sealed with Parafilm, and stored at 4°C for periods of up to eight weeks. To examine the effect of this storage method on the viability of *A. blazei* mycelium over a short term period, plugs were prepared from healthy mycelium stored on YMA and MEA culture plates for periods of 1, 2, 4 and 8 weeks. This was followed by inoculation of the mycelium onto eight plates of the same solid media that was used for storage. The plates were then incubated for 14 days and the radial growth measured every 24 hours. The amount of radial growth and the appearance of the mycelium were used as indictors of mycelial viability.

In preliminary experiments, the plates were incubated at either 25°C or 30°C after resuscitation from storage at 4°C to allow for mycelial growth. This was later revised to an incubation temperature of 25°C for growth of mycelium, as changes to mycelial morphology were noticed in cultures incubated at 30°C. Although mycelial growth on YMA was rapid at 30°C, areas of sectoring mycelium were found to be more prevalent in these cultures compared to cultures incubated at 25°C, as shown in Figure 4.5. Furthermore, there was no growth of mycelium on MEA at 30°C after 2 weeks of storage (Figure 4.6).



(a) **(b)**

Figure 4.5 *(a) healthy growth of* A. blazei *mycelium on YMA after incubation at 25℃ after 2 weeks of storage; (b) sectoring of* A. blazei *mycelium on YMA after incubation at 30℃ after 2 weeks of storage.*



(a) **(b)**

Figure 4.6 *(a) growth of* A. blazei *mycelium on MEA after incubation 25℃ after2 weeks of storage; (b) no growth of* A. blazei *mycelium on MEA after incubation at 30℃ after 2 weeks of storage.*

Mycelial deformation, or sectoring, is an indication that the genetic integrity of the mycelium has been affected or mutated, and is often associated with a loss of viability (Sharma & Brown, 1983; Kirk *et al*., 2001). Sector formation is frequently associated with changes in the ability of the fungus to produce secondary metabolites and enzymes (Ryan *et al*., 2002). The temperature shock due to the transfer of *A. blazei* mycelium from 4°C to 30°C may increase the stress on the growing culture of mycelium to a level that subsequently affects growth of a healthy viable culture. Therefore for an examination of the viability of this storage method, culture plates were incubated at a temperature of 25°C following resuscitation from storage, for a period of 14 days. The

growth of mycelium on YMA and MEA plates for 14 days after 1, 2, 4, and 8 weeks storage on agar at 4°C is shown in Figure 4.7.

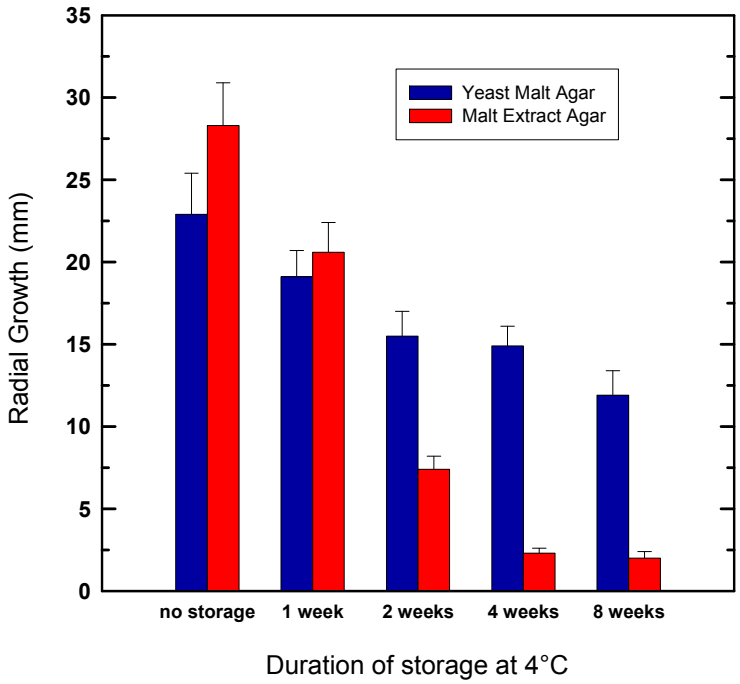


Figure 4.7 *Growth of* A. blazei *mycelium after 1-8 weeks storage on agar plates at 4°C*

Routine subculturing of *A. blazei* on YMA without storage resulted in healthy growth of mycelium on all replicate plates with average growth of 22.9 ± 2.5 mm after 14 days of incubation at 25°C. No sectoring was visible and no lag period evident, with mycelial growth occurring after 24 hours incubation.

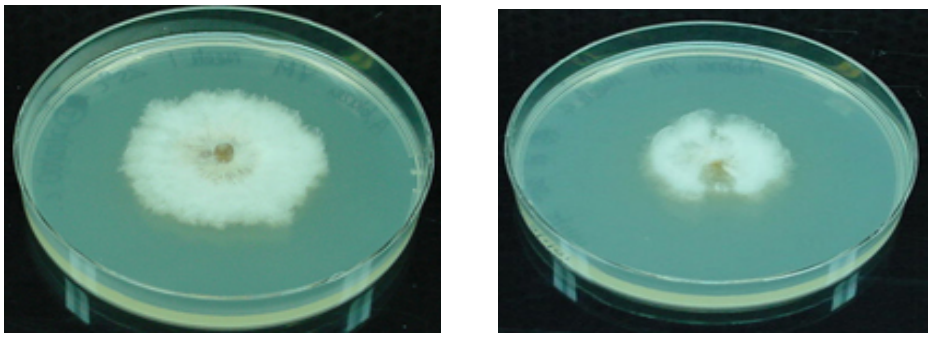
Following one week of storage on YMA at 4°C, all inoculated cultures began to grow immediately, with no lag period evident. The average maximum growth recorded, 19.1 ± 1.6 mm, was slightly less than that observed in cultures not subjected to storage. There was no sectoring observed in the eight replicate plates examined.

Recovery of cultures after two weeks of storage on YMA at 4°C was slightly slower, with an observed lag period of 48 hours before evidence of mycelial growth. All replicate plates grew although there was a further slight reduction in the average growth

obtained 15.5 ± 1.5 mm was recorded after 14 days of incubation. In addition, half of the plates inoculated showed evidence of minor sectoring.

Mycelial growth of *A. blazei* after four weeks of storage on YMA began after a lag period of 48 hours, and the average growth of mycelium was 14.9 ± 1.2 mm, although five out of eight cultures showed evidence of severe sectoring.

After eight weeks of storage on YMA the growth rate was further reduced with an average of 11.9 ± 1.5 mm growth after 14 days of incubation, and a lag period of 4 days was observed before the initiation of mycelial growth. While all cultures grew, severe sectoring was evident in six out of eight cultures. The appearance of sectoring in cultures revived after eight weeks of storage compared to those revived after 1 week of storage is shown in Figure 4.8.



(a) *1 week of storage* (b) *8 weeks of storage*

Figure 4.8 *Growth of* A. blazei *mycelium on YMA (a) 1 week of storage; and (b) 8 weeks of storage on YMA plates at 4 ℃*

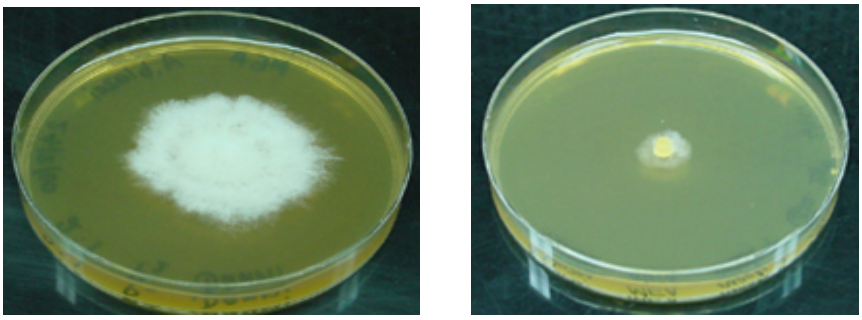
Although a significant decrease in radial growth ($p<0.0001$), and an increase in the recovery period required before growth were observed, storage of *A. blazei* cultures on YMA at 4°C may still be utilised as an appropriate storage for a short-term periods up to eight weeks. While some sectoring occurred after longer storage periods, improved mycelial growth was assured by further subculturing of mycelial plugs cut only from healthy growing parts of the culture where no sectoring was evident.

Good growth of *Agaricus blazei* mycelium was observed on MEA without prior culture storage, with average growth of 28.3 ± 2.6 mm after 14 days of incubation at 25°C. No sectoring was visible and no lag period observed.

Following one week of storage on MEA at 4°C, a lag period of 48 hours was recorded before all inoculated cultures began healthy growth. The average maximum growth recorded after 1 week of storage was 20.6 ± 1.8 mm after incubation for 14 days, with no sectoring observed in the inoculated cultures.

After two weeks of storage on MEA at 4°C the growth of A. *blazei* mycelium was significantly reduced ($p < 0.0001$) with an average growth of 7.4 ± 0.8 mm after 14 days incubation. After a 4-day lag period, six out of eight cultures recorded growth, with minor sectoring evident in all cultures.

Storage and reinoculation of *A. blazei* mycelium on MEA after periods of 4 and 8 weeks resulted in an extremely reduced growth rate, with average growth of 2.3 ± 0.3 mm and 2.0 ± 0.4 mm respectively. Following a 3-4 day lag period, only 50% of inoculated cultures grew. The appearance of *A. blazei* cultures after 8 weeks of storage at 4°C compared to 1 week of storage is shown in Figure 4.9.



(a) *1 week of storage* (b) *8 weeks of storage*

Figure 4.9 *Growth of* A. blazei *mycelium on MEA after (a) 1 week of storage; and (b) 8 weeks of storage on MEA plates at 4 ℃*

These results suggest that MEA is not an appropriate media for storage of *A. blazei* mycelium on agar plates at 4°C for more than 1 week, and YMA is the medium of preference for storage of *A. blazei* using this method.

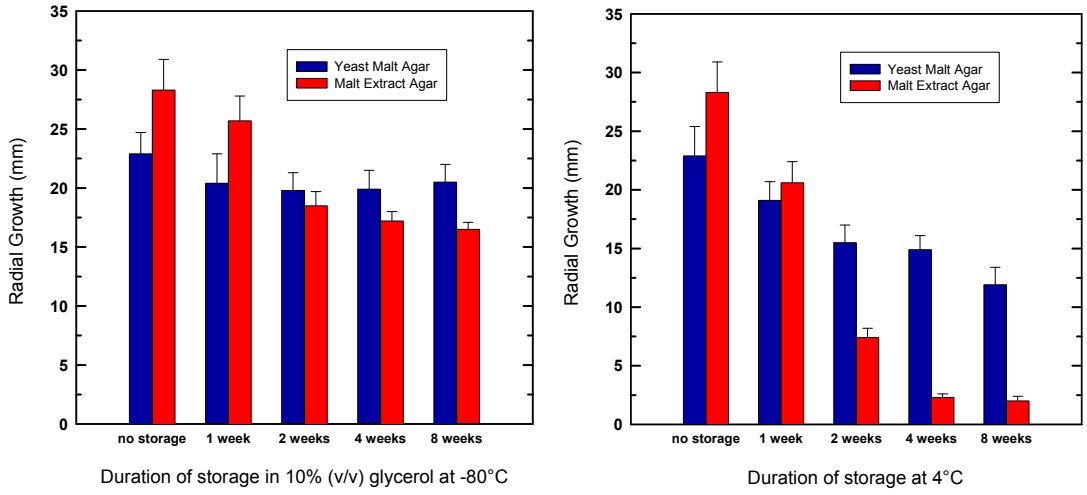
Methods such as the periodic transfer of mycelial cultures stored under refrigeration are commonly used for short term preservation due to the ease of the method and the fact that there is no requirement for expensive equipment. The cold storage lowers the rate of metabolism, and is often referred to as suspended metabolism (Smith & Onions, 1983). The method does however have some disadvantages, such as increased susceptibility to contamination by other microorganisms, which may be prevented by less frequent transfers. Storage in liquid nitrogen at -196°C, however, ensures that metabolism of fungi is effectively halted and is often used if the appropriate facilities are available (Smith, 1982). In addition, it is common practice to suspend the fungi in a cryoprotective solution to improve their viability after resuscitation. (Smith, 1983). Mineral oil has traditionally been used (Wernham, 1946) although 10% (w/v) glycerol is now commonly employed as it is less viscous than mineral oil and therefore easier to use (reference needed here). While fungi can be preserved in liquid nitrogen using cryoprotectants, the storage of *A. blazei* mycelium in 10% (w/v) glycerol was examined with storage in a -80°C freezer.

4.2.2.2 Short-term preservation in 10% glycerol-water at -80°C

Agaricus blazei mycelium was grown on plates of YMA and MEA for 14 days at 30°C. Mycelial plugs were cut from healthy mycelium using a sterile 5 mm diameter cork borer, and transferred to 2 mL vials of sterile 10% (v/v) glycerol. The vials were frozen at -80°C for a period of up to eight weeks to examine the effect of short-term storage, and a period of up to 36 months to assess the effect of long-term storage.

To examine the effect of this storage method on the viability of *A. blazei* mycelium, plugs were thawed at room temperature at specified time intervals and used to inoculate YMA and MEA plates (eight plates /medium). The plates were then incubated for 14 days and the radial growth measured every 24 hours. The amount of radial growth and

the appearance of the mycelium were used as indictors of mycelial viability. The growth of *A. blazei* mycelium on YMA and MEA plates at 25°C after 1, 2, 4, and 8 weeks of storage in 10% (v/v) glycerol at -80°C is shown in Figure 4.10 (a), For comparison, the growth profile of *A. blazei* stored at 4°C is shown in Figure 4.10(b) and is the same data presented in Figure 4.7.



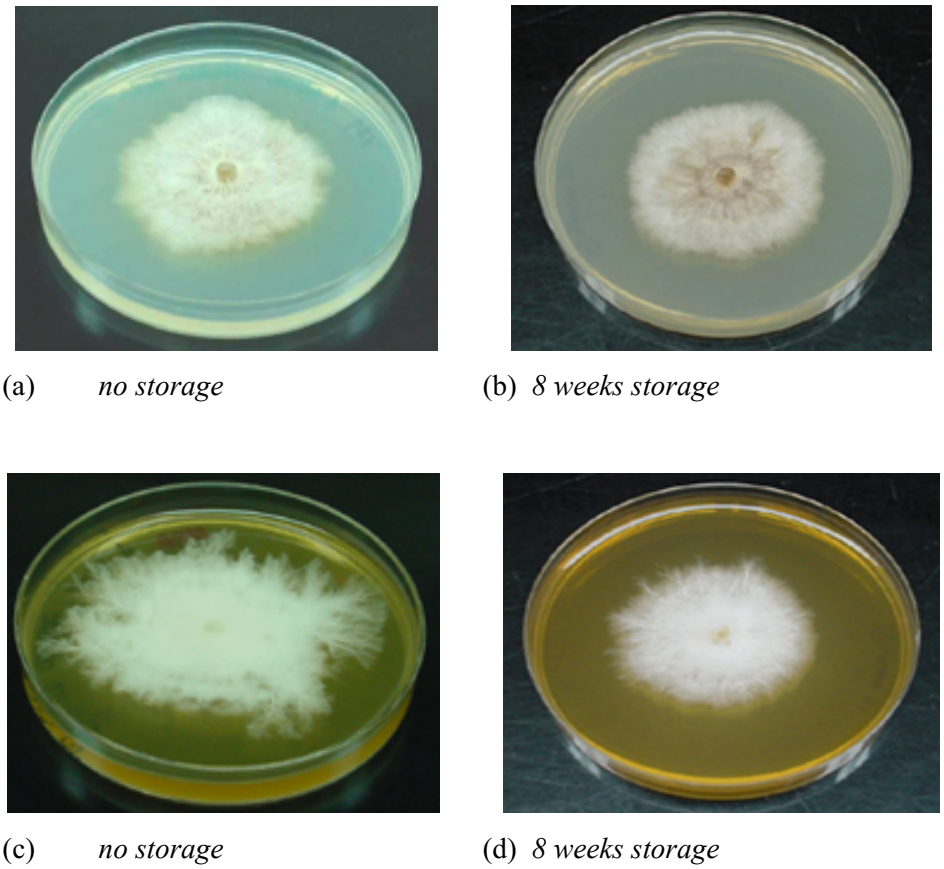
(a) (b)

Figure 4.10 *(a) Growth of* A. blazei *mycelium after storage in 10% (w/v) glycerol in water at -80 °C for 1-8 weeks (b) Growth of* A. blazei *mycelium after storage at 4 °C for 1-8 weeks*

Over a period of 1-8 weeks of storage on YMA at 80 °C in 10% (v/v) glycerol, the average growth of *A. blazei* reduced slightly compared to cultures not subjected to storage. This is in contrast to data obtained using 4°C agar storage (Figure 4.10(b)), which demonstrated a significant reduction in growth after just one week of storage ($p<0.0001$). The average growth of mycelium on YMA after 1, 2, 4 and 8 weeks of storage at -80°C in 10% (v/v) glycerol was 20.4 ± 2.5 mm, 19.8 ± 1.5 mm, 19.9 ± 1.6 mm and 20.5 ± 1.5 mm respectively. No sectoring occurred and the only lag period observed of 48 hours was recorded after eight weeks of storage. This slight reduction was not significant ($p=0.056$).

After one week of storage on MEA at -80°C in 10% (v/v) glycerol, the maximum average growth was 25.7 ± 2.1 mm compared to 28.3 ± 2.6 mm for cultures not subject to storage. After two to eight weeks of storage using this method, the subsequent growth of mycelium remained healthy, although the growth rate decreased significantly over that time ($p<0.0001$), with average growth of 18.5 ± 1.2 mm, 17.2 ± 0.8 mm and 16.5 ± 0.6 mm, after 2, 4 and 8 weeks, respectively. As for YMA, no sectoring was present in any cultures regenerated after 1-8 weeks with the only lag period of one day, again recorded after eight weeks of storage.

The appearance of cultures regenerated after eight weeks of storage at -80°C in 10% (v/v) glycerol on YMA and MEA, compared to cultures not subject to storage is shown in Figure 4.11.



(a) *no storage* (b) *8 weeks storage*

(c) *no storage* (d) *8 weeks storage*

Figure 4.11 *Growth of* A. blazei *mycelium on YMA after 14 days incubation (a) no storage; (b) 8 weeks of storage; Growth* of A. blazei *mycelium on MEA after 14 days incubation (c) no storage; (d) 8 weeks of storage in 10% (v/v) glycerol at -80 ℃*

The method of freezing mycelial plugs at -80°C in 10% (v/v) glycerol was shown to be an effective method for short-term storage of *A. blazei* on both YMA and MEA from 1-8 weeks.

4.2.2.3 Long-term preservation in 10% glycerol-water at -80°C

To examine the effectiveness of this method for long-term storage, the experiment outlined in Section 4.2.2.3 was repeated with mycelial plugs recovered at intervals between 6-36 months. The average growth of cultures regenerated after 6-36 months of storage is shown in Figure 4.12.

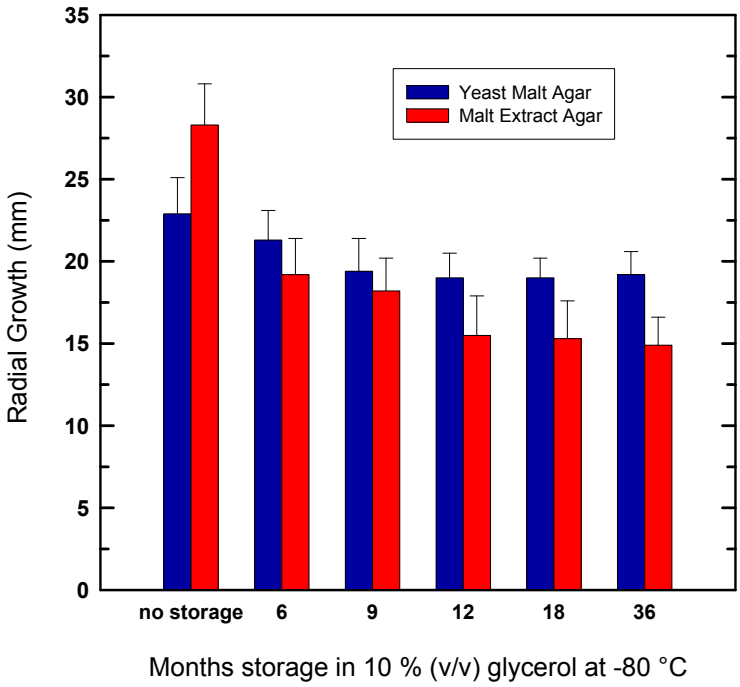
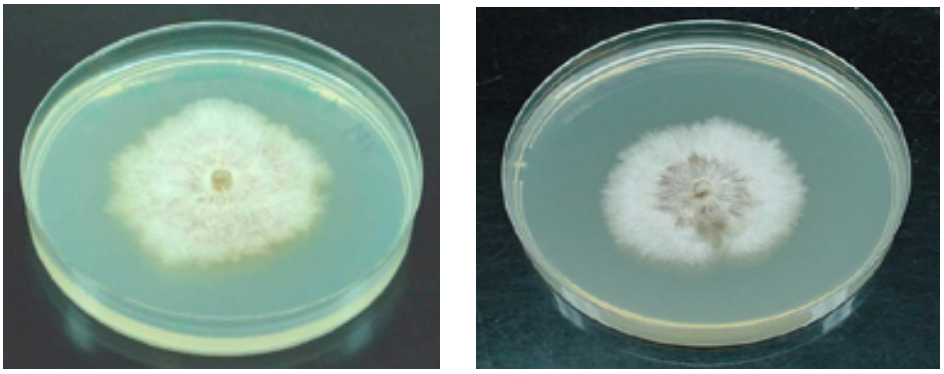


Figure 4.12 *Growth of* A. blazei *mycelium after storage in 10% (v/v) glycerol at -80 ℃ for 6-36 months*

Recovery of *A. blazei* cultures on YMA from storage after 6-9 months resulted in a negligible decrease in the average growth after 15 days of incubation (p=0.003), compared to cultures not subject to storage. After 12, 18 and 36 months, there was no further reduction in growth observed, with the average growth reaching a plateau of

19.0 ± 1.5 mm, 19.0 ± 1.2 mm and 19.2 ± 1.4 mm, respectively. Lag phases were observed for each recovery period: an average of 48 hours for recovery at 6, 12 and 18 months, and 72 hours for recovery at 36 months. The appearance of the mycelial growth on YMA after 36 months of storage compared to no storage is shown in Figure 4.13.

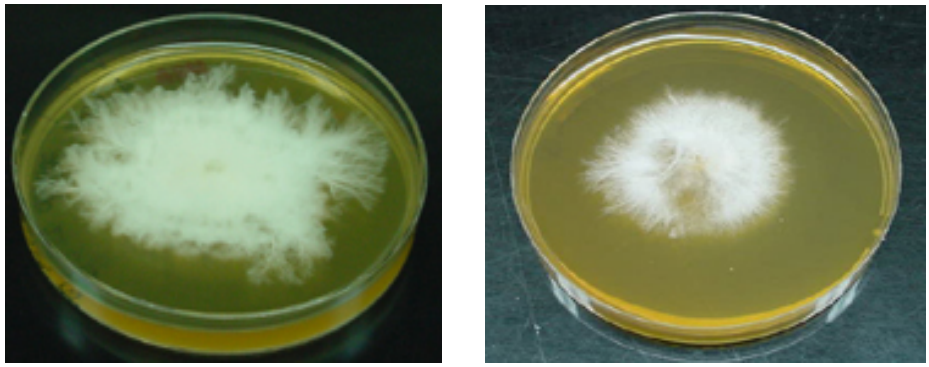


(a) no storage *(b) 36 weeks of storage*

Figure 4.13 *Growth of* A. blazei *mycelium on YMA (a) no storage; (b) 36 weeks of storage in 10% glycerol at -80 ℃*

When regenerated on YMA, good recovery was observed as indicated by the amount, and viability of the mycelium. Minor sectoring, such as that shown in Figure 4.13 (b), was visible in 20% of plates examined, although further subculture of the mycelium from a healthy section of the growth generated mycelial cultures with no sectoring.

When regenerated on MEA, the maximum growth obtainable decreased from 19.2 ± 2.2 mm after 6 months of storage, to 14.9 ± 1.7 mm after 36 months. The majority of cultures grown on MEA showed healthy growth, with 35% of cultures showing minor sectoring after 36 months of storage although there was a significant reduction in fungal growth ($p<0.0001$). The appearance of the mycelial growth on MEA after 36 months of storage compared to that obtained when no storage has taken place is shown in Figure 4.14.



(a) no storage *(b) 36 weeks of storage*

Figure 4.14 *Growth of* A. blazei *mycelium on MEA (a) no storage; (b) 36 weeks of storage in 10% (v/v) glycerol at -80 ℃*

These results indicated that the storage of *A. blazei* mycelium in 10% (v/v) glycerol at -80°C was an effective method for both short-term and long-term periods. While recovery was more effective on YMA, as indicated by a higher average mycelial growth after storage, storage and recovery on MEA also proved to give acceptable results, although more subculturing steps may be needed to ensure healthy fungal growth.

This storage method has been described in detail for the preservation of fungi (Ito & Yokoyama, 1983). Ando (1997) examined the effectiveness of this method for preservation of eight hundred strains of mushrooms from 280 different genera, for one year, and found it to be most effective in all but nine genera studied. In the majority of cases, the viability of strains was 90-100%. Problems arose, however, with more than seven cycles of repeated thawing and freezing. Hence, if the freeze/thaw process is minimised, this method is considered very effective for long-term storage of mushroom cultures, and indeed this has been shown for the preservation of *Agaricus blazei* cultures.

Kitamoto *et al.* (2002) examined the suitability of 10% (v/v) glycerol as a cryoprotectant for maintenance of sixty-six stocks of filamentous fungi, particularly mushrooms. Many fungi, including *A. bisporus,* and medicinal mushrooms such as *L. edodes* and *G. lucidum,* could survive for up to ten years in medium containing 10% (v/v) glycerol.

4.3 Summary

In summary, it was demonstrated that five commercially available agar media supported the growth of *A. blazei* mycelium, indicating this fungus is not particularly fastidious with regard to the nutritional requirements for growth. Yeast malt agar (YMA) was recommended by the ATCC for growth of the particular *A. blazei* strain utilised in this study, and it proved to be one of the more suitable growth media in addition to MEA. Future research may involve an investigation of the growth *A. blazei* using media compositions similar to that used in commercial production of fruiting bodies which utilise less expensive grain materials. The optimal temperature and pH ranges for growth of *A. blazei* mycelium were found to be 28-29°C and pH 5-6, respectively, and varied from the temperature and pH optima of other fungal species examined.

Effective short-term and long-term storage of *A. blazei* mycelium was achieved by freezing healthy mycelial plugs in 10% (v/v) glycerol, followed by storage at -80°C. This method of storage was used for the maintenance of all working cultures of *A. blazei* throughout the course of this research, given the reliability and ease of using this storage method for the resuscitation of healthy mycelium.

This current research highlights the importance of performing a thorough investigation of the physiological requirements of a particular fungal species, given that it is not possible to have one culture medium or set of conditions suitable for maximal growth of different basidiomycetes (Bilay *et al*., 2000). Identification of the appropriate conditions and suitable media for growth, also allows for additional exploitation of conditions when other biotechnological applications, such as the production of enzymes or bioactive compounds, are being considered.

Chapter Five

Biomass and exo-polysaccharide production by *Agaricus blazei* in liquid culture

5.1 Introduction

The growth of fungi in submerged or liquid culture has become a major focus for the discovery of potential bioactive compounds (Pointing, & Hyde, 2001) Whilst the fruiting bodies of medicinal mushrooms have been the main focus for the discovery of such compounds in the past, the fact that they can take several months to culture has lead to an increased focus on the possibility of locating and identifying bioactive substances from cultured mycelia (Tang & Zhong, 2002). In addition, the growth of mycelia is amenable to large-scale production, it is relatively easy to manipulate and control the constituents of the media in which the mycelium is grown, and both the liquid cultured mycelium and the culture filtrate are available for the detection of compounds with potentially medical applications (Yang & Liau, 1998a).

An understanding of the nutritional requirements of a fungus is a prerequisite for successfully growing mycelia in submerged culture (Song, *et al*. 1987). Growth parameters that are important in this process (as well as solid culture) include temperature, pH, culture media constituents, degree of aeration, and agitation speed (Yoneyama *et al*. 1997a, 1997b; Kim *et al*. 2003).

Polysaccharides isolated from liquid cultured mycelium or culture broths that exhibit various biological actions are of particular interest to mushroom researchers. In their review article, Margaritis and Pace (1985) reported that the extent to which organisms produce exo-polysaccharides (EPS) is influenced by a wide range of parameters such as culture medium pH and composition, parameters that also significantly influence the extent of mycelial growth.

Many exo-polysaccharides produced by fungi have been shown to have therapeutic effects including antitumour (Wasser & Weis, 1999), anti-viral and antimicrobial activity (Ooi and Liu, 1999). Accordingly, there is much interest in the generation of EPS by fungal species, and the effect of liquid culture conditions on the rate of production of medicinal compounds.

Research regarding EPS production, and the influence of culture conditions on their production, has been carried out on several fungi (Xu and Yun, 2003; Yang and Liau, 1998; Jonathan & Fasidi, 2001). More recently, Shu *et al*., (2004) examined the effects of culture pH on the production of polysaccharides by *Agaricus blazei* in liquid culture and found that bioactive polysaccharides with higher molecular weights were obtained in cultures with lower pH values, however lower yields were obtained. Conversely, higher yields of bioactive polysaccharides with lower molecular weights were obtained in cultures with higher pH values. It was also found that the extent of polysaccharide production is also influenced by temperature and culture media constituents, two factors investigated in this study.

5.2 Results and Discussion

5.2.1 Biomass production in liquid culture

In this study, the effects of temperature and pH on biomass production by *A. blazei* in 15 day YMB cultures were examined, in addition to investigating the production of biomass over a 30 day period in YMB and PDB. The effect of biomass production in different liquid media formulations was also examined.

5.2.1.1 Effects of temperature and pH on *A. blazei* biomass production

Agaricus blazei mycelium was grown in flasks of YMB according to the method described in Section 3.2.5.2 in an effort to examine the role that temperature plays on the extent of mycelial growth. Triplicate flasks were incubated at temperatures of 15, 20, 25, 30, and 37 °C for 15 days with shaking at 100 rpm. The effect of pH on biomass production was examined according to the method described in Section 3.2.5.3.

Agaricus blazei was grown in YMB media adjusted to pH values in the range pH 3- pH 9. Triplicate flasks for each pH value were incubated for 15 days at 30°C with shaking at 100 rpm.

The biomass produced in each culture flask was removed by filtration, freeze-dried and the average dry biomass at each temperature and pH value was determined. The production of *A. blazei* biomass as a function of temperature and initial media pH are shown in Figures 5.1 (a) and (b) respectively.

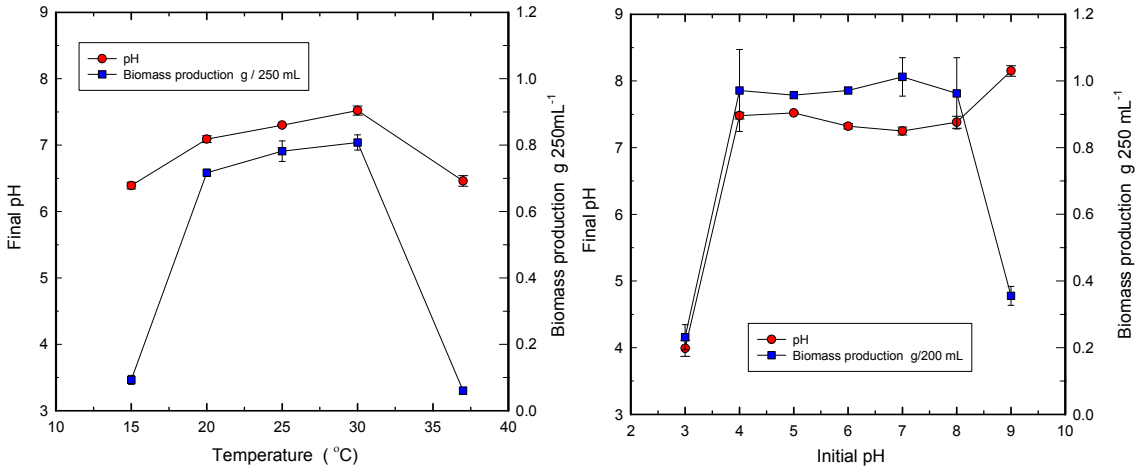


Figure 5.1 *The effect of (a) temperature; (b) pH, on* A. blazei *biomass production in YMB*

For liquid culture in YMB, which has an initial pH of 6.2, *Agaricus blazei* grew well over the temperature range of 20-30 °C, with little growth at 15 °C or 37 °C (Figure 5.1a). This range is a lot narrower than that obtained for growth on solid agar, where *A. blazei* grew at temperatures in the range 15 37 °C. The biomass produced grew in small round pellets similar to the growth of *A. blazei* in liquid culture reported by Akazawa, *et al*. (2002).

The optimum temperature for growth of *A. blazei* was found to be 30 °C, producing 0.8 g biomass per 250 mL culture medium. The pH of the medium was found to have increased from 6.2 to 7.5 during the incubation period. While the optimum temperature for cultivation of *Agaricus blazei* in liquid culture has not been reported for the strain under investigation, researchers investigating growth of *A. blazei* in liquid culture have reported optimal growth temperatures of 25 °C (Yoneyama *et al.* 1997a, 1997b; Jia, *et*

al. 2002; Shu & Wen, 2003; Zou, 2006; Hamedi, *et al*. 2007), 27°C (Lin & Yang, 2006), 28 °C (Shu *et al*., 2004, 2007), 30°C (Akazawa *et al*. 2002), and 32°C (Fan *et al*., 2003).

Cultures incubated at 15 °C and 37 °C exhibited minimal growth. In these instances, no change in pH was observed over the incubation period, which is in contrast to instances where appreciable mycelial growth was observed, where the pH was seen to increase to levels above pH 7 over the period of incubation. This change in pH can be attributed to the production of alkaline substances or the consumption of acidic nutrients in the nutrient media.

It is difficult to identify a single optimal pH value for liquid cultivation of *A. blazei* from the data presented in Figure 5.2(b) since there is some variation within the replicate experiments at some pH values. As a result, an optimal growth pH range of 4-8 was identified. Previous literature reports the optimum pH for growth of A. blazei in liquid culture is pH 5.0 to 6.0 (Zou *et al*. 2002; Kim *et al*. 2004; Fan *et al*. 2003; Lin & Yang, 2006; Hamedi *et al*. 2007). These results concur with the results obtained in this study for growth on solid agar, although on solid culture, moderate growth was also observed at pH 3 and 9 at 30 °C. For submerged culture in YMA, no growth was observed at pH 3 and only minimal growth at pH 9, and there was little change in pH over the incubation period.

The amount of biomass produced over the optimum pH range was 0.9 1.0 g per 250 mL of medium. In these cases, the pH of the medium increased from an initial value of 6.2 to between pH 7.5 - pH 8. A minimal amount of biomass was produced at pH 3 and pH 9, and the final pH of the culture filtrate did not deviate greatly from the initial adjusted pH of the media.

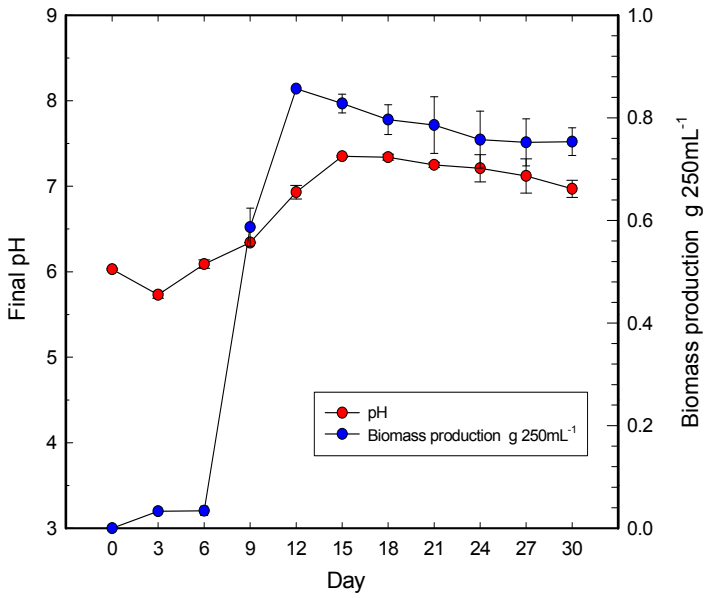
As previously mentioned, Shu *et al*. (2004) also examined the influence of pH on biomass production of *A. blazei* strain ATCC 76739. The extent of biomass production was determined at a temperature of 30°C, shaken at 250 rpm at pH levels ranging from pH 4.0 to pH 7.0 in a culture medium consisting of glucose, peptone, malt extract, and yeast extract, and included additional salts and vitamin B1. Their results also

demonstrated that production of biomass was constant over the pH range examined, with 7.3 7.4 g L^{-1} of biomass produced. In this study, no clear optimal growth pH was identified.

5.2.1.2 Temporal production of biomass by *Agaricus blazei*

A. blazei biomass production in submerged culture was examined using two different liquid media, according to the method described in Section 3.2.5.4. *A. blazei*, previously grown in YMB, was inoculated into flasks of YMB and PDB. Flasks of each media were incubated at 30°C with shaking at 100 rpm. Triplicate flasks of each media were removed from incubation every 3 days for a period of 30 days, and the biomass produced was removed by filtration. The biomass was then freeze-dried, and the average biomass produced over time in each culture media was determined. The production of *A. blazei* biomass as a function of time, in YMB and PDB, is shown in Figure 5.2.

(a)



(b)

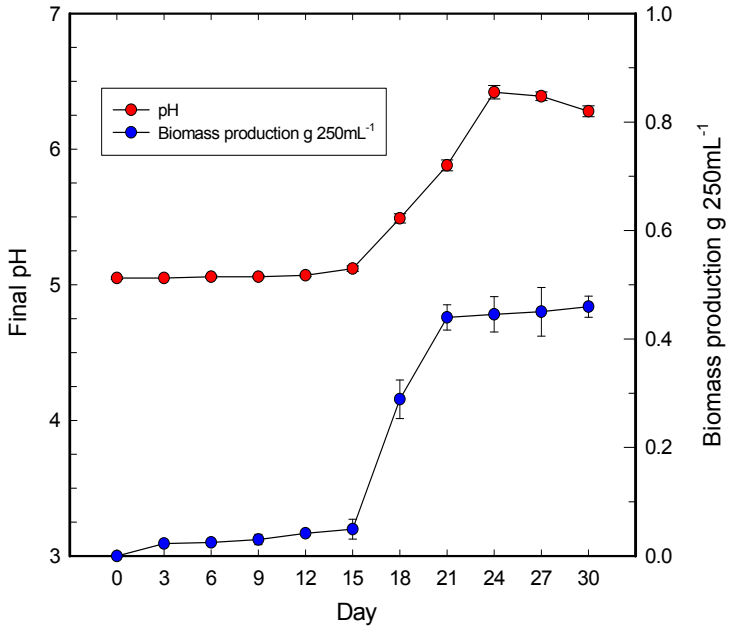


Figure 5.2 *Production of biomass by* A. blazei *in (a) YMB over 30 days ;(b) PDB over 30 days*

After inoculation of *A. blazei* mycelium into the YMB media, a lag period of 6 days was evident before the rate of mycelial growth increased (Figure 5.2a). There was a steady increase of biomass production observed from day 6 to day 12, after which time

biomass production gradually decreased up to day 30. The final pH of the culture filtrate was measured as a function of time, and was seen to increase with the onset of biomass production. The pH of the culture media reached a maximum of pH 8.3 at day 15 after the log phase of biomass production, and slowly declined to approximately pH 7.0 as the extent of biomass production also declined.

A. blazei was also cultivated in PDB, a culture medium that includes in addition to carbon sources, over an incubation period of 30 days. A lag period of 15 days was evident before the rate of mycelial growth increased, more than twice the lag period that was observed for YMB. As discussed earlier, the lag period is essential for the development of digestive enzymes necessary for the breakdown of complex carbohydrates contained in PDB that are not present in YMB. There was a steady increase in biomass production from day 15 to day 21, after which time the rate of biomass production remained approximately constant up to day 30.

The pH of the culture filtrate was seen to increase with the onset of biomass production, and reached a maximum just above pH 6.0 at day 24, three days after biomass production became approximately constant, after which time the pH of the culture media slowly declined.

The growth curves observed for *A. blazei* grown in both YMB and PDB exhibited a distinct lag phase, followed by a period of log phase growth followed by a period of approximately constant growth, show the typical characteristics of fungal growth reported by other researchers (Hwang *et al*. 2003; Hassegawa *et al.* 2005).

Shu *et al.* (2004) examined the growth of *A. blazei* in liquid culture using a media containing the same base ingredients as YMB but also including additional salts and vitamin B1. Growth of *A. blazei* was examined over 24 days at an initial pH of 6.0, and a lag period of approximately 10 days was observed before a log increase in growth over the following 8 days. Biomass production was steady from day 18-20.

When comparing biomass production and the duration of the lag phase in different liquid culture experiments, it is important to consider the inoculum used. The use of

homogenised mycelium from previous liquid cultures as the inoculum for additional culture flasks will reduce the lag phase duration before the onset of exponential growth (Yoneyama *et al*. 1997(b)) compared to similar experiments where a mycelial plugs of solid agar is used as the inoculum. In addition, the amount of homogenised mycelium added to a particular volume of culture medium will profoundly influence the culture media characteristics. More biomass will typically be produced but the production rate will decline at an earlier stage due to exhaustion of nutrients from the culture medium.

Given that longer lag periods were observed for liquid cultures of *A. blazei* mycelium compared to that of agar cultures, incubation periods of 20-30 days were used for future liquid culture experiments involving studies of the production of bioactive compounds. The focus of this research was to screen *A. blazei* extracts obtained from liquid cultured mycelium and culture filtrates for potential bioactivity. Therefore, selection of a cultivation period of 20-30 days allowed an appropriate time for the production of the maximum amount of biomass, and secretion of compounds into the culture media, given that both biomass production and the pH of the culture medium has been seen to rather remain constant or slowly decline after this time.

5.2.2 Exo-polysaccharide production in liquid culture

While there is extensive literature available that reports the bioactive properties, particularly the anti-tumor activity, of *Agaricus blazei* polysaccharides from fruiting bodies and liquid cultured mycelium (Kawagishi, *et al*. (1990); Itoh, et al. (1994); Ito, *et al*. (1997); Fujimiya, et al. (1998); Mizuno, *et al.* (1999)), there have relatively few reports regarding bioactive EPS excreted into liquid culture medium during *Agaricus blazei* growth (Fan *et al.* 2003; Shu *et al*. 2004; Shu & Xu, 2007). The EPS produced by *Agaricus blazei* in liquid culture have been shown to be either beta-glucans (Fan *et al*., 2003; Shu *et al*., 2004) or mannan-protein complexes (Ito *et al*., 1980; Mizuno, 1995). The current research investigated the effect of temperature, pH, and carbon source on the production of EPS by *A. blazei* in liquid culture, and comparisons were made to the amount of biomass produced for each set of culture conditions.

5.2.2.1 Effect of temperature and pH on *A. blazei* exo-polysaccharide production

A. blazei mycelium was grown in flasks of 100 mL basal media according to the method described in Section 3.2.5.5 to examine the effect of temperature on the production of biomass and EPS by in liquid culture. Triplicate flasks were incubated at temperatures of 20, 25, 30 or 37 °C for 15 days with shaking at 100 rpm.

The biomass produced in each culture flask was removed by filtration, freeze-dried and the average amount of biomass produced at each temperature was determined. EPS was extracted from the culture filtrate according to the method described in Section 3.3.3. The production of biomass and EPS by *A. blazei* as a function of incubation temperature is shown in Figure 5.3.

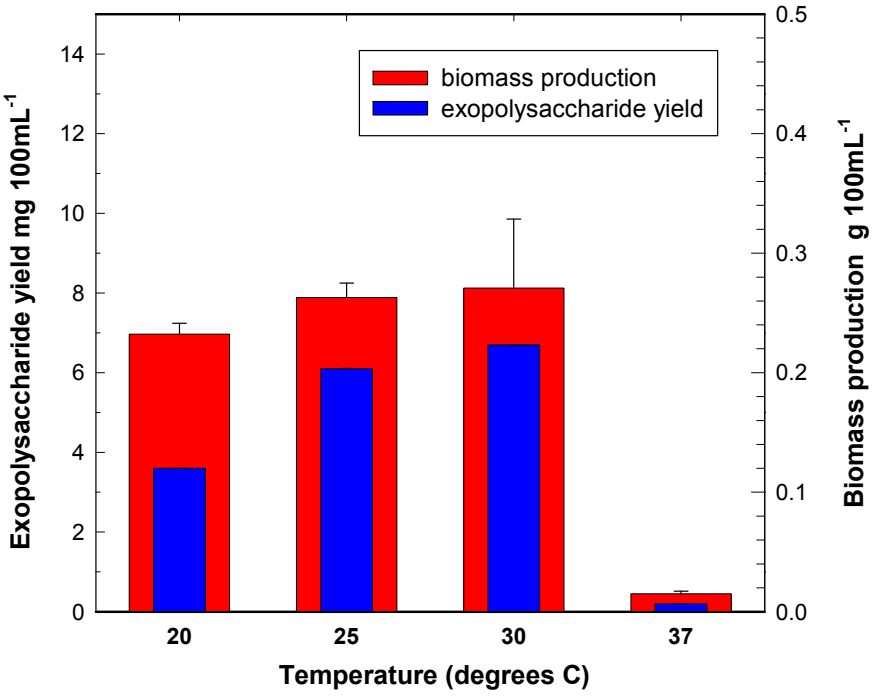


Figure 5.3 *The effect of temperature on the production of biomass and exo-polysaccharide by* A. blazei *in liquid culture.*

The optimum temperature for *A. blazei* production of both biomass and EPS was 30 °C, with 6.7 mg 100 mL^{-1} EPS, and 0.27 g 100 mL^{-1} biomass produced at this temperature. While no other studies have reported a temperature optimum for production of EPS by

Agaricus blazei, the optimum temperature observed of 30 °C is similar to the temperatures used by Fan *et al*. (2003), 32°C, and Shu *et al*. (2004), 28°C, when examining production of EPS by *Agaricus blazei*. For all temperatures studied, the amount of EPS produced was found to be proportional to the amount of biomass produced. This effect has also been observed in the cultivation of other fungi in submerged culture (Yang & Liau, 1998(a); Park *et al*. 2001; Hwang *et al*.2003).

A. blazei mycelium was grown in flasks of 100 mL basal media according to the method described in Section 3.2.5.6 to examine the effect with which the initial pH of the culture media influenced the production of biomass and EPS in liquid culture,. Triplicate flasks of basal media adjusted to pH 3, 4, 5, 6, 7, 8 or 9, were incubated at 30 °C for 15 days at with shaking at 100 rpm.

The biomass produced in each culture flask was removed by filtration, freeze-dried and the average biomass produced at each temperature was determined. EPS were extracted from the culture filtrate according to the method described in Section 3.3.3. The production of biomass and EPS by *A. blazei* as a function of initial pH of the culture media is shown in Figure 5.4.

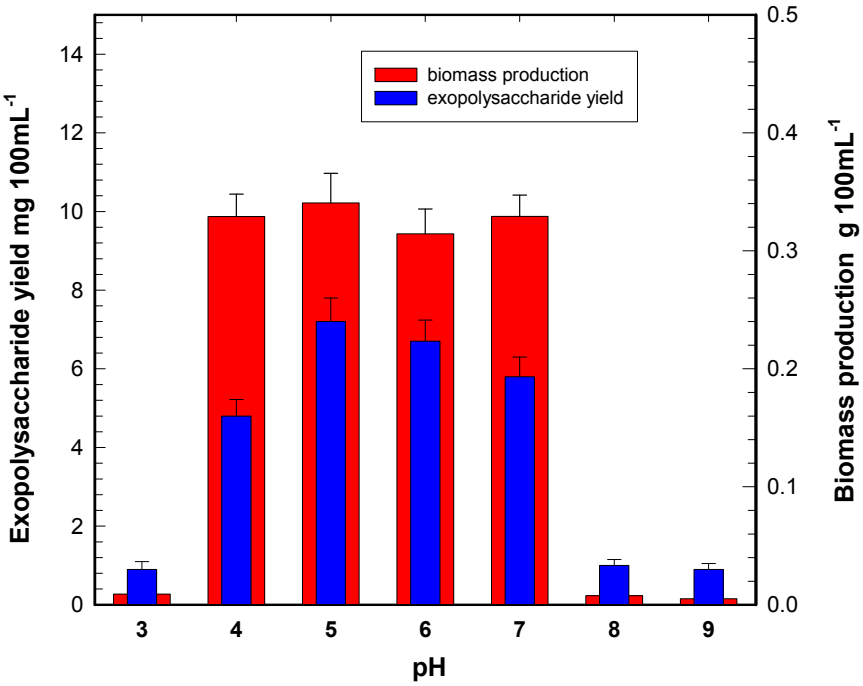


Figure 5.4 *The effect of pH on the production of biomass and exo-polysaccharide by* A. blazei *in liquid culture.*

The results in Figure 5.6 show that the optimal pH for production of *A. blazei* biomass is within the range of pH 4-7, with 0.32- 0.34 g 100 mL^{-1} over the pH range. The greatest EPS production occurred in a culture medium that had been adjusted to an initial pH of 5, at a rate of 7.2 mg 100 mL^{-1}. A lower degree of EPS production was observed at pH 4.0 with only 4.8 mg 100 mL^{-1}. These results are generally consistent with the data reported by Shu *et al*. (2004), who reported that the polysaccharide formation by *A. blazei* in liquid culture was favoured by a higher culture pH, however they achieved maximum production of EPS at pH 7.0. It should be noted however that their experiments were carried out using batch fermentation with the pH maintained at a constant level throughout the incubation period. In addition, the data were collected daily over a 20-30 day period and the reported maximum biomass and EPS production for each pH was quoted for different incubation periods. At pH 7.0 they observed a faster rate of biomass and EPS production with data reported at Day 21, and this growth rate decreased as the pH of incubation was reduced. Data for pH 6.0, pH 5.0 and pH 4.0 were reported at Day 24, 26 and 34 respectively. This current experiment reports data at Day 15 for all media pH values investigated. The results obtained in this study however

are in concordance with the observations that the optimal pH for polysaccharide formation is slightly higher than that required for biomass production (Kim *et al*. 2004; Hamedi *et al*. 2007).

Shu *et al*. (2004) reported that the molecular weight of the polysaccharides produced by the fungus decreased from 1080 kDa to 600 k Da as the culture pH was increased from 4.0 to 7.0. Researchers studying the production of polysaccharides by other mushroom species have reported similar observations (Madi, et al. 1996), while others have described the opposite observation that high molecular weight polysaccharide species are produced at higher incubation pH values around pH 6.5 (Lee *et al*. 2001).

5.2.2.2 Effect of carbon source on *A. blazei* exo-polysaccharide production

A. blazei mycelium was grown in flasks of 100 mL basal media according to the method described in Section 3.2.5.7 to examine the effect of the carbon source on the production of biomass and EPS in liquid culture,. Triplicate flasks of basal media containing 1% glucose, sucrose, mannose or arabinose as the carbon sources were incubated at 30 °C for 15 days with shaking at 100 rpm.

The biomass produced in each culture flask was removed by filtration, freeze-dried and the average biomass at each temperature was determined. EPS were extracted from the culture filtrate according to the method described in Section 3.3.3. The production of biomass and EPS by *A. blazei* as a function carbon source is shown in Figure 5.5.

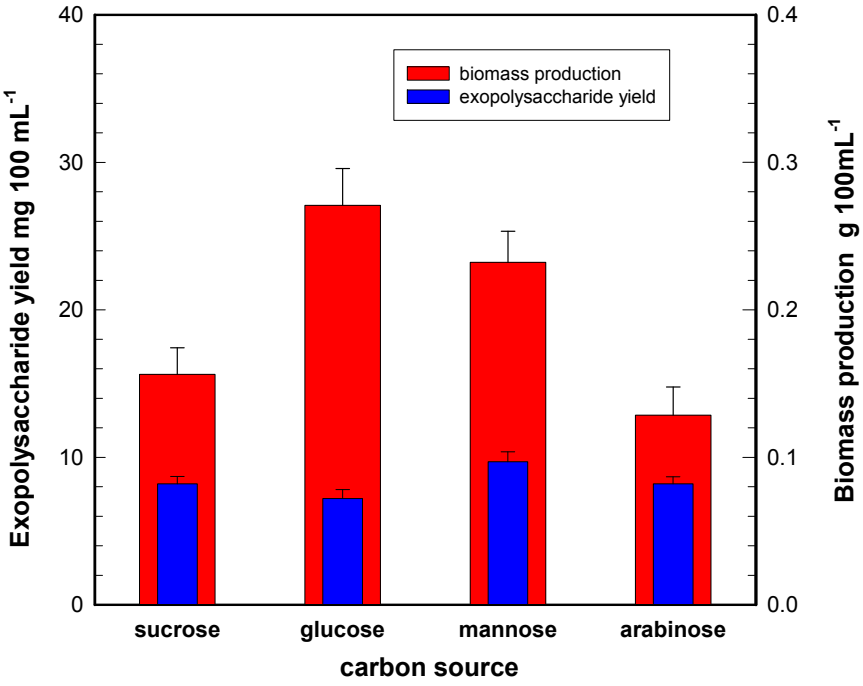


Figure 5.5 *The effect of carbon source on the production of biomass and exo-polysaccharide by* A. blazei *in liquid culture.*

The results indicated that optimum carbon source for biomass production by *A. blazei* was glucose, with 0.27 g produced per 100mL of medium, followed by mannose, sucrose and arabinose with 0.23 g 100 mL^{-1}, 0.16 g 100 mL^{-1}, and 0.13 g 100 mL^{-1}, respectively. Production of EPS was greatest when mannose was included as the carbon source with 9 mg $100mL^{-1}$, although only marginally less EPS were produced with the three other carbon sources examined with 8 mg $100mL^{-1}$ for arabinose and sucrose, and 7 mg EPS 100 mL^{-1} for glucose.

Glucose is thought to be the most effective energy source from a biological perspective, (Cochrane, 1958), and is the most widely utilised carbon source by fungi (Griffin, 1994), so it was not surprising that media containing glucose as a carbon source produced the highest yield of biomass. Zou *et al.* (2006) also found that using glucose as a carbon source for *A. blazei* growth resulted in a greater level of biomass than when sucrose, maltose and fructose was used, with 12.25 g L^{-1} biomass produced compared to 9.75 g L^{-1}, 10.32 g L^{-1}, and 9.44 g L^{-1} respectively, and this effect has also been observed by other research groups ((Jia *et al*. 2002; Kim *et al*., 2004; Shu & Xu., 2007).

Fan *et al.* (2003) observed maximum biomass production by *A. blazei* using fructose as the carbon source, although the amount produced was not significantly different from that obtained using glucose, maltose, and sucrose ($p>0.05$). Furthermore, the glucose media formulation used in the study by Fan *et al.* (2003) yielded less EPS (2.2 mg 50 mL^{-1}) compared to that of sucrose and maltose, with 3.5 and 3.1 mg 50 mL^{-1} respectively. Only 0.27 mg 50 mL^{-1} of EPS was produced when fructose was used as the carbon source. This observation was also reported by Zou (2006), who found that use of sucrose as the carbon source resulted in a greater level of EPS being produced (223 mg L^{-1} day^{-1}) compared to glucose, maltose and fructose with 163, 177, and 171 mg L^{-1} day^{-1} respectively.

5.2.2.3 Composition of exo-polysaccharides produced by *A. blazei*

Samples of the EPS produced in the previous experiments were analysed by TLC according to the method described in Section 3.7.6, for the presence of monosaccharides glucose and mannose. The amount of protein in EPS produced was determined according to the method described in Section 3.7.4.

The amount of protein and species of monosaccharide present in the exo-polysaccharides produced is shown in Table 5.1.

Condition	% Protein	Monosaccharide present
Basal	2.2	Mannose
20 °C	2.4	Mannose
25 °C	2.2	Mannose
30 °C	2.8	Mannose
pH 4	2.5	Mannose
pH 5	1.9	Mannose
pH 6	2.2	Mannose
pH 7	1.8	Mannose
Sucrose	0.7	Mannose
Glucose	2.2	Mannose
Mannose	5.3	Mannose
Arabinose	1.5	Mannose

Table 5.1 *The % protein and monosaccharides present in exo-polysaccharides produced by* A. blazei *under different culture conditions and media constituents.*

For all growth media formulations studied, mannose was found to be the only monosaccharide present as detected by TLC. Temperature and pH did not affect the amount of protein produced, with an average yield of approximately 2-3 % protein for all temperatures and pH values examined. However, the carbon source of the media formulation did influence the amount of protein within EPS produced. EPS from media containing mannose or sucrose consisted of 5% and 0.7% protein respectively. While Fan *et al*. (2003) targeted β-glucan production by concentrating on EPS produced with glucose as a carbon source, Mizuno,T (1995) found that EPS produced by *A. blazei* with glucose as a carbon source were mannan-protein complexes, which is consistent with the results of the current study.

5.2.3 Production of biomass by *Agaricus blazei* in different liquid culture media

In an effort to scale up production of biomass and culture filtrates to enable sufficient extracts to be obtained for bioactivity screening, *A. blazei* mycelium was grown in a range of culture media containing different components, the composition of which is described in Section 3.2.2.1. Flasks containing 1 L of YMB, CMM, MEB, PMP, GLUC

and SUCR liquid media were prepared according to the method described in Section 3.2.5.10. Duplicate flasks of each media were incubated at 30 °C for 30 days with shaking at 100 rpm. An incubation period of 30 days was selected to allow direct comparison with similar studies regarding the extraction of bioactive compounds from *A. blazei* culture filtrate (Mizuno, T., 1995; Shu, *et al*., 2004).

The biomass produced in each culture flask was removed by filtration, freeze-dried and the average biomass produced per litre for each culture media was determined. The production of biomass by *A. blazei* as a function of culture media is shown in Figure 5.6, and the final pH of the culture media and deviation from the original pH is given in Table 5.2.

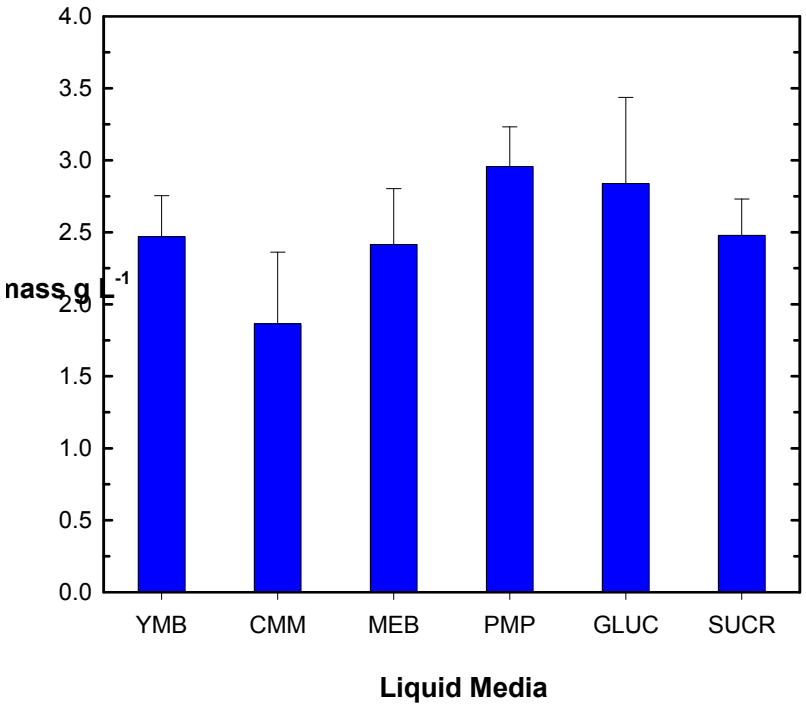


Figure 5.6 Agaricus blazei *biomass production in different liquid media*

Liquid media	Initial pH	Final pH	Difference in pH
YMB	6.12 ± 0.28	7.08 ± 0.36	+ 0.96
CMM	6.94 ± 0.20	6.24 ± 0.24	- 0.7
MEB	5.38 ± 0.10	8.00 ± 0.19	+ 2.62
PMP	5.10 ± 0.15	5.91 ± 0.36	+ 0.81
Glucose	6.01 ± 0.05	7.02 ± 0.33	+ 1.01
Sucrose	5.97 ± 0.15	8.25 ± 0.11	+ 2.28

Table 5.2 *Initial and final pH values of 30 day* Agaricus blazei *cultures in different media*

Each of the six culture media used for the growth of *A. blazei* mycelium produced between 1.5-3.0 g biomass per litre of culture, with the highest average growth observed for PMP and glucose medium. In addition to the lowest average growth observed for the CMM medium, it was the only one that recorded a reduction in pH over the incubation period (Table 5.1). This observation is in contrast to the data reported by Yang & Liau (1998), who suggested that the lower the final pH, the greater the amount of mycelial growth that was obtained. The reduction in pH observed by Chen (Chen, 1999) when cultivating fungi in liquid culture was not observed in this study, where an increase in pH was observed over the incubation period for five of the six media studied, particularly for when using MEB and sucrose media. The slight decrease in pH over the incubation period for CMM may be the result of the presence of potassium salts in the media formulation having a buffering effect.

5.3 Summary

The production of biomass by *A. blazei* in liquid culture was examined as a function of temperature and pH over a 15 day period in order to identify the optimal conditions for growth. The optimum temperature for biomass production was found to be 30°C, and while no single optimum growth pH was identified, the highest level of biomass was produced over the pH range 4-8, which is consistent with the data reported by Shu *et al.* (2004), where an optimal pH range was quoted for the growth of *A. blazei* in liquid culture rather than a specific pH. Most commercially available media formulations for

fungal growth have an initial pH of 5-6 which lies in the middle of the optimal range reported in this study.

The production of biomass by *A. blazei* over a 30 day period was also examined in YMB and PDB. While the characteristic fungal growth phases were identified (a lag phase followed by exponential growth or log phase growth, followed by a plateau or senescence of growth), the duration of these phases varied depending on the culture media formulation used.

EPS production by *A. blazei* at different growth temperatures and pH levels was found to be directly proportional to the amount of biomass produced. The effect of different carbon sources for growth and production of EPS, however, indicated that the amount of EPS produced was not proportional to the amount of biomass produced. Whilst the sucrose medium resulted in approximately half the biomass than, for example, the glucose medium, the sucrose medium resulted in a greater level of EPS production, an observation consistent with the work reported by Fan *et al*. 2003; Zou *et al*., 2006). EPS produced by *A. blazei* under different conditions were found to be mannan-protein complexes consisting of 2-3 % protein, except for those produced with sucrose and mannose as a carbon source with 0.7% and 5.3% protein respectively.

Production of *A. blazei* mycelia was scaled up to 1 L cultures in media formulations containing different carbon sources and media constituents (Section 5.2.3). Mycelium from all six culture formulations were freeze dried and stored for further solvent extraction experiments. The examination of the potential bioactivity of the solvent extracts obtained is described in Chapter 6.

Chapter Six

Bioactivity of *Agaricus blazei* solvent extracts

6.1 Introduction

Fungal biomass consists of thousands of chemical compounds that can be divided into two classes, primary and secondary metabolites (Brown, 2001). Primary metabolites constitute the bulk of the biological material and include compounds such as DNA, proteins and carbohydrates. These compounds are necessary for the maintenance of the fundamental processes of life. The secondary metabolites are small molecules which are diverse in chemical structure and are involved in mediating ecological interactions between organisms. It is these secondary metabolites that are of interest when searching for novel bioactive compounds as in most cases, some kind of biological activity is attributable to them.

Since the bulk of fungal biomass consists of primary metabolites, the secondary metabolites, which constitute approximately 1% by weight of the original material, must be released and solubilised from the cellular matrix by extraction into organic solvents or water. The most common technique for the extraction of secondary metabolites from fungi is pulverising frozen or dry mycelium into a fine powder, then subjecting them to extraction processes using various solvents in a Soxhlet extractor. Alternatively, supercritical fluid extraction may be employed, This method requires smaller volumes of solvent and is more efficient, but requires the availability of specialised equipment (Brown, 2001).

A number of extracts from fruiting bodies and liquid cultured mycelium of various fungal species have been reported to display antimicrobial and cytotoxic activity (Park, *et al.* 1995a; 1995b; Hirasawa *et al*., 1999; Suay *et al*., 2000; Gao *et al*., 2002; Ofodile *et al.*, 2005; Turkoglu *et al*., 2006). To date, extracts from *Agaricus blazei* fruiting bodies have been the main focus for investigation into antibacterial and cytotoxic substances. Bernardshaw *et al*. (2005) found that an aqueous extract from the fruiting bodies of *A. blazei* protected against systemic *Streptococcus pneumoniae* infection in

mice, but there have been no reports on the antibacterial activity of organic solvent extracts from *A. blazei* fruiting bodies or liquid cultured mycelium, however a cytotoxic steroidal compound has been identified in a hexane extract from fruiting bodies of *A. blazei* which displaying *in vitro* activity against HeLa cells (Kawagishi *et al*., 1988).

This current study investigated the presence of potentially bioactive substances from liquid cultured *A. blazei* mycelium. Freeze-dried mycelium from liquid cultures of *A. blazei* cultivated in different culture media was extracted using a range of solvents of increasing polarity in a Soxhlet apparatus. The yield of each solvent extract was then determined. The resulting extracts were then screened for antibacterial activity against a range of Gram positive and Gram negative bacteria, and for cytotoxicity against two tumour cell lines *in vitro*.

6.2 Results and Discussion

6.2.1 Effect of liquid culture media on organic solvent extract yield

Agaricus blazei mycelium was cultivated in six different growth media, YMB, CMM, MEB, PMP, GLUC and SUCR (Section 5.2.3). Samples of ground, freeze-dried mycelium cultivated in each growth medium (5-6 g), were extracted with a cascade of four organic solvents of increasing polarity- hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH), using a Soxhlet apparatus (Section 3.3.1). The average yield of each extract (milligrams per gram of freeze-dried mycelium) is shown in Table 6.1.

	Yield solvent extract (mg g^{-1} mycelium)*				Total crude extracts (mg g^{-1} mycelium)
	Hexane	**DCM**	**EtOAc**	**MeOH**	
YMB	14 – 3	4 – 1	7– 4	130 – 20	155 – 20
CMM	14 – 3	6 – 2	8 – 4	170 – 50	198 – 20
MEB	9 – 3	4 – 1	2 – 1	160 – 20	175 – 20
PMP	8 – 3	5 – 1	11 – 5	270 – 80	294 – 20
GLUC	12 – 4	4 – 1	6 – 3	170 – 20	192 – 20
SUCR	11 – 2	4 – 3	2 – 1	260 – 40	277 – 20

* Average and standard deviation of five replicate extractions

Table 6.1 *Yield of solvent extracts from* A. blazei *mycelium grown in different culture media*

For each of the six biomass samples extracted, the largest extract yield was obtained with methanol, although a wide range in yield was observed (130 270 mg g^{-1} mycelium). Methanol was the most polar solvent used for extraction, indicating that there are a high proportion of extractable polar compounds in *A. blazei* mycelium. It is interesting to note that the highest yield of MeOH extract was obtained from mycelium grown in PMP and SUCR media. While the other media formulations have glucose as the sole carbon source, PMP also contains potato extract and sucrose is the sole carbon source in SUCR media. These alternative carbon sources may lead to the production of more highly polar compounds.

For all culture media except PMP, the second largest mass of extract was obtained with hexane (9-14 mg g^{-1} mycelium). For PMP, the ethyl acetate extract had the second largest mass (11 mg g^{-1} mycelium). Generally, ethyl acetate and dichloromethane gave the extract yields (2-11 mg g^{-1} of mycelium).The largest mass of extractable compounds was obtained from PMP and SUCR (294 and 277 mg g^{-1} mycelium respectively), and was mainly due to the larger mass of the methanol extract compared to the other culture media. Extracts obtained from CMM and GLUC totalled 198 and 192 mg g^{-1} mycelium,

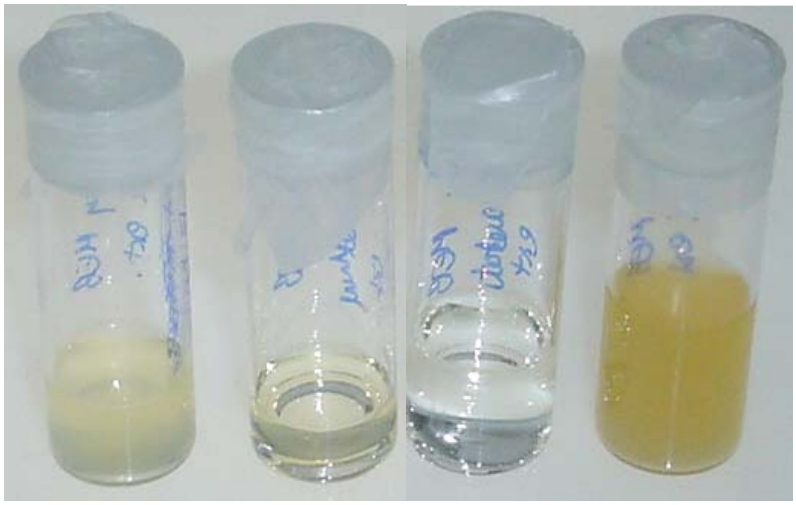
respectively, followed by MEB with 175 mg g^{-1} mycelium. YMB yielded the lowest total of crude extract with 155 mg g^{-1} mycelium.

In addition to different yields for each solvent, the appearance and colour of the extracts also varied depending on the culture medium used for fungal biomass production. The extracts obtained for YMB, CMM, SUCR and GLUC were very similar and contrasted to those obtained from MEB and PMP (Table 6.2 and Figure 6.1).

Extract	Colour and consistency of solvent extract	
	YMB, CMM, GLUC, SUCR	MEB, PMP
Hexane	Dark yellow; opaque	Pale yellow; opaque
DCM	Dark orange; opaque	Pale yellow; transparent
EtOAc	Pale orange/red; transparent	Colourless; transparent
MeOH	Dark orange/red; opaque	Dark yellow; opaque

Table 6.2 *Colour and consistency of solvent extracts from* A. blazei *mycelium grown in different culture media*

(a)



(b)

Figure 6.1 *Colour and consistency of solvent extracts from* A. blazei *mycelium grown in (a) YMB; (b) MEB*

Most extracts were opaque and consisted of two distinct phases which were identifiable when the extracts were cooled to room temperature following Soxhlet extraction. This was particularly apparent for the methanol extracts. A solid precipitate became evident and the two phases, designated as supernatant and precipitate , were screened separately for cytotoxic activity (Sections 6.2.3.1 and 6.2.3.2). Differences in colour and appearance of solvent extracts have been reported (von Wright, *et al.* 1992). Together with the range of yields obtained above, this indicates differences in the chemical composition of mycelial samples cultivated using different liquid media.

Extracts were screened for activity against a variety of bacteria and cytotoxicity against two tumour cell lines. The ethyl acetate extracts from MEB and SUCR mycelium could not be screened for bioactivity due to the insufficient yields obtained(<2 mg g^{-1} of mycelium) which prevented the preparation of extracts with the concentration required for screening.

6.2.2 Screening of organic solvent extracts for antibacterial activity

Solvent extracts obtained from *Agaricus blazei* mycelium cultivated on six different media were tested for antibacterial activity against a range of Gram positive and Gram negative bacteria. Bacteria under investigation included the Gram positive cocci *S. aureus*, and *S. pyogenes*, the Gram positive rod, *B. cereus*, the Gram negative cocci, *B. catarrhalis* and Gram negative rods, *E. coli*, *S. entrica* serovar Typhimurium, and *Ps. aeroginosa*.

Antibacterial activity was determined using the disc diffusion method (Section 3.4.3). Briefly, stock solvent extracts were prepared at concentrations of 100 mg/mL in the corresponding extraction solvent. Ten μL aliquots of each extract were impregnated on test filter discs (6 mm diameter), giving a total of 1000 μg (1 mg) of extract on each disc. The discs were then placed on agar plates inoculated with the test bacterium, and the plates incubated for 24-48 hours. The plates were then examined for the presence of zones of inhibition, with zones of inhibition greater than 6 mm recorded (Table 6.3).

EXTRACT		Gram – ve bacteria				Gram + ve bacteria		
		B. catarrhalis	***E. coli***	***Ps. aeroginosa***	***S. enterica***	***B. cereus***	***S. aureus***	***S. pyogenes***
		Zones of inhibition*						
YMB	hexane	12 ± 4	-	-	-	-	-	-
	DCM	18 ± 3	-	-	-	7 ± 0	9 ± 1	10 ± 1
	EtOAc	13 ± 0	-	-	-	7 ± 0	-	8 ± 0
	MeOH	11 ± 2	-	-	-	-	-	-
CMM	hexane	10 ± 1	-	-	-	-	-	-
	DCM	15 ± 4	-	-	-	8 ± 1	9 ± 1	10 ± 1
	EtOAc	13 ± 1	-	-	-	7 ± 0	-	8 ± 0
	MeOH	10 ± 0	-	-	-	-	-	-
MEB	hexane	9 ± 0	-	-	-	-	-	-
	DCM	14 ± 1	-	-	-	8 ± 0	7 ± 0	-
	MeOH	10 ± 1	-	-	-	7 ± 0	-	-
PMP	hexane	11 ± 1	-	-	-	-	-	-
	DCM	14 ± 1	-	-	-	8 ± 1	9 ± 1	9 ± 1
	EtOAc	14 ± 0	-	-	-	7 ± 0	16 ± 1	7 ± 0
	MeOH	10 ± 2	-	-	-	-	-	-
GLUC	hexane	16 ± 4	-	-	-	-	-	-
	DCM	17 ± 2	-	-	-	7 ± 0	8 ± 1	9 ± 1
	EtOAc	16 ± 2	-	-	-	8 ± 0	-	8 ± 1
	MeOH	11 ± 1	-	-	-	-	-	-
SUCR	hexane	12 ± 2	-	-	-	-	-	-
	DCM	16 ± 2	-	-	-	7 ± 0	-	-
	MeOH	8 ± 0	-	-	-	-	-	-

* Results are the average of triplicate experiments

\- = no zone of inhibition measured

Table 6. 3 *Results of antibiotic screening-diameter (mm) zones of inhibition.*

Interestingly, all extracts showed some antibacterial activity against *B. catarrharlis*, with DCM extracts giving the largest zones of inhibition (14-18 mm). This may be due to the presence of the same or different (but bioactive) compounds. Theoretically, different compounds should be extracted into solvents of different polarity in the

Soxhlet process, however complete separation is rarely achieved and varied proportions of the same compound may be recovered in several fractions (Harbourne, 1973). Figure 6.2 depicts the zones of inhibition observed for DCM extracts from YMB and MEB (Control discs consisting of DCM and showing no zones of inhibition are located in the centre of the agar plate).

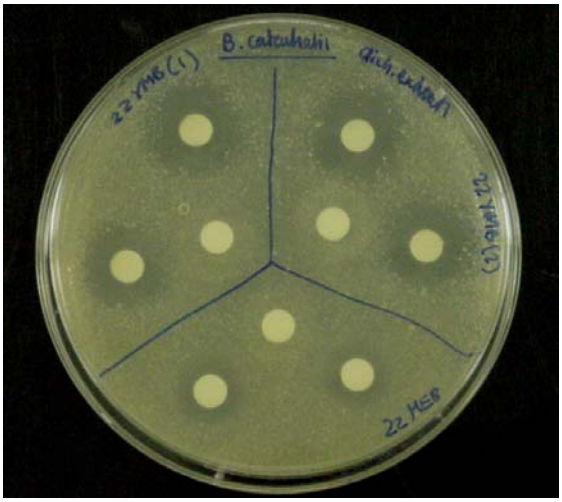


Figure 6. 2 *Zones of inhibition observed against* B. catarrhalis *for YMB DCM extract (top left and top right) and MEB DCM extract (bottom).*

While the YMB DCM extracts gave complete zones of inhibition, i.e. a completely clear zone surrounding the impregnated disc, incomplete zones of inhibition were observed for MEB DCM extracts with some small isolated bacterial colonies visible in the zone surrounding the disc. Ethyl acetate extracts were also quite active against this bacterium with complete zones of inhibition (13-16 mm). Methanol extracts showed the least inhibition and were only found to be active against *B catarrhalis* (zones of inhibition of 8-11 mm), except for the methanol extract from MEB which had some activity against *B cereus*. *B. catarrhalis* was also the only Gram-negative bacterium that was susceptible to the action of the *A. blazei* extracts, with inhibition observed for *E. coli*, *Ps aeroginosa*, and *S. enterica*. Similar observations have been made previously, and extracts from mushrooms are commonly less active against Gram-negative bacteria (Beltran-Garcia, *et al*. 1997, Smania *et al*. 1999). This may be due to the presence of a lipid-polysaccharide complex in the outer membrane of Gram-negative bacteria which

confers greater resistance to environmental substances including antibiotics (Tortora *et al*. 2001).

For the Gram-positive bacteria examined, small zones of inhibition (7-10 mm diameter) were observed for DCM extracts from mycelium grown in YMB, CMM, PMP ad GLUC against *B. cereus*, *S. aureus* and *S. pyogenes*. The DCM extracts from MEB mycelium only showed inhibition against the former two bacteria, while DCM extracts from SUCR media only showed weak inhibition against *B. cereus*. EtOAc extracts from YMB, CMM, PMP, and GLUC weakly inhibited the growth of *B. cereus* and *S. pyogenes* (zones of inhibition of 7-8 mm diameter), while the EtOAc extract from PMP mycelium showed additional strong activity against *S. aureus* (16 mm diameter zone of inhibition).

While the zones of inhibition observed against *B. catarrhalis* and the Gram-positive bacteria studied are evidence of antibacterial substances present in *A. blazei* liquid cultured mycelium, the size of the zone of inhibition is not a true reflection of the potency of compounds within the extract that are causing inhibition. The amount of extract impregnated on the disc will affect the size of the zone observed, and the method used in this study for identifying potential antibacterial compounds, whilst easy to perform, relies on the ability of the extract/compound to diffuse readily through the media (Barry & Thornsberry, 1985). Other methods, such as determining the Minimal Inhibitory Concentration (MIC) (Smania Jr, *et al*., 1999; Hirasawa *et al.,* (1999)), involve diluting the extract in liquid media and determining bacterial growth by nephelometry, thus eliminating the need for the diffusion of compounds through solid media. This method does however require that the extracts/compounds be soluble in the medium used for the experiments. If the extracts are particularly non-polar, this method may not be suitable.

There has been a great deal of literature published on the antibacterial activity of fruiting body and mycelial extracts of many species of mushroom against both Gram-negative and Gram-positive bacteria (Beltran-Garcia *et al*., 1997; Suay *et al*., 2001; Hatvani, 2001; Rosa *et al*., 2003: Hearst *et al.*, 2009). Bernardshaw *et al*. (2005) reported *in vivo* antibacterial activity of an aqueous extract from *Agaricus blazei* fruiting

bodies against systemic *Streptococcus pneumoniae* infection in mice, and two previous reports have demonstrated *in vitro* antibacterial activity in *A. blazei* extracts from fruiting bodies (Osaki *et al*. (1994); Ye & Lin, (2001), but this study is the first account of *in vitro* antibacterial activity in extracts from liquid cultured *A. blazei* mycelium.

The broad spectrum of activity amongst different *A. blazei* extracts against *B. catarrhalis* warrants further examination given that this bacterium (also known as *Moraxella catarrhalis*) causes upper and lower respiratory tract infections, is an important cause of otis media and sinusitis in the young, and can cause pneumonia in elderly patients who have underlying respiratory pathology (Paykel, 2002). The bacterium has rapidly acquired resistance to many β-lactam antibiotics previously used to treat infection. Therefore, the identification of novel compounds that may combat infection would be most advantageous. It would also be valuable to test these extracts against closely the related pathogenic *Neisseria* sp., such as *Neisseria gonorroheae* and *Neisseria meningitidis*.

Ideally, further investigations into the antibacterial activity of the *A. blazei* solvent extracts identified in this study would require that preliminary fractionation of the extracts should carried out using methods used previously such as thin layer chromatography (Nostro *et al.*, (2000), bio-autography (Saxena *et al*., 1995) or flash chromatography (Brown, 2001) to isolate active components. Subsequently, a thorough characterisation of the activity and potency of active compounds, and their potential for antibacterial therapy, could be achieved.

6.2.3 Screening of organic solvent extracts for cytotoxicity against tumour cell lines

Solvent extracts were tested for cytotoxic activity using the MTT cell viability assay (Section 3.5) against two human tumour cell lines, the human cervical carcinoma cell line, HeLa, and a B-cell lymphoma cell line, Raji,. Each stock extract solution (50 mg mL^{-1} in DMSO) was diluted to concentrations of 12.5, 25, 50, 100, and 200 μg mL^{-1} in the appropriate medium for the cell line under investigation. The concentration of DMSO in the final assay procedure was less than 0.4 % and hence not toxic to the cell lines tested (data not shown). The cell concentration used in the assay was optimised for each cell line investigated. In order to detect cytotoxicity, the response of the cell control (with no extract added) must be such that a reduction in cell viability can be clearly identified. In addition, it is important that the cells under investigation are actively dividing, i.e. in the log phase of growth (Mosmann, 1983).

6.2.3.1 Screening for cytotoxicity against HeLa cells

HeLa cell solutions and *A. blazei* solvent extracts were prepared in DMEM. The optimal seeding concentration for HeLa cell cytotoxicity testing was determined by the preparation of a standard curve. HeLa cell solutions were prepared by 10-fold serial dilutions, with a final concentration range of 10^1 - 10^6 cells mL^{-1}. The cell solutions were seeded in 96 well plates and the MTT viability assay was carried out over a period of 48 hours (Section 3.5.4). The absorbances of different concentrations of HeLa cell solutions are shown in Figure 6.3.

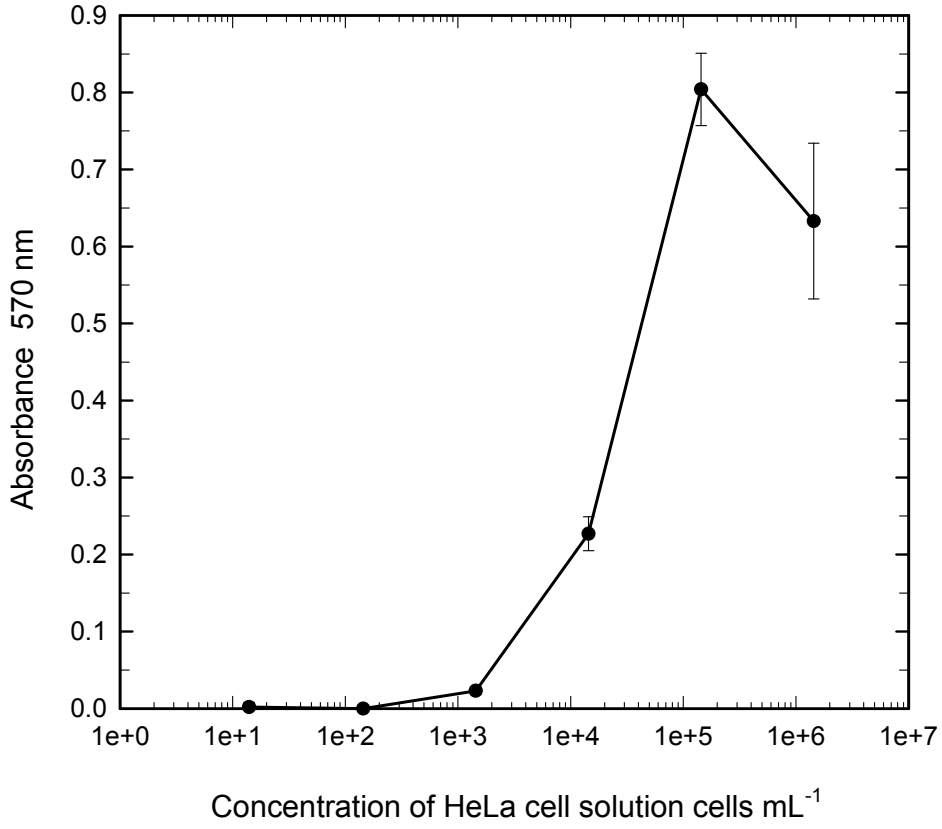


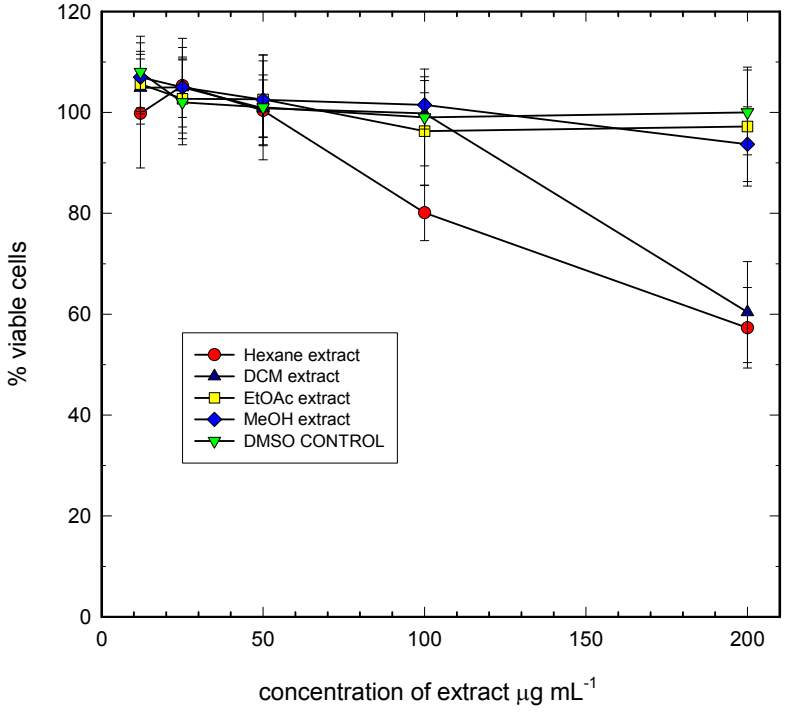
Figure 6.3 *HeLa cell MTT viability assay standard curve*

The results shown in Figure 6.3 indicate that a HeLa cell solution of less than 1 x10^4 cells mL^{-1} is not of sufficient density to produce an optimal response in the MTT viability assay with a maximum absorbance of 0.2 at 570 nM. In addition, cells at this concentration were too sparsely dispersed and not in log phase growth. At a concentration of 1 x10^5 cells mL^{-1}, the cells had reached a plateau of growth, with higher concentrations showing a reduction in the MTT response. A cell solution of 5 x10^4 cells mL^{-1} was determined as the optimum concentration for HeLa cell cytotoxicity testing using the MTT viability assay (Section 3.5.5.1). Assays were performed using three replicate extracts obtained from independent extractions. The proportion of viable cells remaining after treatment was calculated by dividing the absorbance at 570 nm (optical density (OD)) of the test extract by the absorbance at 570 nm (OD) of the cell only control, and expressing the ratio as a percentage, according to the following equation:

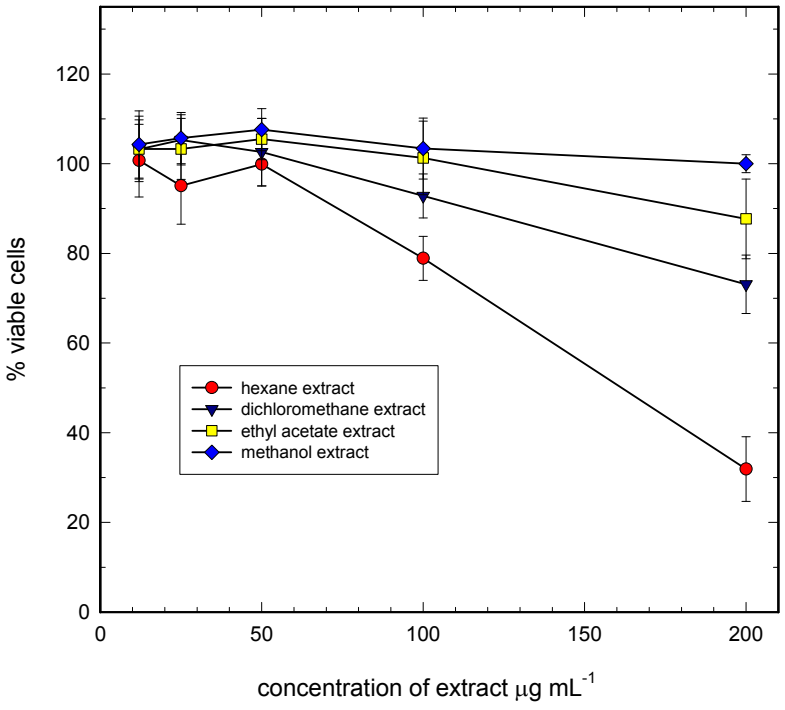
% viable cells = (OD_{test} OD_{blank}) / ($OD_{control}$-OD_{blank}) x 100

Cytotoxic activity was quantified by the calculation of the Cytotoxic Concentration 50 (CC_{50}). This descriptor gives the concentration of the extract required to induce a 50% reduction in cell viability compared to the untreated cell control.

The cytotoxicity of *A. blazei* extracts from mycelium cultured in six media is shown in Figures 6.4-6.6.



(a)



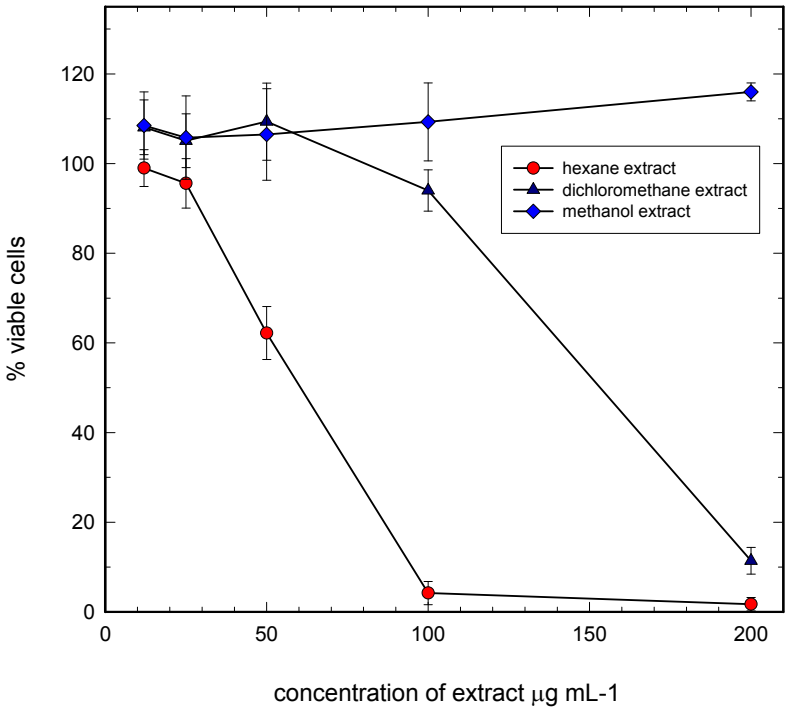
(b)

Figure 6.4 *Cytotoxicity against HeLa cells – (a) YMB solvent extracts; (b) CMM solvent extracts*

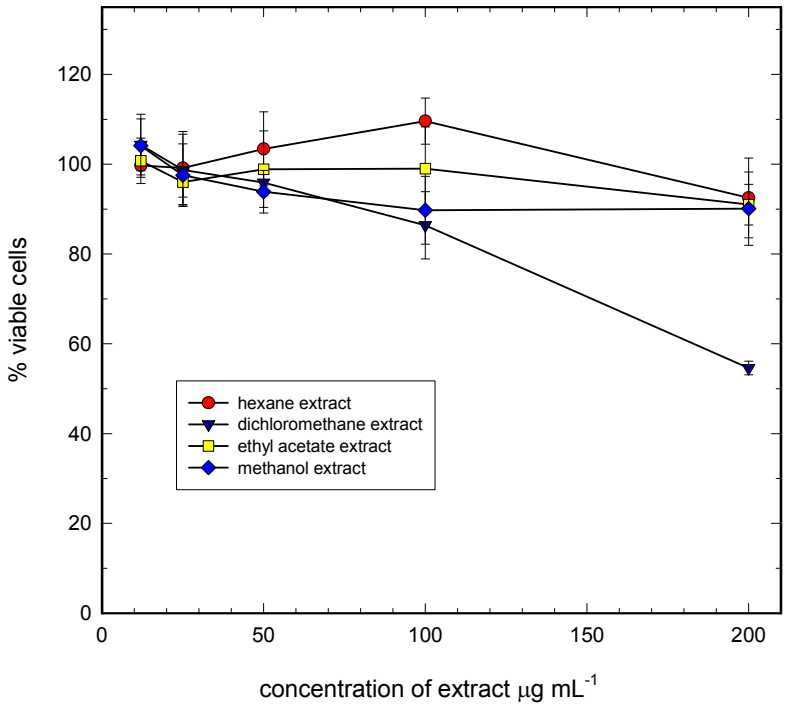
The results in Figure 6.4(a) show that the most non-polar extracts hexane and DCM, showed some inhibition of HeLa cell growth (CC_{50} >200 μg mL^{-1}) with the viability of cells for both extracts at 200 μg mL^{-1} at 57 % and 60% respectively. Neither the EtOAc or methanol extract showed cytotoxic activity, with 98% and 94% cell viability at the highest concentration tested.

The hexane extract from mycelium grow in CMM media had a CC_{50} of 161 μg mL^{-1} (Figure 6.4(b)), whereas all other extracts examined did not show cytotoxic activity (CC_{50} >200 μg mL^{-1}).

Figure 6.5 shows the results of cytotoxicity testing of extracts obtained from mycelium grown in MEB and PMP media.



(a)



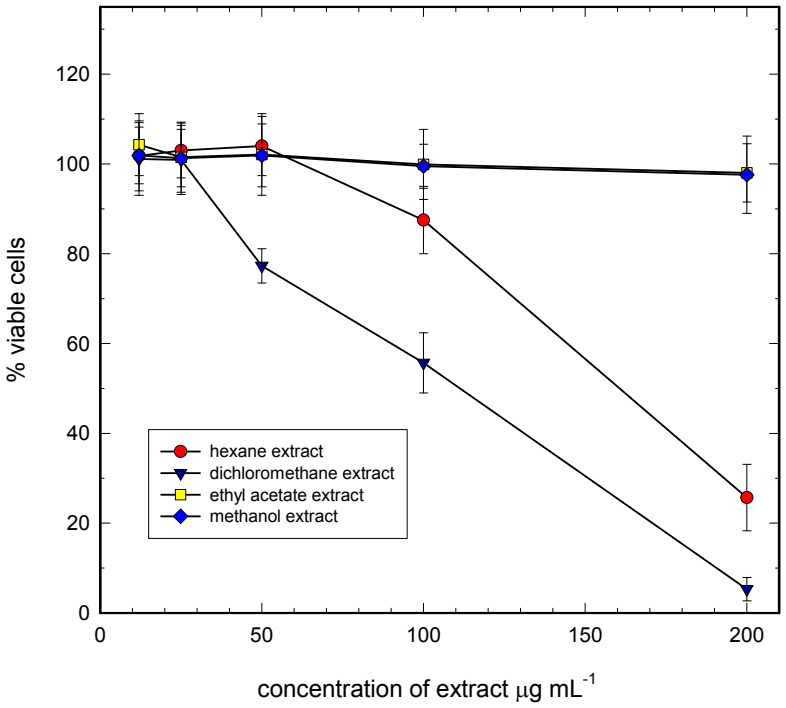
(b)

Figure 6.5 *Cytotoxicity against HeLa cells – (a) MEB solvent extracts; (b) PMP solvent extracts*

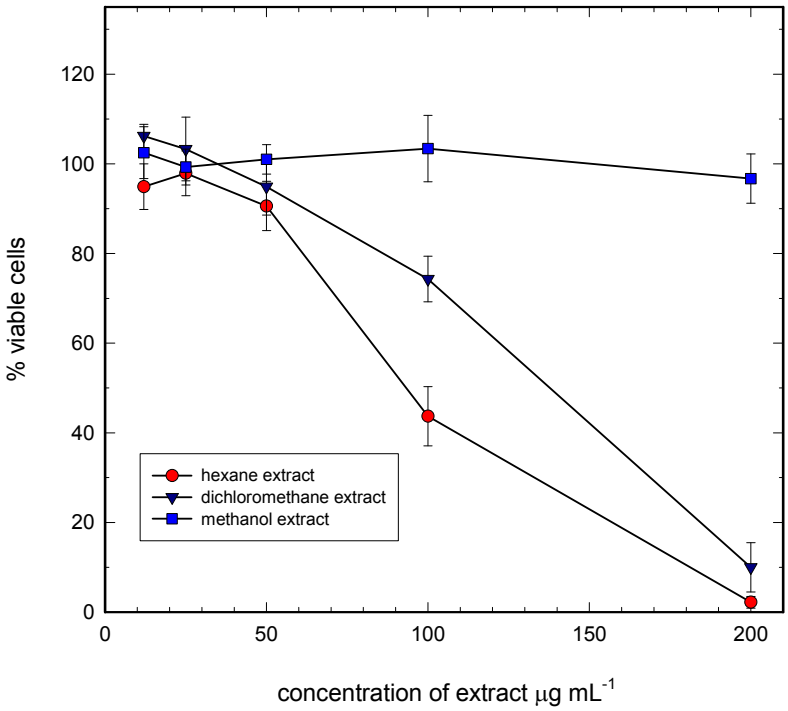
Both the hexane and DCM extracts from MEB showed marked cytotoxic activity against HeLa cells with CC_{50} values of 62 μg mL^{-1} and 153 μg mL^{-1} respectively. In contrast, the methanol extract did not have any effect on cell proliferation (Figure 6.5(a)).

Interestingly, the hexane extract from PMP mycelium did not show any effect on cell proliferation with 93% of cells still viable compared to the cell only control (Figure 6.5(b)). The DCM extract showed some activity with a 45% reduction in cell viability compared to the control, while the methanol extract had no cytotoxic activity.

The results of cytotoxicity testing of GLUC and SUCR mycelial extracts are shown in Figure 6.6.



(a)



(b)

Figure 6.6 *Cytotoxicity against HeLa cells – (a) GLUC solvent extracts; (b) SUCR solvent extracts*

As has been the case for all mycelial extracts (except PMP), the hexane and DCM extracts from GLUC mycelium also inhibited the growth of HeLa cells, with CC_{50} values of 158 μg mL^{-1} and 112 μg mL^{-1} respectively (Figure 6.6(a)). The EtOAc and methanol extracts did not inhibit cell growth with 99% cell viability remaining for both extracts at the highest concentration tested.

The hexane and DCM extracts from SUCR media also showed cytotoxic activity against HeLa cells with CC_{50} values of 109 μg mL^{-1} and 134 μg mL^{-1}, respectively (Figure 6.6(b)). As previously observed for all other methanol extracts, the methanol extract from SUCR media did not inhibit the growth of HeLa cells.

Due to the high extract yields obtained for methanol extracts for all mycelial samples studied, these extracts were further fractionated and re-tested in order to determine whether any potential cytotoxic activity was being masked by other compounds, even at the highest concentration tested (200 μg mL^{-1}). The results presented in Section 6.2.1 highlighted that two distinct phases were present in mycelial methanol extracts, designated as supernatant (MeOH-S) and precipitate (MeOH-P) . These extracts were separated by removal of the supernatant layer and screened separately for cytotoxic activity against HeLa cells (Figure 6.7).

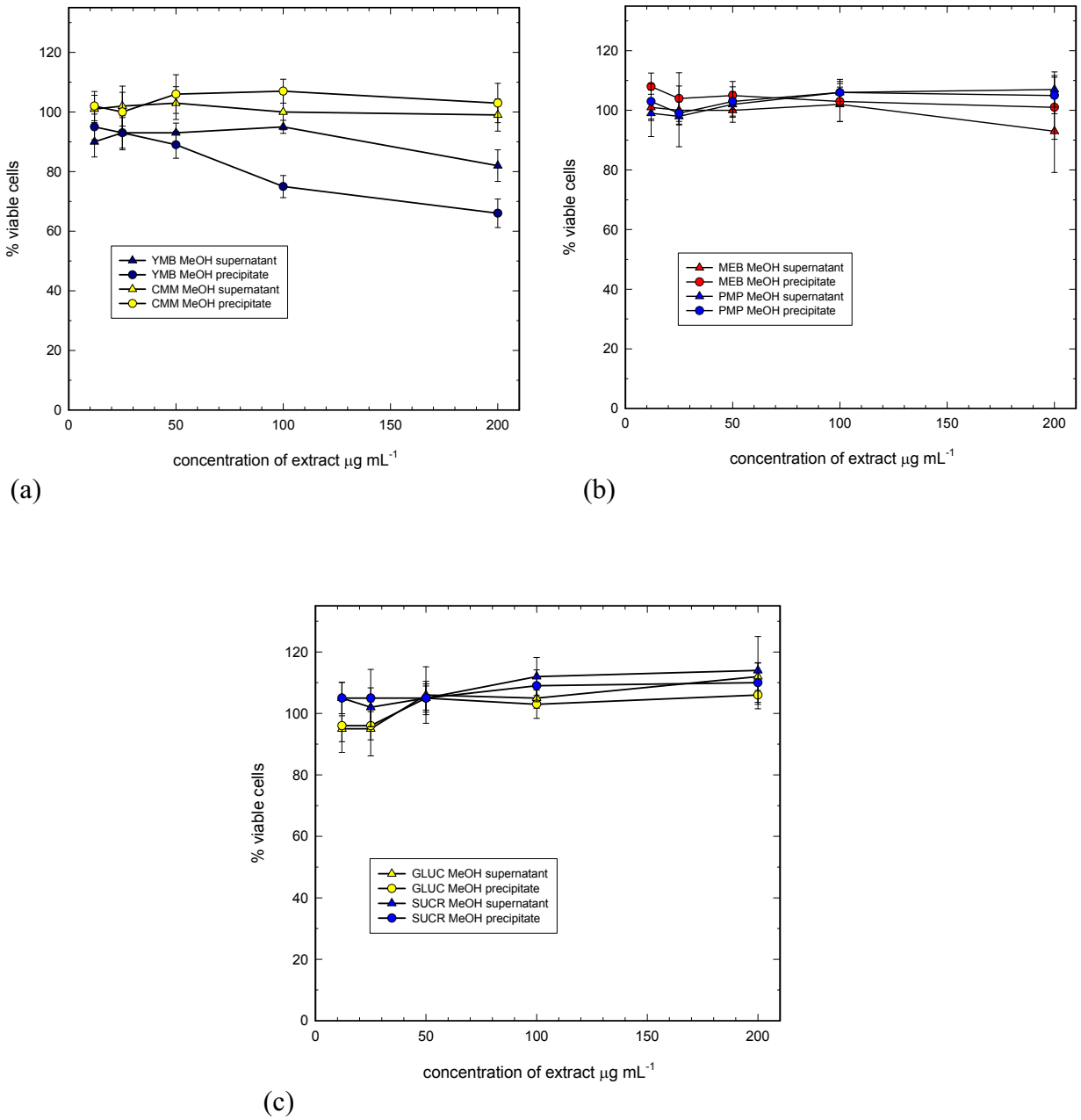


Figure 6.7 *Cytotoxicity against HeLa cells fractionated MeOH extracts - (a) YMB & CMM; (b) MEB & PMP; (c) GLUC & SUCR.*

The MeOH-P extract from YMB showed some degree of cytotoxic activity against HeLa cells, with only 65% of cells viable at 200 μg mL^{-1} (Figure 6.7(a)), indicating the presence of cytotoxic compounds in the extract. The MeOH-S extract from the same media did not appear to inhibit cell proliferation with 82% cells viable at the highest concentration tested. No cytotoxic activity was observed for MeOH-S or MeOH-P extracts from CMM mycelium (Figure 6.7(a)), as was also the case for fractionated methanol extracts from MEB and PMP (Figure 6.7(b)), and extracts from GLUC and SUCR (Figure 6.7(c)).

In summary, CC_{50} values were obtained for Hexane and DCM extracts from MEB, GLUC and SUCR mycelium indicating cytotoxic activity against HeLa cells, while no activity was observed for the more polar extracts, EtOAc and methanol. A small degree of activity was identified in the MeOH-P extract from YMB, although further fractionation of the extract is required for an assessment of the significance this result.

Previously, Kawagishi *et al*. (1988) reported that three steroids from an acetone extract of the fruiting bodies of *A. blazei* demonstrated cytotoxic activity against HeLa cells *in vitro*. The minimum concentrations giving complete growth inhibition were between 8-63 μg mL^{-1}. The results of this study compare favourably considering that relatively crude extracts were investigated and the compounds responsible for the activity observed have not been isolated. For example, the MEB hexane extract completely inhibited the growth of HeLa cells at 100 μg mL^{-1}, while the MEB DCM extract, the GLUC hexane and DCM extracts, and the SUCR hexane and DCM extracts all completely inhibited the growth of HeLa cells at approximately 200 μg mL^{-1}.

The results of this study are also encouraging in comparison to other mushroom species tested against HeLa cells. Zhu *et al.* (2000) reported that an alcohol extract from *Ganoderma lucidum* spores had a CC_{50} of 750 μg mL^{-1}, while an ethyl acetate extract from *Paxillus involutus* showed cytotoxic activity against HeLa cells with CC_{50} values in the range 125- 250 μg mL^{-1} (Habtemariam, 1996). In addition, Kawagishi *et al*. (1990b) isolated two novel cytotoxic phenols from *Hericium erinaceum* fruiting bodies

that caused complete inhibition of HeLa cells at concentrations of 6.3 μg mL^{-1} and 100 μg mL^{-1}.

6.2.3.2 Screening for cytotoxicity against Raji cells

The extracts tested in the previous section for cytotoxic activity against HeLa cells were further examined for there ability to inhibit the growth of Raji cells, a B-cell lymphoma cell line that is grown in suspension *in vitro*.

Raji cell solutions and *A. blazei* solvent extracts were prepared in RPMI 1640. Prior to carrying out the cytotoxicity assay, the optimal cell concentration was determined as described above for HeLa cells (Section 6.2.3.1). The absorbances of Raji cell solutions at different concentrations are shown in Figure 6.8.

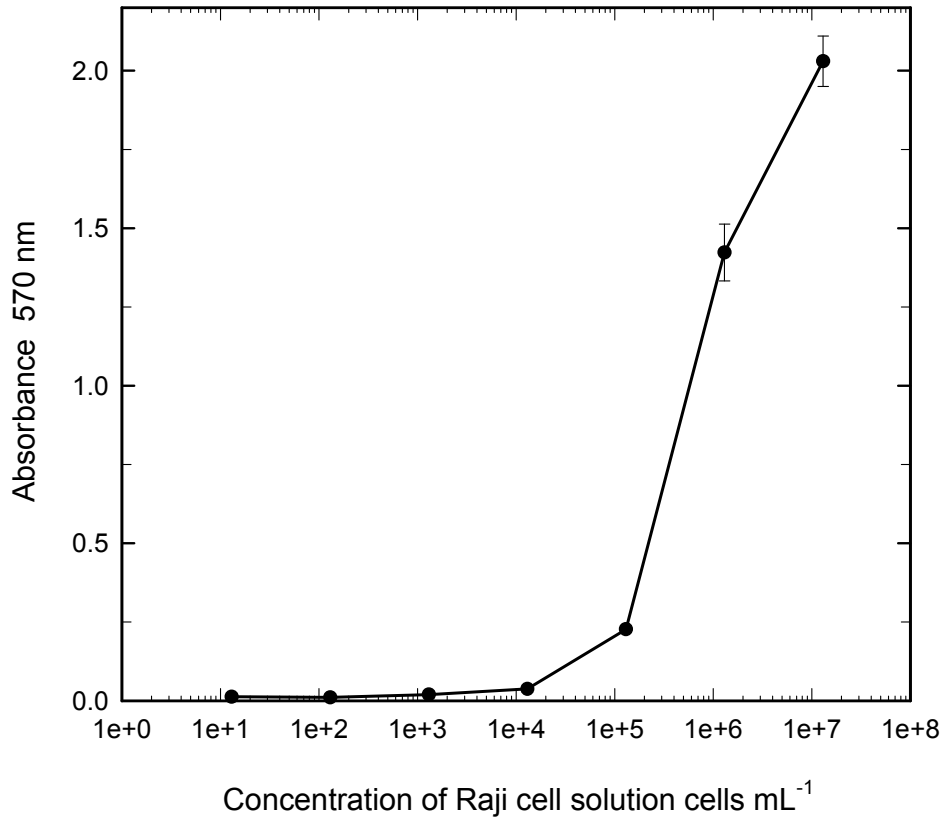
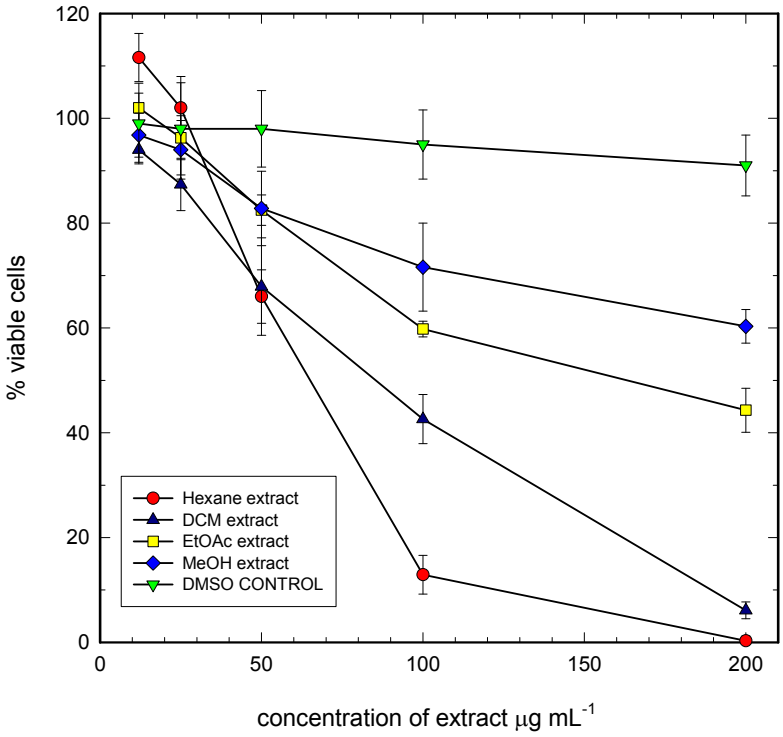


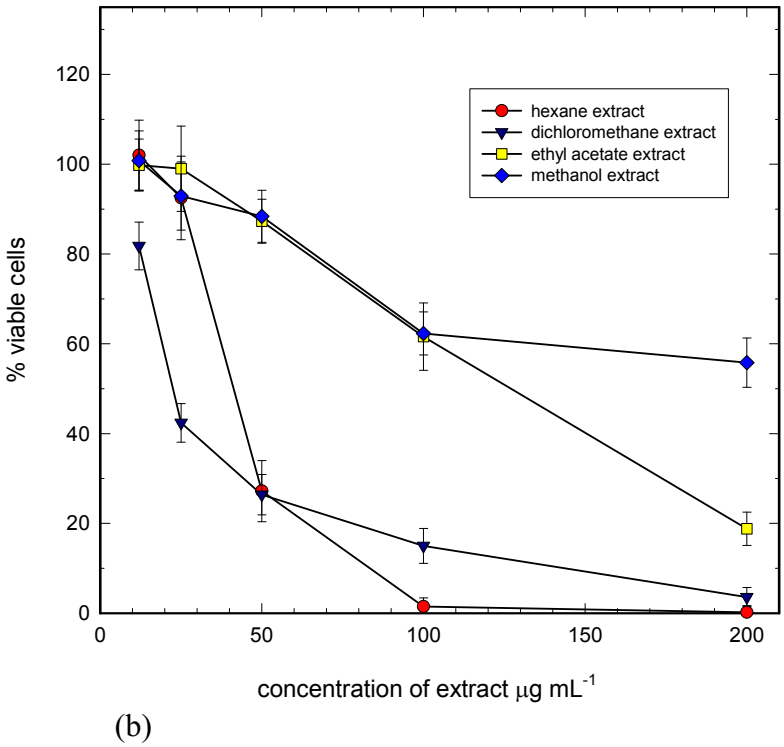
Figure 6.8 *Raji cell MTT viability assay standard curve*

The results shown in Figure 6.8 indicate that a Raji cell solution of less than 1x 10^5 cells mL^{-1} is not of a sufficient density to produce an optimal response in the MTT viability assay (maximum absorbance of 0.22). A cell density of 5 x 10^5 cells mL^{-1} was determined as the optimal concentration for Raji cell cytotoxicity testing.

The cytotoxic activity of *A. blazei* solvent extracts was determined against Raji cells as described in Section 3.5.5.2. The results were obtained from quadruplicate wells from using extracts obtained from two independent extractions and are shown in Figures 6.9-6.12.



(a)

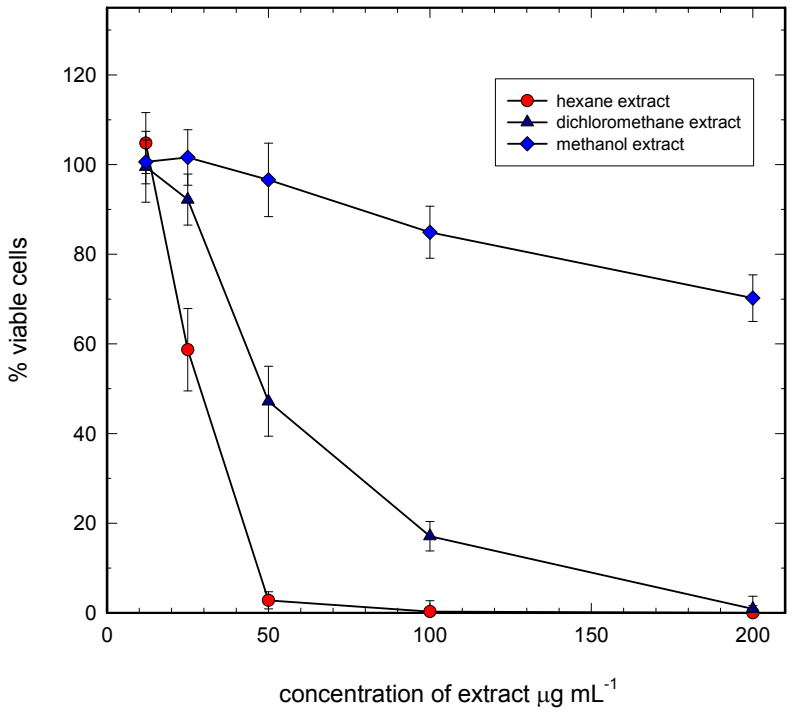


(b)

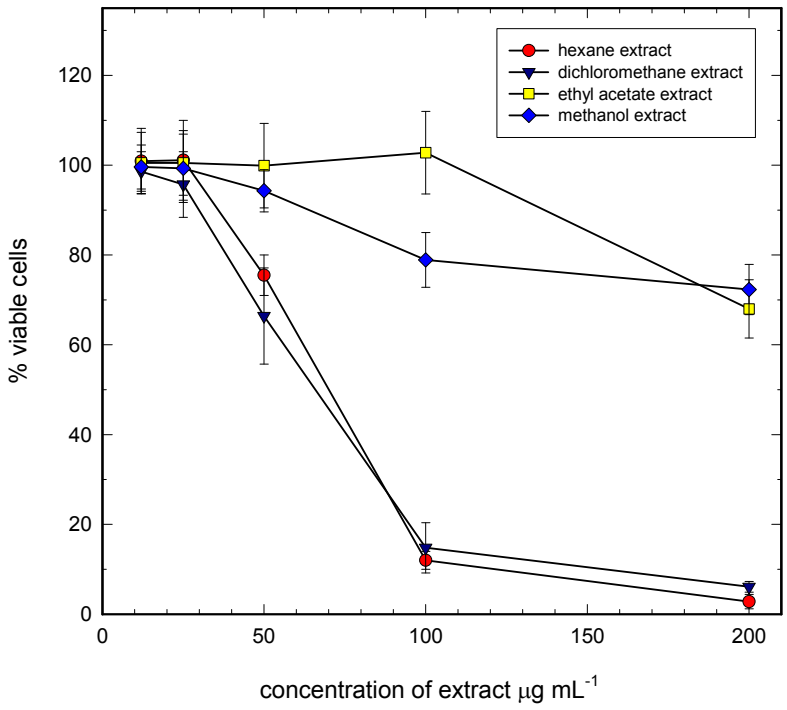
Figure 6.9 *Cytotoxicity against Raji cells – (a) YMB solvent extracts; (b) CMM solvent extracts*

The hexane, DCM, and EtOAc extracts from YMB mycelium all had marked cytotoxic activity against Raji cells (Figure 6.9(a)). The activity observed decreased with the increased polarity of the solvent, with CC_{50} values for the three extracts of 67 μg mL^{-1}, 98 μg mL^{-1} and 167 μg mL^{-1}, respectively. The methanol extract from YMB mycelium also showed some cytotoxicity with only 61% of the cells viable compared to the control at 200 μg mL^{-1}.

A similar observation was made for CMM mycelium, with hexane, DCM and EtOAc extracts having CC_{50} values of 41 μg mL^{-1}, 29 μg mL^{-1} and 130 μg mL^{-1}, respectively. The methanol extract also showed some activity with 57% cell viability at 200 μg mL^{-1}. The cytotoxicity activity of extracts from both the MEB and PMP mycelium is shown in Figure 6.10.



(a)



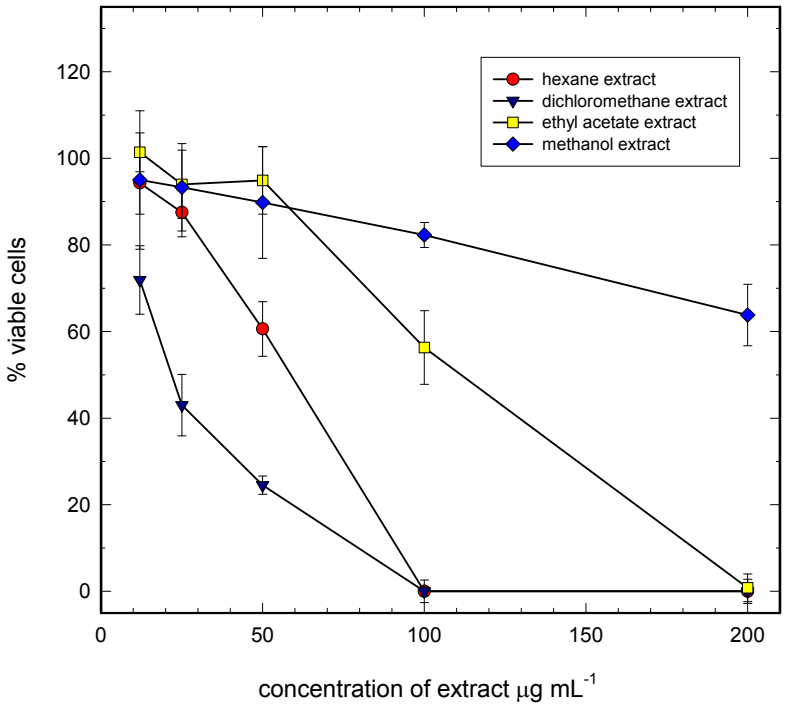
(b)

Figure 6.10 *Cytotoxicity against Raji cells – (a) MEB solvent extracts; (b) PMP solvent extracts*

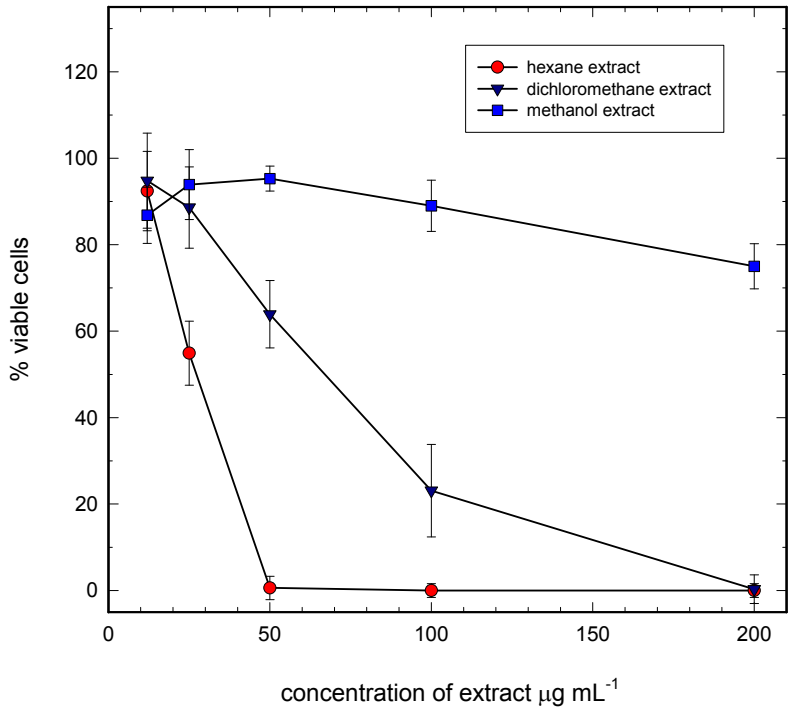
The hexane and DCM extracts from MEB mycelium showed the most cytotoxic activity with CC_{50} values of 31 μg mL^{-1} and 61 μg mL^{-1}, respectively (Figure 6.10(a)), which is similar to the observations made for YMB and CMM extracts. The methanol extract showed marginal activity with 70% viability at 200 μg mL^{-1}.

Hexane and DCM extracts from PMP mycelium (Figure 6.10(b)) showed similar activity with CC_{50} values of 69 μg mL^{-1} and 67 μg mL^{-1}, respectively. Only marginal activity was observed for the EtOAc and methanol extracts, with 68% and 73% viability, respectively, at 200 μg mL^{-1}.

Analysis of the GLUC and SUCR media extracts for cytotoxic activity against Raji cells is shown in Figure 6.11.



(a)



(b)

Figure 6.11 *Cytotoxicity against Raji cells – (a) GLUC solvent extracts; (b) SUCR solvent extracts*

The results obtained for GLUC and SUCR media were similar to those of the mycelial extracts discussed above, with all but the methanol extracts showing considerable cytotoxic activity. For GLUC media, the hexane, DCM, and EtOAc extracts had CC_{50} values of 58 μg mL^{-1}, 52 μg mL^{-1} and 29 μg mL^{-1} respectively, with 64% of cells viable at 200 μg mL^{-1} for the methanol extract (Figure 6.11(a)). Interestingly, the EtOAc extract from mycelium grown in this media was the most potent of all EtOAc extracts analysed thus far.

The hexane extract from SUCR mycelium was very potent with a CC_{50} value of 26 μg mL^{-1}, while the DCM extract had a CC_{50} of 68 μg mL^{-1} (Figure 6.11(b)). Again, the methanol extract was not active with 76% of cell viability at 200 μg mL^{-1}.

The lack of activity of the methanol extracts against Raji cells was investigated further using MeOH-S and MeOH-P samples, as described for HeLa cells, and the results displayed in Figure 6.12.

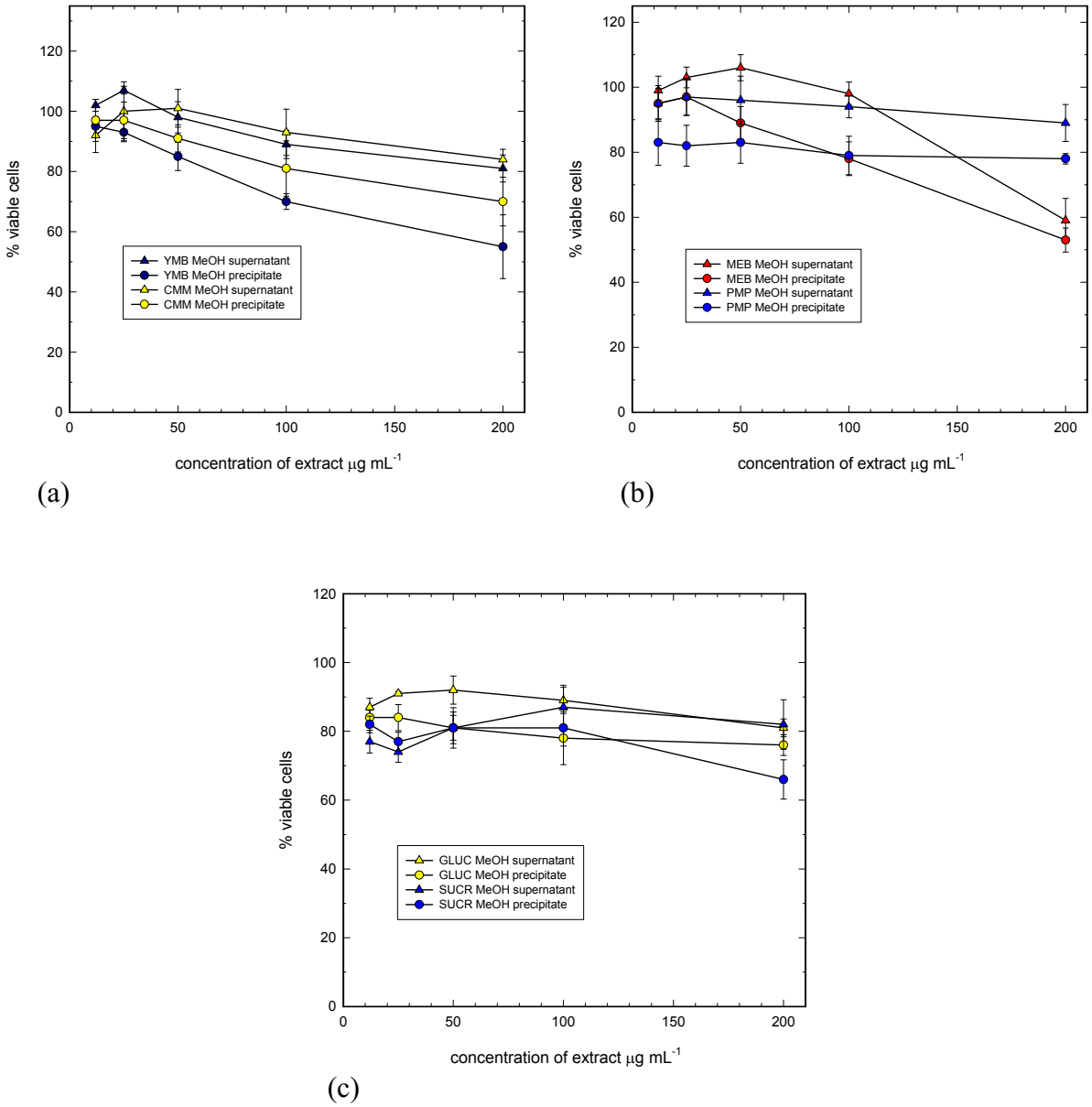


Figure 6.12 *Cytotoxicity against Raji cells fractionated MeOH extracts - (a) YMB & CMM; (b) MEB & PMP; (c) GLUC & SUCR.*

It is evident from the data in Figure 6.12 that the fractionated MeOH extracts did not reveal more enhanced cytotoxic activity against Raji cells, with similar results to the un-fractionated extracts (Figures 6.9-6.11). The percentage viability of cells at 200 μg mL^{-1} was between 53-89% for all extracts and no CC_{50} values were determined, indicating

that any cytotoxic principles were present at low concentrations, and only a more extensive fractionation procedure would reveal the presence of any compounds worthy of further investigation.

The activity seen against Raji cells is very promising for the development of potential anti-cancer treatments. Given the variation observed with mycelial extracts from different media, and the fact that activity was strong in hexane extracts and gradually became less potent as polarity of the solvent decreased, it would be interesting to determine if the activity was due to one compound being extracted at different concentrations across various solvents, or different compounds. As previously mentioned, varied proportions of the same compound may be recovered in several fractions (Harbourne, 1973). Further fractionation of extracts will help clarify this issue for the extracts analysed in this study.

A summary of the results of cytotoxicity testing against HeLa and Raji cells, including CC_{50} values and the percentage viability of extracts at 200 μg mL^{-1} (%V) where no CC_{50} was obtained, is outlined in Table 6.4.

Extract		HeLa cell line		Raji cell line	
		% V	CC_{50} (µg mL^{-1})	% V	CC_{50} (µg mL^{-1})
YMB	Hexane	57	>200	-	67
	DCM	60	>200	-	98
	EtOAc	98	>200	-	167
	MeOH	94	>200	61	>200
CMM	Hexane	-	161	-	41
	DCM	74	>200	-	29
	EtOAc	89	>200	-	130
	MeOH	100	>200	57	>200
MEB	Hexane	-	62	-	31
	DCM	-	153	-	61
	MeOH	118	>200	70	>200
PMP	Hexane	93	>200	-	69
	DCM	55	>200	-	67
	EtOAc	91	>200	68	>200
	MeOH	90	>200	73	>200
GLUC	Hexane	-	158	-	58
	DCM	-	112	-	52
	EtOAc	99	>200	-	29
	MeOH	99	>200	64	>200
SUCR	Hexane	-	109	-	29
	DCM	-	134	-	68
	MeOH	97	>200	76	>200

Table 6.4 *Summary of cytotoxicity testing of* A. blazei *solvent extracts*

Overall, Raji cells were more sensitive to *A. blazei* solvent extracts than HeLa cells, with hexane, DCM, and EtOAc extracts giving considerably lower CC_{50} values compared to the same extracts against HeLa cells. The more non-polar extracts, hexane and DCM, showed greater cytotoxic activity than more polar extracts, EtOAc and methanol. In particular, the methanol extracts tested in this study appear to be inactive and did not show considerable cytotoxic activity. Ajith *et al.* (2003) reported cytotoxic activity of methanol extracts from *Phellinus rimosus* fruiting bodies against Dalton s lymphoma ascites, and Ehrlich s ascites carcinoma cells, although these extracts were tested from 1 mg mL^{-1}, with corresponding CC_{50} values in the range 100-550 µg mL^{-1}.

Other polar, and in particular water-soluble, mushroom extracts from *Lentinus edodes* have also been reported to be active *in vitro* against human breast adenocarcinoma cells (Israilides *et al*. (2008)), although the CC_{50} values reported were again comparatively higher at 5-6 mg mL^{-1}. Therefore, testing of *A. blazei* extracts at a higher concentration may identify cytotoxic activity, although prior fractionation of extracts would more be more beneficial in the identification of any cytotoxic compounds from methanol and polar *A. blazei* extracts.

Previous research has identified a vast number of cytotoxic and antibacterial extracts and compounds, from both fungi and plants, which have been isolated using organic solvents rather than water (Valisolalao, *et al*., 1983; Cowan, 1999; Hirasawa *et al*., 1999; Rosa *et al*., 2003; Wang & Weller, 2006). The majority of compounds extracted using organic solvents, and reported to have cytotoxic or anti-bacterial activity *in vitro*, are terpenes. These include triterpenes (Toth, *et al*., 1983; Obuchi, *et al.*, 1990; Wasser and Weis, 1999; Gao *et al.*, 2002), and their derivatives such as sesquiterpenes (Anke & Sterner, 1991; Ishikawa *et al.*, 2001), lanostanoids (Keller *et al*., 1996; Gan *et al.*, 1998; Smania Jr *et al*., 1999), as well as phenolic compounds (Vogel *et al*., 1974; Kawagishi *et al*., 1990b).

Following further fractionation of *A. blazei* extracts, and identification of the compound(s) responsible for the activity observed, and studies that further characterise the activity and investigate the mode of action should be performed. For example, studies could be undertaken to investigate whether the inhibition of growth of bacteria is bactericidal or bacteriostatic (Hirasawa *et al*., 1999). Previous studies of the cytotoxic activity of *A. blazei* extracts, have shown that the activity can be attributed to the induction of apoptosis (Gan *et al.*, 1998; Chung *et al.*, 2001; Yassin *et al.*, 2003), effects on the cellular cycle (Gan *et al*., 1998; Zhu *et al.*, 2000), interference of cellular signal transduction (Zhu *et al*., 2000; Chung *et al*., 2001), and genotoxicity (von Wright *et al*., (1992). It is also important to characterise the selectivity of the cytotoxic activity by carrying out cytotoxicity assays on non-cancerous cell lines.

This study is the first report of *in vitro* antibacterial activity and cytotoxic activity in *Agaricus blazei* extracts obtained from liquid cultured mycelium. As previously

discussed, further fractionation and identification of the active components is required so that a further understanding of these observations is obtained, perhaps with a view to the development of products for biomedical applications.

Chapter Seven

Bioactivity of *Agaricus blazei* protein extracts

7.1 Introduction

There have been few, if any, investigations into the extraction and bioactivity of *Agaricus blazei* proteins, with the majority of the attention to date being focussed on the extraction of *A. blazei* polysaccharides and their reputed strong anti-tumour activity (Kawagishi *et al*., 1990a; Itoh *et al*., 1994; Ito *et al*., 1997; Fujimiya *et al*., 1998; Mizuno *et al*., 1999).

Proteins extracted from other species of mushroom have been investigated for their bioactivity, and have been reported to possess various properties, including antifungal activity (Lee *et al.*, 1999; Lam & Ng, 2001a; Chu *et al*., 2005; Wang & Ng, 2006a), immunomodulation (Kino *et al*., 1989; Ko *et al*., 1995; Hsu *et al*., 1997), insulitis prevention (Kino *et al*., 1990), *in vitro* cytotoxicity (Lam & Ng, 2001b; Lam *et al.*, 2001; Ng *et al*., 2003), and anti-viral activity (Piraino & Brandt, 1999; Wang & Ng, 2000, 2001, 2006b). The majority of these bioactive proteins have been identified as being of low molecular weight, in the range of 8 39 kDa.

In this current study, proteins were extracted from *A. blazei* mycelium cultivated in different culture media. The types of protein extracted, together with its yield, were compared, and selected extracts were screened for anti-viral activity against simian rotavirus SA11, and cytotoxic activity against HeLa and Raji tumour cell lines.

7.2 Results and Discussion

7.2.1 Effect of liquid culture medium on extraction of protein from *Agaricus blazei*

Agaricus blazei mycelium was cultivated in six different growth media, YMB, CMM, MEB, PMP, GLUC and SUCR (Section 5.2.3), harvested, and freeze-dried. Protein was

extracted from each sample of mycelium, according to the method described in Section 3.3.2. Briefly, approximately one gram of ground, freeze-dried mycelium was mixed with 100 mL of saline (0.85% NaCl), and left stirring overnight at 4 °C to allow the protein to be solubilised. Ammonium sulphate was added to the resulting supernatant at a saturation level of 80% to precipitate the protein, which was then collected by centrifugation, and the crude protein extract dialysed extensively against dH_2O.

In order to examine the size and relative amounts of proteins extracted from each mycelial sample, crude protein extracts (5 mg mL^{-1} in dH_2O), were analysed using SDS polyacrylamide gel electrophoresis (SDS-PAGE), according to the method described in Section 3.7.1. Protein extract solutions (20 μL) were subjected to electrophoresis and the proteins visualised by staining with Coomassie Brilliant Blue solution. The resulting gel is shown in Figure 7.1.

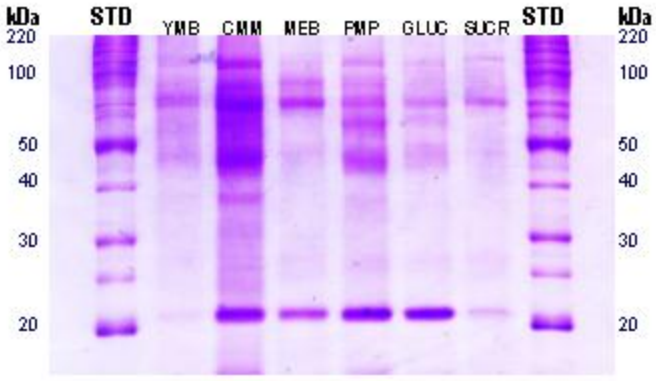


Figure 7.1 *SDS-PAGE of crude protein extracts from* A. blazei *mycelium grown in six different culture media.*

The SDS-PAGE gel shown in Figure 7.1 indicates that the amount and type of protein extracted varies depending on the growth media used for cultivation. The crude protein extract from YMB mycelium consisted of a number of proteins in the range of approximately 40-150 kDa, with very few lower molecular weight proteins present. In fact, for all the protein extracts examined, the majority of proteins extracted were in the size range of 40-150 kDa. Each protein extract, however, differed in the number and amount of each species of protein extracted.

Some lower molecular weight proteins of less than 40 kDa were also identified. In particular, a protein of approximately 20 kDa was extracted from each mycelium sample, with an intense band observed for all protein samples, except for those from YMB and SUCR media.

The highest yield of protein was obtained from mycelium grown in CMM, indicated by the greatest band intensity on the gel. Crude protein from CMM mycelium also contained the most diverse range of protein species, shown by the greater number of bands compared to other protein extracts, followed closely by protein extract from PMP media. For this reason, this extract was chosen for further analysis and screening for bioactivity.

Replicate extractions of protein from CMM mycelium were performed, and the reproducibility of the amount, and number of species of protein extracted, in addition to the reproducibility of the extraction procedure, was determined. Crude protein extracts from replicate extractions were analysed by SDS-PAGE, the resulting gel of which is shown in Figure 7.2.

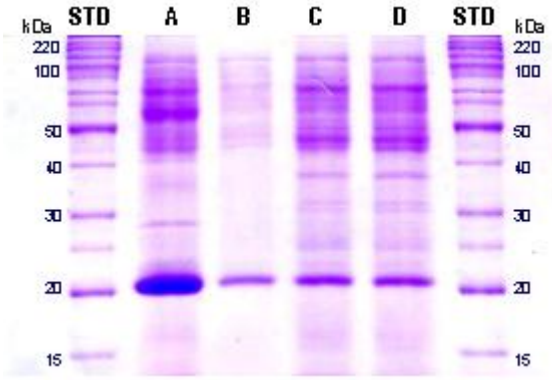


Figure 7.2 *SDS-PAGE of replicate crude protein extracts from CMM mycelium.*

Lanes A, B, and C contain samples of crude protein extracted from three different samples of *A. blazei* mycelium cultivated in CMM medium. Although there are slight variations in the amount of protein extracted, the number of different species of protein obtained in each extract is relatively constant. However, a lower yield of protein was contained in the protein extract in Lane B compared to the other protein extracts. Slight variations in the conditions of each culture flask (such as pH of medium, health of

mycelium used to inoculate the culture fluid, or premature exhaustion of essential nutrients) may account for the reduced yield in this sample.

Lanes C and D contain two replicate protein extracts from the same sample of *A. blazei* mycelium grown in CMM media. The amount of protein and variety of protein species in each extract appears to be identical, confirming the reproducibility of the extraction procedure.

Replicate protein extracts were analysed for their protein, glucose and polysaccharide content in order to characterise the components of the extracts. It is unlikely that the extracts consisted solely of protein as addition of ammonium sulphate to precipitate the protein may also result in the precipitation of other compounds, such as polysaccharides and simple sugars such as glucose.

The protein content of the extracts was determined using the Bradford protein assay (Section 3.7.4), polysaccharide content was examined using a modified method of the phenol sulphuric acid method (Section 3.7.3), and glucose content was analysed using Megazyme Glucose Assay Kit (Section 3.7.5). The results are summarised in Table 7.1 and are presented as the average and standard deviation of four replicate protein extracts and characterisation experiments.

Component	% of crude CMM protein extract
Protein	67 ± 5
Polysaccharide	6 ± 6
Glucose	7 ± 1
Other	19

Table 7.1 *Percentage composition of CMM media crude protein extracts*

The results in Table 7.1 highlight that the main component of the crude extract was protein, followed by polysaccharide and glucose. It is not surprising that some polysaccharide was be extracted into saline in the initial protein extraction due to their presence in fungal mycelium. By difference, 19% of the mass of the extract was not identified.

Experiments were then undertaken to determine whether the extracts contained bioactive components by screening for anti-viral activity against rotavirus using an MTT-based anti-viral method described by Pauwels *et al*. (1988), in addition to an examination of cytotoxic activity against two tumour cell lines: HeLa and Raji.

7.2.2 Screening of protein extracts for anti-viral activity against Rotavirus

Rotaviruses are the most frequent cause of infantile gastroenteritis worldwide and a significant cause of death, following severe diarrhoea and dehydration, in infants and young children of developing countries (Gray & Desselberger, 2000). A study by Parashar *et al*. (2003) identified rotavirus causes approximately 111 million episodes of gastroenteritis, 25 million clinic visits, 2 million hospitalisations, and an average 440,000 deaths in children less than five years of age annually. This large number of infant disease and deaths highlights the urgent need for interventions, such as the development of vaccines to prevent childhood deaths. Two vaccines are currently available in Australia (Schulz *et al*., 2007) although there is still a need for alternative strategies, such as the development of antiviral and chemotherapeutic agents for the treatment of rotavirus infection (Kiefel & von Itzstein, 2003).

To aid the investigation of anti-rotaviral agents *in vitro*, the African Green Monkey kidney epithelial cell line, MA104, was employed for this study. This cell line is routinely used and supports the growth of a wide variety of strains of rotavirus (Ward *et al*., 1984; Patton *et al*., 2000). In this investigation, CMM protein extract from *Agaricus blazei* mycelium was screening for antiviral activity against simian rotavirus, SA11.

7.2.2.1 Optimisation of MA104 cell seeding density for anti-viral assays

MA104 cell solutions and *A. blazei* solvent extracts were prepared in MEM. The optimal seeding concentration for MA104 cells for anti-viral testing was comparison to standards. MA104 cell solutions were prepared by 10-fold serial dilutions, with a final concentration range from 10^1 10^7 cells mL^{-1}. The cell solutions were seeded in 96 well plates and the MTT viability assay was carried out over a period of 72 hours (Section 3.5.4). The absorbance at 570 nm of the MA014 cell solutions at different concentrations is shown in Figure 7.3.

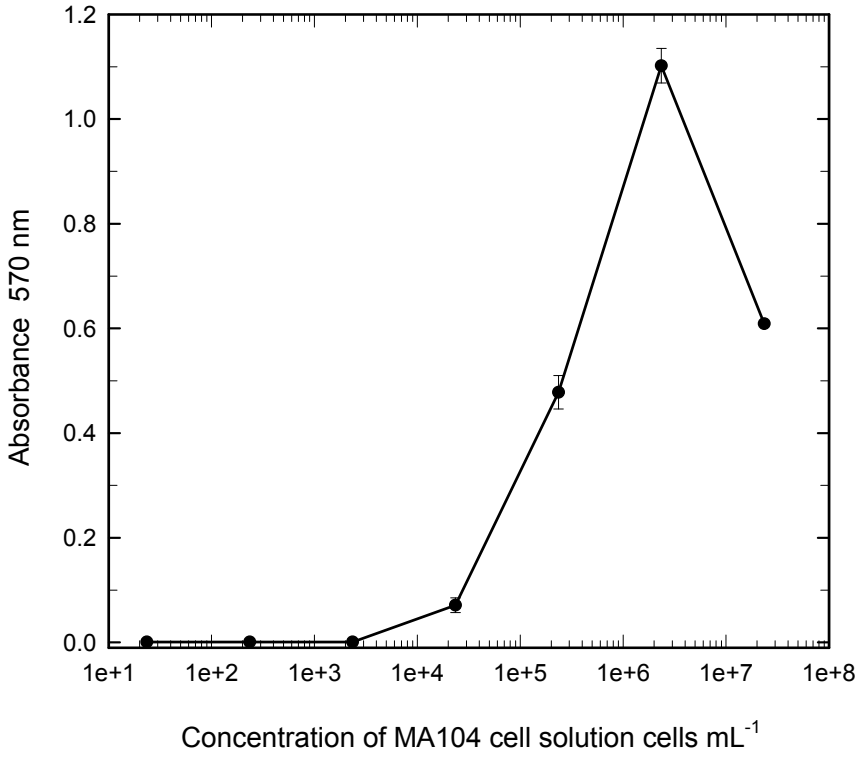


Figure 7.3 *MA104 cell MTT viability assay standard curve*

The results shown in Figure 7.3 indicate that a MA104 cell solution of 1 x10^4 cells mL^{-1} or less is not of a sufficient density to produce an optimal response in the MTT viability assay, with a maximum absorbance 0.8 at 1 x10^4 cells mL^{-1}. At a concentration of 1 x10^6 cells mL^{-1} the cells seeded in 96-well plates have reached a plateau of log phase growth, with a reduction in the MTT response at 1 x10^7 cells mL^{-1}. As a result of the above observations, a MA104 cell solution of 8 x10^5 cells mL^{-1} was chosen as the optimal cell count, and used for all anti-rotaviral screening experiments.

7.2.2.2 Optimisation of virus titre

The optimal concentration of virus required to produce the appropriate level of cytopathic effect (CPE) was determined prior to the commencement of anti-viral assays against rotavirus. If the concentration was too low, a high proportion of virus-infected cells would survive the duration of the assay making it difficult to observe anti-viral activity when the cells are treated with test extract. Conversely, if the concentration of

virus was too high, the concentration at which the compounds are tested may not be sufficient to observe a decrease in CPE on the cells, and thus identify anti-viral activity.

The optimisation of the virus concentration for use in the screening assays was determined according to the method described in Section 3.6.3., and was based on the procedure described by Woo *et al*. (1997). Briefly, ten-fold dilutions of SA11 were prepared (10^{-1} 10^{-9}) and added to previously-seeded MA104 cells in 96-well plates, at the optimal cell concentration. A neat virus solution was also included, and the plates were incubated for 72 hours, and complete CPE was observed in the cells treated with neat virus. An MTT assay was then performed to assess cell viability of the cells treated with different concentrations of virus, and the results are shown in Figure 7.4.

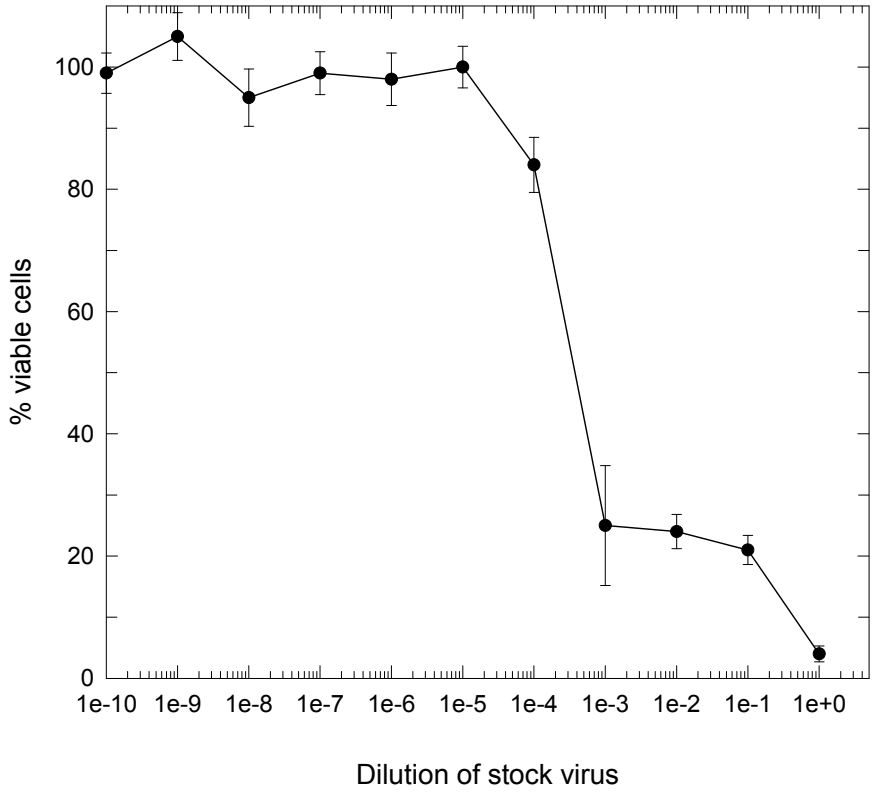


Figure 7.4 *MA014 Cell viability after treatment with increasing concentration of rotavirus SA11*

The data shown in Figure 7.4 indicate that the SA11 virus at a concentration of 10^{-5} 10^{-10} was too dilute for use in the assay as minimal CPE was observed. A virus dilution

of 10^{-2} was deemed most appropriate for use in screening for anti-viral activity as the cell viability was 20% after 72 hours. In addition, this result was reproducible compared to those of other dilutions (e.g. 10^{-3} dilution) which showed some variability between replicate wells. This virus dilution was used for the preliminary screening assay and mode of action experiments described in the following sections. All the anti-viral assays described were carried out in triplicate, with duplicate wells for each condition, and the results are presented as averages with standard deviation for the complete set of experiments. Porcine trypsin at 10 μg mL^{-1} (the concentration used to pre-activate SA11 virus) was not found to have an effect on the viability of MA014 cells.

7.2.2.3 Preliminary anti-viral screening assay

A preliminary anti-viral screening assay was performed by adding the test extract over a concentration range of 12.5-1000 μg mL^{-1} to samples of activated SA11 virus. A pre-incubation step (addition of virus to the cells for a fixed period of time to allow for viral attachment, followed by removal of the viral inoculum) was not performed in this preliminary screening assay. In this case, virus and extract were added at the same time with viral attachment presumably taking place in the first hour of the 72 hour assay duration period.

In parallel, cells were incubated with extract at the various concentrations without virus infection in order to identify any cytotoxic effects of the extracts on MA104 cells that may also cause non-viral CPE. The results of the preliminary screening assay are shown in Figure 7.5.

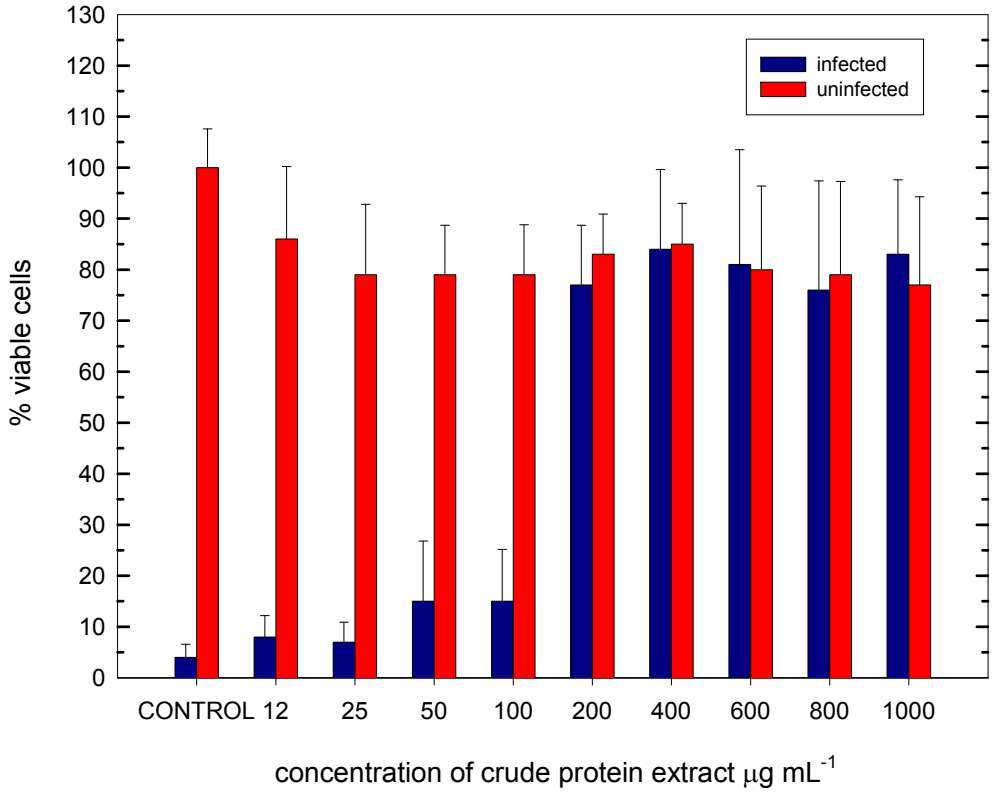


Figure 7.5 *Preliminary antiviral screening assay of crude* Agaricus blazei *CMM protein extract*

The results presented in Figure 7.5 indicate that the protein extract from CMM mycelium displays an inhibitory effect on the infection of rotavirus SA11 in MA104 cells well below cytotoxic concentrations. The cytotoxicity of MA014 cells is indicated by the cell viability in the presence of protein extract only, as shown by the red bars in Figure 7.5. While it was quite variable at all extract concentrations examined, it is clear that the extract does not have a marked cytotoxic effect on MA014 cells with an average of 75% of cells still viable at 1000 μg mL^{-1}.

When protein extract was added in addition to rotavirus (blue bars in Figure 7.5), extract concentrations of 200 μg mL^{-1} and above provided protection against rotavirus infection, compared to the uninfected control ($p<0.0001$). . In the presence of extract at concentrations below 200 μg mL^{-1}, the cell viability decreased markedly in infected cells and a significant difference was observed compared to the uninfected control ($p>0.0001$), indicating that no antiviral effect was apparent at these concentrations. An effective concentration 50 (EC_{50}), defined as the concentration that causes a 50%

inhibition of viral infection compared to the control, was calculated using a regression analysis of the dose response curve generated from the data in Figure 7.5. The EC_{50} for the CMM protein extract was calculated at 145 μg mL^{-1}.

Ebina, *et al*. (1990) have previously reported *in vitro* anti-rotaviral activity of a protein bound polysaccharide (designated PSK), from the mycelia of the medicinal mushroom *Coriolus versicolor*, but there are no other accounts of other fungal extracts, particularly proteins, that have anti-rotaviral activity.

Given this preliminary evidence of an anti-viral effect against SA11 virus, the protein extract was tested in further anti-viral assays designed to investigate the mode of action of the extract on the virus. Based on methods reported by others, investigations were carried out to determine whether the extract was acting by inhibiting viral attachment or penetration into cells (Eo *et al*., 2000; Kaneko *et al*., 2001; Hernandez-Corona *et al*., 2002), as well as potential virucidal activity (Mucsi *et al*., 2001; Apers *et al*., 2001). These experiments were carried out by varying the timing of addition of extract and virus to the cells, as well as the temperature for the pre-incubation step necessary for attachment of the virus to the cell surface. CMM protein extract was tested at a maximum concentration of 600 μg mL^{-1} in the following experiments as this range was considered sufficient to detect antiviral activity given the EC_{50} observed in the preliminary screening.

7.2.2.4 Attachment assay at 4°C

In order to investigate whether the CMM protein extract was producing an anti-viral effect by inhibiting the attachment of SA11 virus to the cell surface, a 1 hour pre-incubation step at 4°C was included in the initial viral attachment phase (Section 3.6.5). At a temperature of 4°C, virus will attach to the cell membrane surface but will not penetrate the cell surface. Following removal of the viral inoculum and incubation at 37°C for 72 hours, any virus successfully attached to the cell surface will then penetrate the cell surface and proceed with the infection process leading to CPE. If the protein extract is included in the 4°C pre-incubation step and interferes with the attachment process, no virus particles will adhere to the cell surface. Therefore, following removal

of the virus-extract inoculum, no penetration of virus will take place and no CPE will be observed. The results of the attachment assay at 4°C are shown in Figure 7.6.

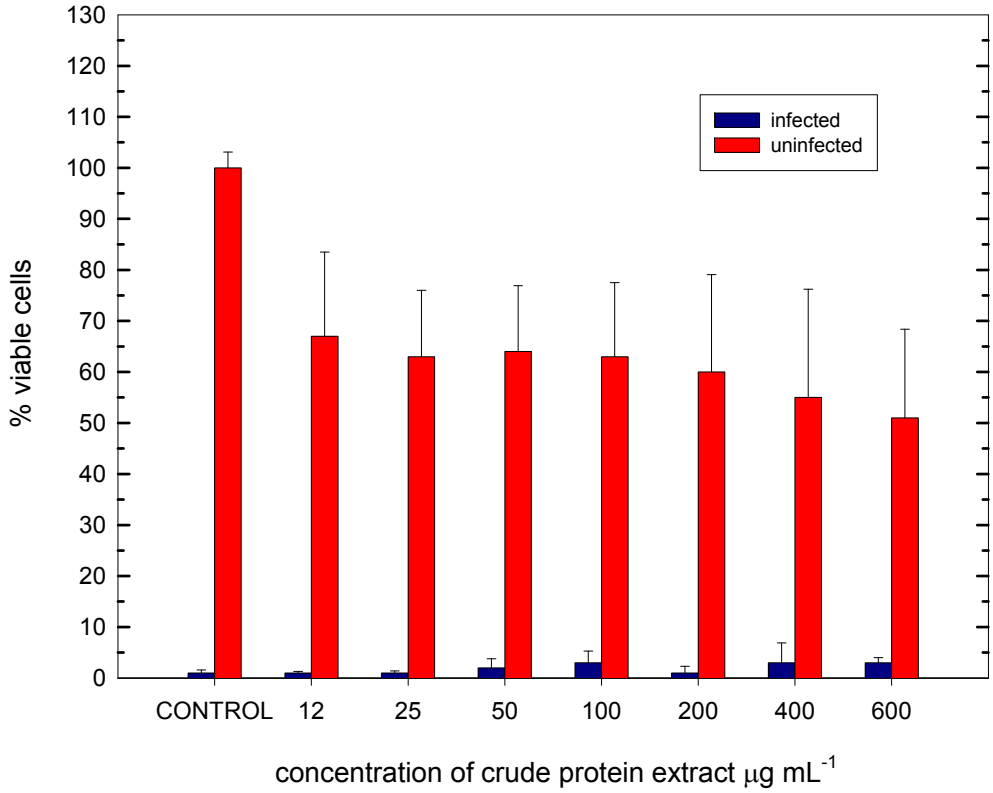


Figure 7.6 *Screening for inhibition of viral attachment to MA104 cells by CMM protein extract.*

The results in Figure 7.6 indicate that the CMM protein extract does not interfere with the attachment of SA11 to the surface of MA104 cells as no protection of viral infection was observed. Complete CPE was observed at all extract concentrations tested similar to the control. Some cytotoxicity was observed in uninfected cells exposed to extract at different concentrations although the cell viability observed was quite variable and no clear response to the dose of extract was observed.

7.2.2.5 Viral entry assay at 37°C

A similar experiment was performed by carrying out the pre-incubation step at 37°C (Section 3.6.6), rather than 4°C to examine whether the extract was having an effect on

the entry of SA11 virus into MA104 cells. At this temperature, virus particles will attach and penetrate the cell surface. Therefore, if CPE is prevented when incubation is performed in the presence of the protein extract, (also considering the results at 4°C), it could be concluded that the extract inhibits viral entry into the cell. The results of the assay for viral entry at 37 °C are shown in Figure 7.7.

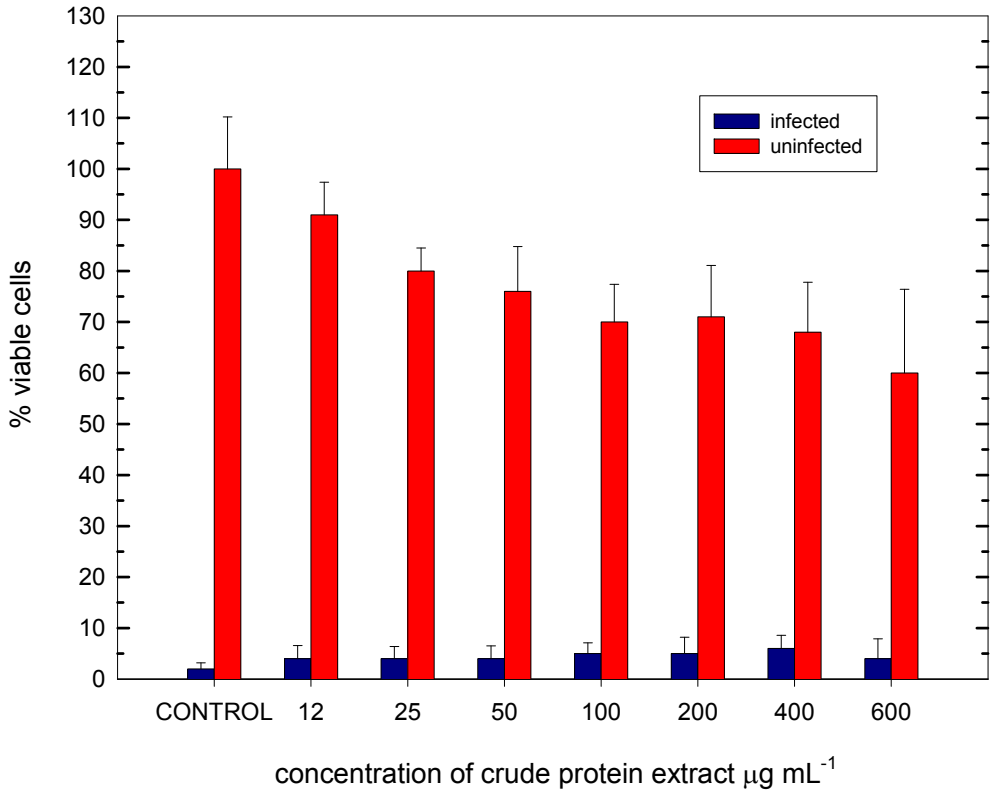


Figure 7.7 *Screening for inhibition of viral entry to MA104 cells by CMM protein extract.*

As was observed for the viral attachment assay, no inhibition of CPE was observed when examining the effect on viral entry (Figure 7.7). CPE was observed at all extract concentrations with cell viability less than 10% in each case. Again a small amount of cytotoxicity was observed in MA104 cells in a dose dependant manner, although this was variable at all extract concentrations and the average cell viability did not decrease below 60%. The results in Figure 7.7 indicate that CMM protein extract did not inhibit SA11 infection of MA104 cells by preventing entry of the virus across the cell membrane.

7.2.2.6 Virucidal assay

Extract (12.5 600 μg mL^{-1}) was pre-incubated with SA11 virus for 1 hour prior to addition to MA104 cells in 96-well plates, as described in Section 3.6.7 to examine whether the CMM protein extract may be conferring inhibition of SA11 virus through direct virucidal action. The results obtained are shown in Figure 7.8.

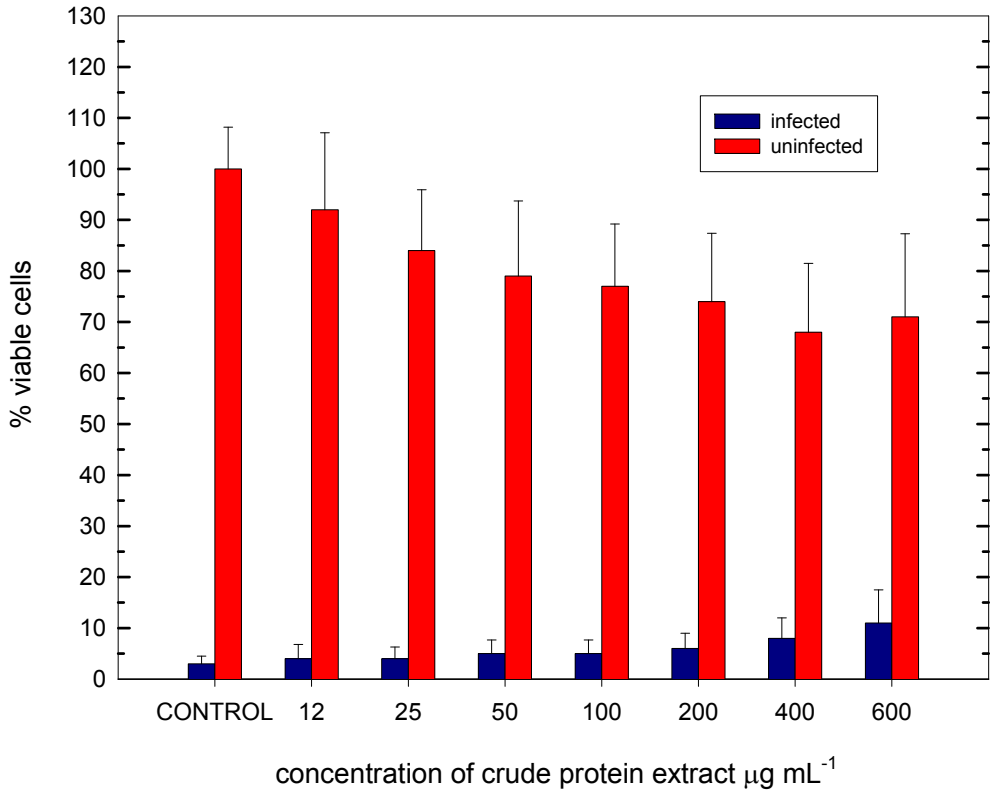


Figure 7.8 *Virucidal activity of* Agaricus blazei *CMM protein extract*

The data in Figure 7.8 indicate that *A. blazei* protein extract may have a virucidal action on SA11 virus, with a gradual but slight increase in MA104 cell viability as the concentration is increased from 100 600 μg mL^{-1} extract, although with cell viability at an average of 12% at 600 μg mL^{-1} compared to 3% for the control, this effect was not significant ($p>0.0001$). Further assays would need to be carried out to confirm whether this was in fact the case by testing the extract at higher concentrations, or alternatively, pre-incubating the extract with the virus for a longer period than 1 hour.

From the results of the experiments described in Sections 7.2.2.4 7.2.2.6, the mechanism of action of the CMM protein extract from *A. blazei* was not clearly elucidated, and further experimentation is required to determine if the extract does in fact have virucidal activity.

Other anti-viral screening assays can be employed for the identification of antiviral activity including plaque reduction assays (Ramia & Sattar, 1979; O Mahony *et al*., 2000), and virus yield reduction assays (Oxford *et al.*, 1999). Given that there is inherent variability in the anti-viral screening assay used in this study, further assay optimisation or the use of other anti-viral screening assays would be beneficial to confirmation of the antiviral activity observed in the preliminary screening assay (Section 7.2.2.3). Other methods that have been employed for the identification of anti-rotaviral activity include plaque reduction assays (Smith *et al*., 1979) and neutralisation of infectivity assays (Woode *et al*., 1987).

One hypothesis to explain the results obtained is that proteins in the extract may in fact be inhibiting the effect of trypsin, which plays an important role in the infectivity of rotavirus. Trypsin enhances the infectivity of most rotaviruses, not by affecting the efficiency or rate of attachment of virus to cells, but rather by increasing levels of uncoated virus within cells (Clark, *et al*., 1981). Almeida & Hall (1978) reported that 1000-fold more bovine rotavirus is obtained when trypsin is incorporated in the maintenance medium and allowed to remain throughout the growth cycle, an effect also indicated for human rotaviruses.

Complete infectious particles (virions) of rotavirus, also called triple-layered particles, are composed of three concentric layers of proteins surrounding a genome of 11 segments of double-stranded RNA. Six of the genome segments code for structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7), with the remaining segments coding non-structural proteins. The outermost layer of the virion is composed of two proteins, VP4 and VP7, which are involved in the initial interaction between the virus and the host cell (Estes & Kapikian, (2007).

Cultivation of most viruses requires the addition of exogenous proteases to the culture medium, and in the case of rotavirus, the addition of trypsin ensures activation of viral activity by cleaving the outer capsid protein VP4 (Estes & Kapikian, (2007)). Virus attachment is by VP4 (Crawford *et al.* 1994), though binding of rotavirus particles to cells does not require cleaved VP4 (Fukuhara *et al*. 1988). Trypsin cleavage of VP4 has been shown to enhance viral penetration and infectivity (Estes & Kapikian, (2007). The mechanism for this enhancement is not known, but trypsinised particles enter cells more rapidly than those not trypinised (Keljo *et al*. 1988; Kaljot *et al*. 1988).Therefore the presence of a potential trypsin inhibitor in the assay medium of a cell based assay, such as the presence of the CMM protein extract in the screening assays described in this study, may produce an anti-viral effect.

Trypsin inhibitors from breast milk have been shown to reduce the susceptibility of neonates to rotavirus infection (McLean & Holmes 1981; Jayashree *et al*., 2005), and soybean trypsin inhibitors have also demonstrated a protective effect from rotavirus infection in infant mice (Katyal *et al*., 2001). While trypsin inhibitors from crude plant (Birk, 1985) and fungal extracts (Wang & Ng, 2001) have demonstrated antiviral activity against HIV, there are no previous reports of fungal trypsin inhibitors that inhibit or prevent rotavirus infection. Further investigation is required to examine whether such compounds are present in the CMM protein extract from *A. blazei*.

7.2.3 Screening for cytotoxicity against tumour cell lines

CMM protein extract was also tested for cytotoxic activity against two human tumour cell lines, the human cervical carcinoma cell line, HeLa, and a B-cell lymphoma cell line, Raji. Each stock extract solution (10 mg mL^{-1} in PBS or dH_2O) was diluted to concentrations of 12.5, 25, 50, 100, 200, 400, 600, 800 and 1000 μg mL^{-1} in the appropriate medium for the cell line under investigation. The concentration of PBS or dH_2O in the extract solutions was not toxic to the cell lines tested. PBS and dH_2O controls were included in every assay; an example of the lack of cytotoxicity shown by these solutions is given in Figure 7.9. The cell concentration used in the assay procedure for both cell lines was according to the previously optimised value (Sections 6.2.3.1 and 6.2.3.2).

Raji cell solutions and *A. blazei* CMM protein extracts for testing with this cell line were prepared in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution. Raji cells at a density of 5 x 10^5 cells mL^{-1} were seeded into each well. For testing against HeLa cells, HeLa cell solutions and *A. blazei* CMM protein extract solutions were prepared in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution. HeLa cells at a density of 5 x 10^4 cells mL^{-1} were seeded into each well. Cytotoxic activity was determined using the MTT cell viability assay described in Section 3.5.5.1.Cytotoxic activity was determined using the MTT cell viability assay described in Section 3.5.5.2.

The results are the average and standard deviation of three separate experiments using extracts from three separate extractions. The proportion of viable cells remaining after treatment with CMM protein extract at different concentrations was calculated as previously described (Section 6.2.3.1), by dividing the absorbance at 570 nm of the test extract by the absorbance at 570 nm of the cell only control, and expressing the ratio as a percentage. Cytotoxic activity shown by extracts that reduced the cell viability to at least 50% was quantified by the calculation of the 50% Cytotoxic Concentration (CC_{50}). The cytotoxicity of *A. blazei* crude protein extract from CMM mycelium against Raji cells and HeLa cells is shown in Figures 7.9 and 7.10, respectively.

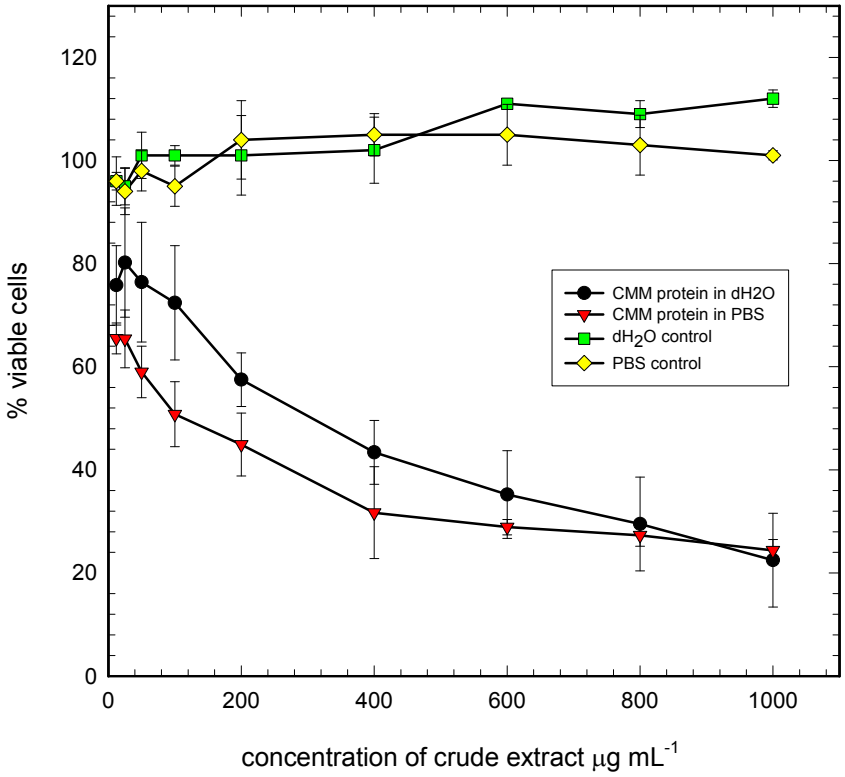


Figure 7.9 *Cytotoxicity of CMM crude protein extract against Raji cells*

The CMM protein demonstrated cytotoxic activity against Raji cells, as shown by the decline in the % of viable cells in Figure 7.9. Crude protein extracts dissolved in both dH_2O and PBS produced a dose response in cytotoxic activity, although at concentrations above 600 μg mL^{-1} the inhibitory effect appeared to reach a plateau. Complete inhibition of cell growth was not observed with the maximum reduction in cell viability at 1000 μg mL^{-1} of approximately 25% for extracts dissolved in dH_2O or PBS. The calculated CC_{50} values for the protein extract dissolved in PBS and dH_2O were 100 μg mL^{-1} and 300 μg mL^{-1}, respectively.

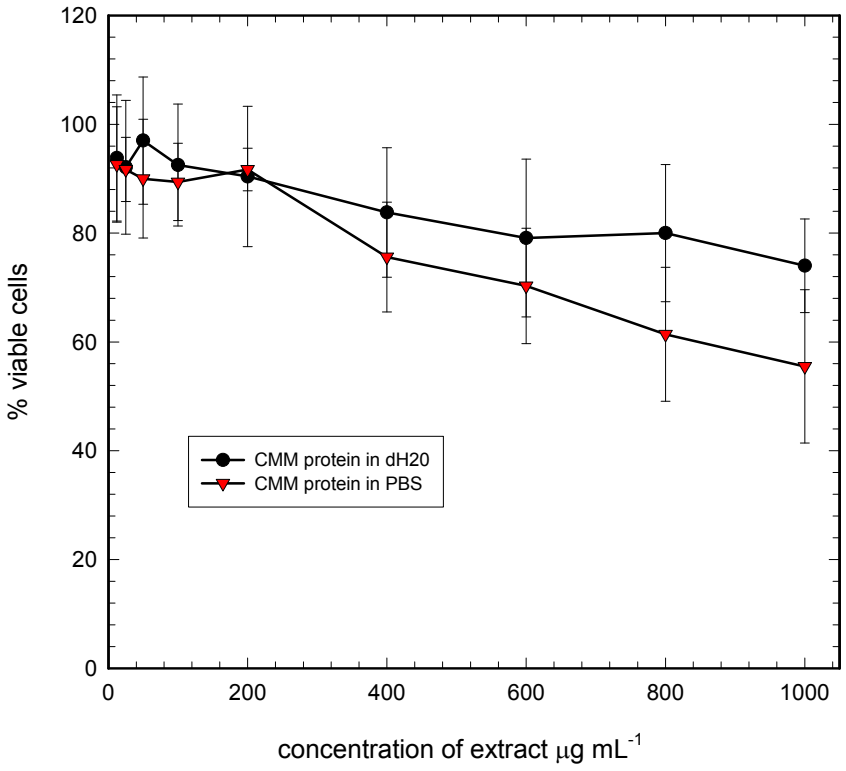


Figure 7.10 *Cytotoxicity of CMM crude protein extract against HeLa cells*

The results in Figure 7.10 indicate that the crude protein extract obtained from *A. blazei* mycelium grown in CMM medium had a slight cytotoxic effect against HeLa cells, particularly when the extract was dissolved in PBS. A dose response was evident over the concentration range tested for protein extracts dissolved in both PBS and dH_2O. At 1000 μg mL^{-1}, the highest concentration of protein extract tested, the percentage of viable cells was found to be 78 % and 58% when dissolved in dH_20 and PBS, respectively. The fact that PBS-solubilised protein extract produced a stronger cytotoxic effect than the H_2O-solubilised extracts may be due to the fact that PBS is closer to physiological pH, and similar to the cell media used for the assay. The buffering action of PBS may alleviate minor pH fluctuations, which may have caused variation and adverse growth conditions in test wells, and provided more favourable conditions for active proteins in the extracts. Further fractionation of the extract and re-testing may

increase the level of cytotoxic activity against HeLa cells, if the protein/s responsible for the reduction in cell viability were present in the extract in low concentrations.

There was a large standard deviation between the results for replicate extracts against both HeLa and Raji cells. This may be due to the fact that a large number of protein species are extractable from *A. blazei* mycelium and these other active species may be present in different concentrations in the replicate extracts. In addition, the effect of cytotoxic proteins may by masked by the presence of a higher proportion of other proteins, effectively resulting in a dilution of cytotoxic proteins and a varied response depending on the extract.

Fractionation of the protein extract to concentrate groups of proteins, followed by re-examination of the cytotoxic activity of the different fractions, should be carried out to further isolate the compound or compounds responsible for the cytotoxic activity against the cell lines tested. Performing gel filtration procedures to fractionate the extract due to size of proteins will help identify whether the protein active against the lymphoma cell line Raji, has a low molecular weight similar to other cytotoxic proteins previously reported.

Lam *et al.* (2001) identified an 8kDa protein from the puffball mushroom *Calvatia caelata*, which showed very strong cytotoxic activity against breast carcinoma cells *in vitro*, and reported IC_{50} of 100 nM. The mechanism of action was thought to be related to translation-inhibiting activity but has yet to be elucidated. This protein was also found to have anti-mitogenic activity. Another protein from *Calvatia caelata* with a molecular weight of 39 kDa was also reported to reduce the viability of breast cancer cells with an IC_{50} of 2.3 μM (Ng *et al*., 2003). This protein also had anti-mitogenic activity.

Lam & Ng (2001b) found that a 20 kDa protein from fruiting bodies of *Hypsizigus marmoreus* inhibited the proliferation of the mouse leukaemia cell line L1210, with a CC_{50} of approximately 6 μM, and to a lesser extent human hepatoma cells (HepG2) and human leukemic cells (HL60), with CC_{50} values of approximately 25 μM and 35 μM respectively. This protein had a broad spectrum of bioactivity, with anti-fungal and anti-

mitogenic activity also reported, in addition to the protein s ability to inhibit HIV-1 reverse transcriptase.

In summary, the protein extract from *Agaricus blazei* mycelium grown in CMM media appeared to have an antiviral effect on simian rotavirus SA11, although the mechanism of action could not be elucidated. It was hypothesized that the extract may contain a trypsin inhibitor that was causing the antiviral effect observed in Section 7.2.2.3. Further experiments are required to determine if this is the case.

The CMM protein extract exhibited cytotoxic activity against the leukemic cell line Raji, with CC_{50} values for the extract dissolved in PBS and dH_2O of 100 μg mL^{-1} and 300 μg mL^{-1}, respectively. Further fractionation and isolation is required to characterise the protein species responsible for the cytotoxic activity against this cell line. No marked cytotoxic effect was observed against the cervical cancer cell line HeLa. It would be important to establish that cytotoxicity against non-tumour cells lines for the CMM extract is negligible if these extracts were to be used in clinical applications.

In addition to cytotoxic and antiviral activity, other reported bioactivities of fungal proteins from popular medicinal mushrooms include the immunomodulation effect of LZ-8 from *Ganoderma lucidum* (Kino *et al*., 1989; Tanaka *et al.*, 1989; van der Hem *et al*., 1995), and Flammulin, isolated from *Flammulina velutipes* (Ko *et al*., 1995; Lindequist *et al*., 2005). Pleurostrin, from the oyster mushroom *Pleurotus ostreatus* (Chu *et al*., 2005), and ganoderin from *Ganoderma lucidum* (Wang & Ng, 2006) have been shown to have antifungal activity. Given the range of bioactivity of fungal proteins, it would be valuable to screen *Agaricus blazei* proteins further for other important medicinal properties, such as those described above, in addition to antitumour and antibacterial activity.

Chapter Eight

Bioactivity of *Agaricus blazei* exopolysaccharide extracts

8.1 Introduction

The majority of the published research into the medicinal properties of *Agaricus blazei* has focussed on substances isolated from the mushroom fruiting bodies, particularly polysaccharides. By far the most widely studied and reported medicinal properties of these polysaccharides is *in vivo* antitumour activity (Kawagishi, *et al*., 1989, 1990; Mizuno, T., *et al*., 1990a, 1990b; Itoh *et al*., 1994; Mizuno M., *et al*., 1998; Fujimiya, *et al*., 1998, 1999; Ohno, *et al*., 2001; Oshiman, *et al*., 2002; Ebina, T., 2005; Chen *et al*., 2006; Gonzaga *et al*., 2009). This research, which has suggested that consuming these fruiting bodies has the ability to stimulate the immune system and protect against tumours has led to an increased production and consumption of *A. blazei* in China and Japan (Firenzuoli *et al*. 2007). Other bioactive properties possessed by polysaccharides from *A. blazei* fruiting bodies include antimutagenicity (Angeli, *et al*., 2006; 2009), cytotoxicity against tumour cells *in vitro* (Shen *et al*., 2001; Chen *et al*., 2007), antioxidant activity (Soares *et al*., (2009), immunomodulation (Hashimoto *et al.*, 2002) and antidiabetic activity (Kim *et al*., 2005).

The cultivation of *Agaricus blazei* fruiting bodies on a large scale requires carefully controlled conditions, and can take 4-5 months before mature fruiting bodies are ready to harvest (Iwade & Mizuno, 1997). Therefore, investigating liquid culture cultivation of *A. blazei* has become a focus for bio-prospecting institutions and companies. It has been reported that in some cases, polysaccharides extracted from liquid cultured *A. blazei* mycelium exhibit some of the medicinal effects identified in those extracted from the fruiting body. These properties include antitumour activity (Ito *et al*., 1987, 1997; Mizuno, *et al*., 1999), cytotoxicity against tumour cell lines *in vitro* (Jiang & Gu, 2005), antioxidant activity (Ker *et al*., 2005) and antimutagenic activity (Sun *et al.*, 2006).

Numerous studies pertaining to the production and yield of *Agaricus blazei* exopolysaccharides (those secreted into the medium during liquid culture) have been

reported (Fan *et al*., 2003; Kim *et al.*, 2004; Shu *et al*.,2004, 2007; Zou, *et al*., 2006; Lin & Yang, 2006; Shu & Xu, 2007), however the medicinal properties of *A. blazei* exopolysaccharides have not been extensively examined.

Exopolysaccharides displaying anti-tumour activity against the solid tumour Sarcoma 180 have been extracted from the medium of liquid cultured *Agaricus blazei* (Mizuno, 1995; Fan *et al*., 2003). Immunomodulatory exopolysaccharides have been identified which stimulated the release of tumour necrosis factor-alpha (TNF-α) by the macrophage cell line RAW 246.7, and increased phagocytic function of macrophages respectively (Shu & Xu, 2007; Zhang *et al*., 2007). More recently, Yu *et al*. (2008) identified cytotoxic activity of exopolysaccharides from *A. blazei* against prostate cancer cells.

In order to further examine the degree of bioactivity of *A. blazei* exo-polysaccharides, this study examined the spectrum of *in vitro* cytotoxic activity by screening against cervical cancer and leukemic cell lines. In addition, the potential antiviral effects of exopolysaccharides were examined against simian rotavirus SA11.

8.2 Results and discussion

Exopolysaccharides extracted from 30 day *Agaricus blazei* CMM culture filtrate were used for cytotoxicity and antiviral screening. While methods for the microwave extraction of *A. blazei* polysaccharides have recently been described (Tang *et al.*, 2006; Zhang, *et al*., 2006), ethanol precipitation is commonly used for the precipitation of polysaccharides from aqueous extracts from mushroom fruiting bodies (Mizuno, 1999; Wasser & Weis, 1999b; Reshetnikov *et al.*, 2001).widely in these studies.

8.2.1 The effect of ethanol volume on precipitation of exopolysaccharides from *Agaricus blazei* culture filtrate

Ethanol extraction is the method commonly employed for the extraction of exopolysaccharides from mushroom culture filtrates, however different volumes of ethanol have been used for precipitation. Culture filtrates from various mushroom

species have been extracted with a 1x volume of ethanol (Babitskaya *et al.*, 2000), 2x volumes of ethanol (Mizuno, 1995), and 4x volumes of ethanol (Tang & Zhong, 2002; Yang & Liau, 1998) in order to induce the precipitation of polysaccharides. The volume of ethanol used for the precipitation of polysaccharides will affect the types and yield of polysaccharides extracted from the culture filtrate, and the presence of bioactive species (Sorimachi *et al*., 2001). Therefore, the mass and composition of *Agaricus blazei* exopolysaccharides extracted using increasing ethanol volumes from 1x to 7x was examined.

The extraction of exopolysaccharides was carried out according to a modified method of Mizuno *et al.* (1995), as described in Section 3.3.3. *Agaricus blazei* was grown in CMM for 30 days at 30°C. The biomass was removed by filtration, and a 1 L volume of the culture filtrate was freeze-dried and diluted to a volume of 200 mL with dH_2O. This effected a 1:5 concentration of the culture filtrate. Absolute ethanol was added to 15 mL samples of the concentrated culture filtrate in increasing volumes from 15 mL (1x volume), to 105 mL (7x volumes). The filtrates were kept at 4°C with constant stirring overnight to allow for precipitation of polysaccharides. The polysaccharide solutions were then centrifuged, and the resulting precipitate was washed with acetone and ether, and dissolved in dH_2O. The exopolysaccharide solutions were extensively dialysed against dH_2O and lyophilised. The resulting precipitates constituted the crude exopolysaccharide extracts. The mass of crude exopolysaccharide extract as a function of ethanol volume addition is given in Figure 8.1. The results are presented as the average and standard deviation of triplicate extractions at each ethanol concentration.

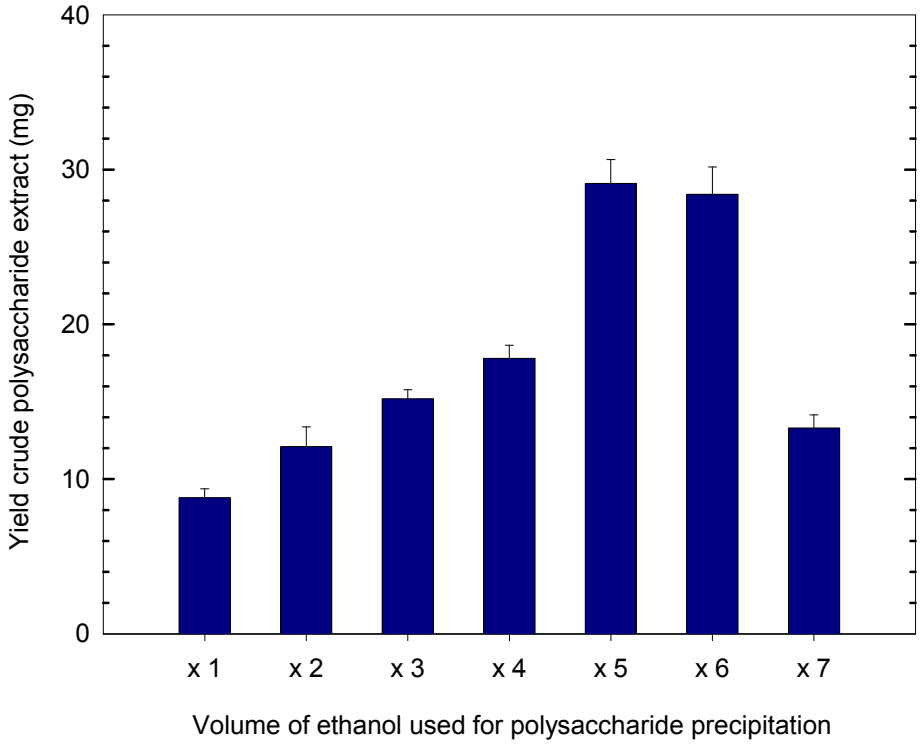


Figure 8.1 *Mass of exopolysaccharides precipitated from* Agaricus blazei *CMM culture filtrate with increasing volumes of ethanol.*

The results in Figure 8.1 indicate that the average mass of crude exopolysaccharide produced increased with increasing ethanol proportion, with 9 mg/15 mL filtrate at 1x ethanol volume, up to a maximum of 29 mg/15mL filtrate using a 5x volume of ethanol. The mass of crude extract was 28 mg/15mL filtrate with the addition of a 6x volume, while increasing the ethanol to a 7x volume, decreased the mass of crude polysaccharide to 13 mg/15mL filtrate. While these results indicated that the greatest mass of crude polysaccharide extract occurred when a 5x volume of ethanol was used for precipitation, it is also important to consider the composition of the crude extracts precipitated with each ethanol volume fraction. To characterise the crude polysaccharide extracts at each ethanol volume, the polysaccharide, protein and glucose content of each was determined (Figure 8.2) using the methods described in sections 3.7.3, 3.7.4 and 3.7.5 respectively.

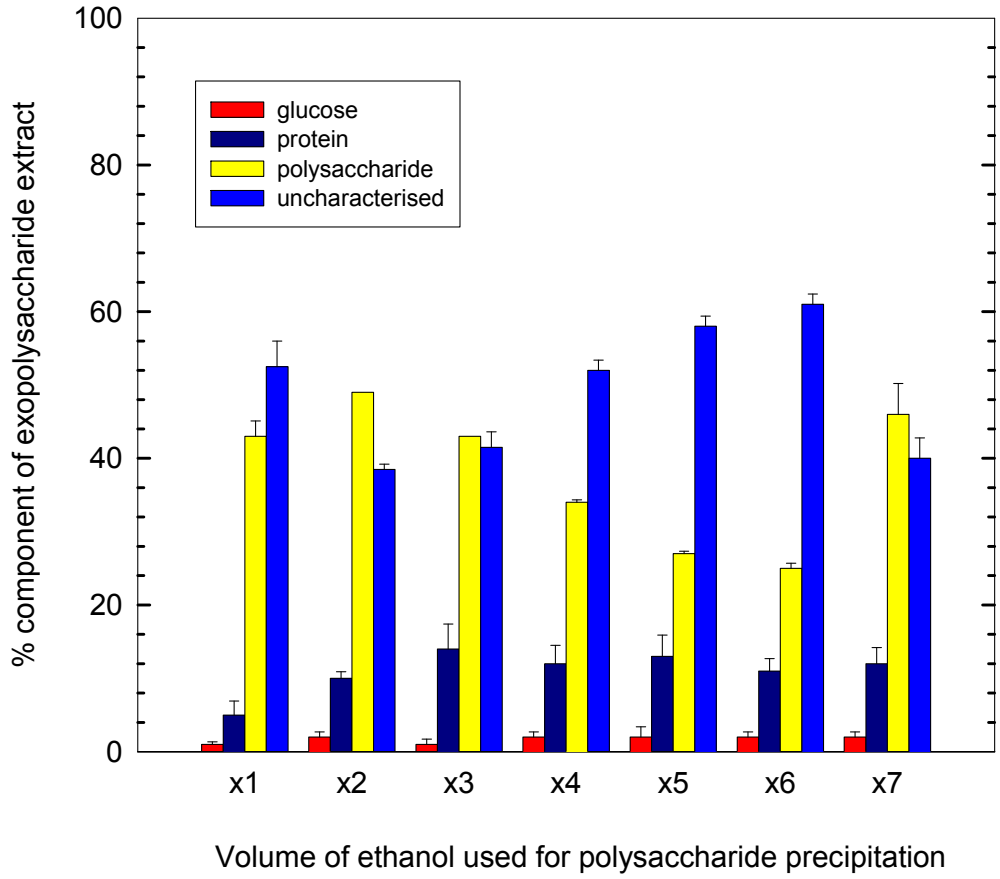


Figure 8.2 *Composition of* Agaricus blazei *exopolysaccharide extracts precipitated with increasing volumes of ethanol.*

The results in Figure 8.2 indicate that there was only a small amount of glucose (less than 4%) detected in each exopolysaccharide extract. This was expected as residual glucose should have been removed during the dialysis procedure. In addition, ethanol is employed for the purpose of eliminating low molecular weight species and isolating higher molecular weight species from the extracts (Reshetnikov *et al.*, 2001). Precipitation with 1x, 2 x, and 3x volumes of ethanol resulted in crude extracts containing between 40-50% polysaccharide. Addition of higher proportions of ethanol resulted in extracts with progressively less polysaccharide content, reducing to 26% when a 6x volume of ethanol was used for precipitation. Interestingly, the extract

obtained with a 7x volume of ethanol did not follow this trend, resulting in a polysaccharide content of 46%.

The protein content of the extract obtained using 1x volume of ethanol was 6%. Increasing this to 2x ethanol resulted in extracts with 10% protein, and there was no appreciable further increase in the percentage protein of the extracts as the volume of ethanol was further increased, with the remaining extract containing between 11-14% protein.

Polysaccharide, protein and glucose accounted for approximately 50% of the mass of the extracts, with the remainder being uncharacterised. The proportion of the uncharacterised mass obtained using 1x, 2x, and 3x volumes of ethanol was found to be 52%, 38% and 42%, respectively. The proportion of the extract that was composed of uncharacterised substances increased as the volume of ethanol used for extraction increased from 4x to 6x ethanol. As previously highlighted, the polysaccharide content gradually decreased as the ethanol volume increased in these extracts, suggesting that while these extracts gave a higher yield of extract (Figure 8.1), they could not be used for screening exopolysaccharide activity due to the high content of compounds. The extract obtained using a 7x volume of ethanol had a very similar profile to that obtained with a 2x volume, however this was not used due to the large volume of ethanol required for preparation of the extracts.

A 2x volume of ethanol for precipitation of polysaccharides was selected for bioactivity screening of *A. blazei* exopolysaccharide due to the fact that it contained the highest proportion of polysaccharide and the lowest proportion of uncharacterised material . While polysaccharides of varied size and structure have been extracted from liquid cultured mycelium and fruiting bodies of *A. blazei* (Kawagishi *et al*., 1989; Mizuno *et al*., 1999; Yuexin *et al*., 2002; Gonzaga *et al*. 2005), no further fractionation or characterisation (molecular weight or structure of the polysaccharide species) of the extracts was carried out. To give a preliminary indication of whether different species or quantities of active product were present in different extracts, the 1x ethanol volume extract was also examined for bioactive properties for comparison .

The polysaccharide extracts obtained using 1x and 2x volumes of ethanol were screened for anti-viral activity against simian rotavirus SA11, and cytotoxicity against the two tumour cell lines, HeLa and Raji.

8.2.2 Screening for anti-viral activity

Agaricus blazei exopolysaccharide extracts precipitated from a 1x volume (designated 1xEPS), and 2x volumes of ethanol (designated 2xEPS), were tested for antiviral activity against rotavirus using the MTT cell viability assay described in Section 3.6.4. Stock exopolysaccharide extract solution (10 mg mL^{-1} in PBS) was diluted to concentrations of 12.5, 25, 50, 100, 200, 400, 600, 800 and 1000 μg mL^{-1} in MEM. The concentration of PBS in the final assay procedure was less than 0.4 % and was not toxic to the cell line used to propagate virus. The cell concentration and virus dilution used in the assay procedure was previously optimised for the cell line investigated (Section 7.2.2.1 and 7.2.2.2 respectively.)

The results of antiviral screening of *A. blazei* CMM culture filtrates 1xEPS and 2xEPS are shown in Figures 8.3 and 8.4, respectively. The results presented are the average of three experiments, from extracts obtained from three separate extractions.

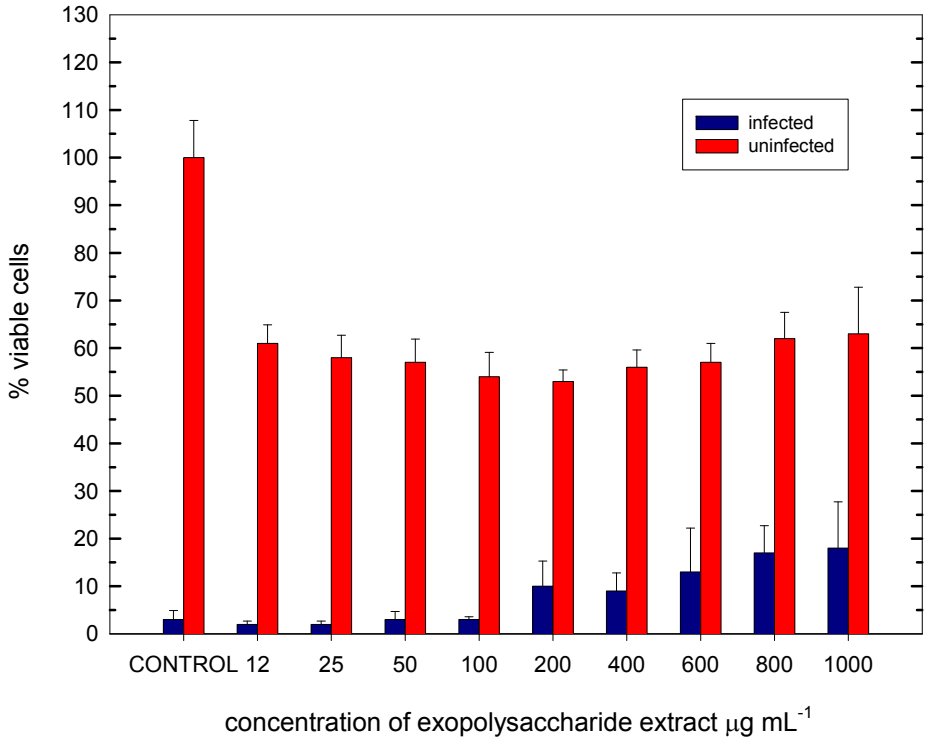


Figure 8.3 *Effect of 1xEPS on rotavirus infection in MA104 cells*

The results in Figure 8.3 show that addition of 1xEPS and SA11 virus to MA104 cells at the same time produces a slight antiviral effect when tested at concentrations of up to 1000 μg mL^{-1}. The cell viability of MA104 cells infected with the virus increased in a dose-dependant manner from 200-1000 μg mL^{-1}, although significant protection of virus infection was not observed. The average maximum cell viability of 18 –10% was not significantly different from the control which had an average cell viability of 4% ($p > 0.001$). A slight cytotoxic effect was observed in uninfected MA104 cells treated with extract, although it was not dose-dependant, with the % viability of cells averaging 54-64% across the extract concentration range tested. Figure 8.4 below, summarises the results of the antiviral assay of 2xEPS against rotavirus in MA104 cells.

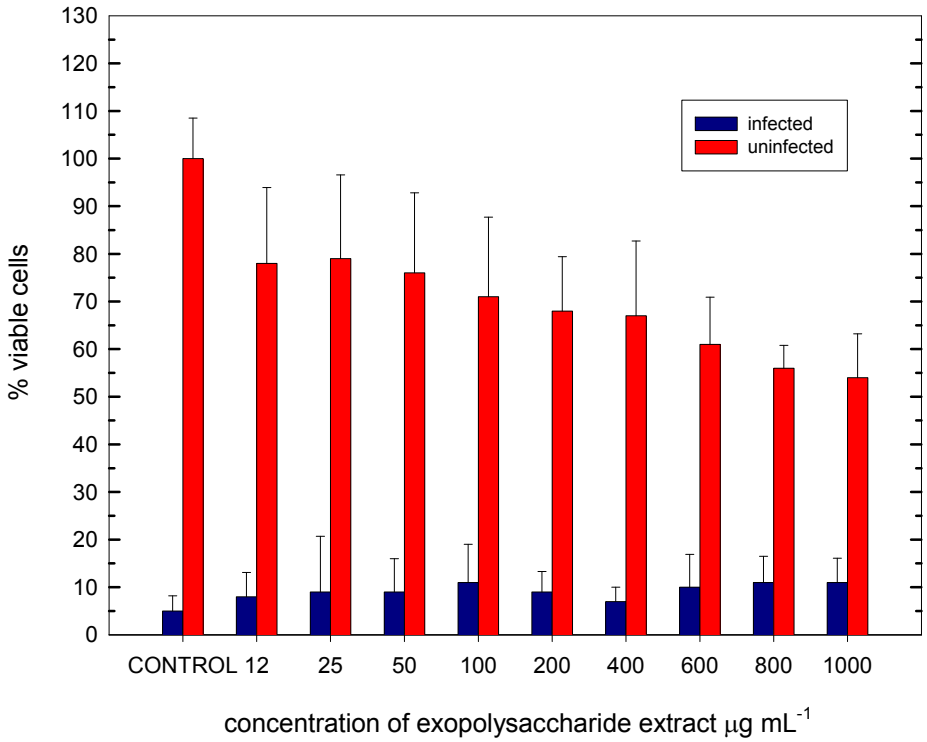


Figure 8.4 *Effect of 2xEPS extract on rotavirus infection in MA104 cells*

The results in Figure 8.4 indicate that addition of 2xEPS in combination with SA11 did not have an appreciable effect on virus infection in MA104 cells, with no significant increase in cell viability as the extract concentration was increased. In addition, a slight dose-dependant cytotoxic effect was observed for 2xEPS in uninfected cells .

Antiviral activity has not previously been reported from *A. blazei* exopolysaccharides from liquid culture filtrate. Antiviral activity of polysaccharides from *A. blazei* fruiting bodies, however, has previously been reported. Faccin *et al.* (2007) found that an aqueous extract (AqE), ethanol extract (EtOHE), and an isolated polysaccharide (PLS) from *A. blazei* demonstrated antiviral activity against poliovirus type 1 *in vitro*. They carried out time-of-addition studies using a plaque assay in HEp-2 cells, and identified a concentration-dependant reduction in the number of plaques up to 50%, 67% and 88% for the AqE, PLS, and EtOHE respectively. In addition, the selectivity indexes defined as the antiviral activity (EC_{50}) divided by cytotoxic activity (CC_{50}) were 5.4, 9.9 and 12.3 respectively, indicating that the antiviral effect observed was not likely to be due to

cell death. The authors hypothesized that the substances acted at the initial stage of the replication of poliovirus as their addition had little effect on reducing viral adsorption, did not show any virucidal effect and were more effective when added during poliovirus infection.

An *in vivo* study in humans by Grinde *et al.* (2006) examined the effect of *A. blazei* fruiting body extract on chronic hepatitis C patients. The authors examined the gene expression in peripheral blood cells of patients, and found that the viral load was slightly reduced after 1 week of *A. blazei* extract treatment, although the decrease was not significant, indicating that the extract, presumed to have a high β-glucan content, did not have an antiviral effect against hepatitis C *in vivo*.

Sorimachi *et al.* (2001) used increasing amounts of ethanol to extract both *A. blazei* fruiting bodies and liquid cultured mycelium, and tested the extracts obtained at a concentration of 100 μg mL^{-1} against western equine encephalitis virus (WEE), herpes simplex virus (HSV) and poliovirus. While extracts obtained using 29 50% ethanol from *A. blazei* fruiting body had a slight effect on poliovirus, they did not show any antiviral effect on WEE or HSV. The extracts obtained from liquid cultured mycelium using a 44% and 50% ethanol volume, however, resulted in complete inhibition of CPE induced by WEE and also had a slight effect on CPE induced by HSV.

The results of the current study indicate that *A. blazei* exo-polysaccharide from culture filtrate obtained with a 1x volume of ethanol (50% ethanol volume), may contain species that inhibit the growth of simian rotavirus *in vitro*, although the effect was not significant. The slight antiviral effect was observed at concentrations 10 times greater than the 100 μg mL^{-1} observed for complete inhibition of virus growth for 50% ethanol *A. blazei* mycelia extracts, as reported by Sorimachi *et al.* (2001). The concentrations of polysaccharide in dried mycelium, however, may be expected to be higher than that of concentrated filtrate broth due to the dilution effect of the higher water content. The bioactive compound content a 50% ethanol extraction of mycelium could not be considered comparable to that of a 50% ethanol extraction from culture filtrate, unless the filtrate was evaporated to dryness, which was not the case in this study. In order to identify if anti-rotaviral polysaccharides indeed exist in the 1xEPS extract, further

fractionation is required to concentrate different species of polysaccharide, followed by re-examining the 1xEPS fractions for both antiviral activity for inhibition of rotavirus infection, and cytotoxic activity in MA104 cells, to determine the selectivity index and consolidate whether true antiviral activity exists.

8.2.3 Screening for cytotoxicity against tumour cell lines

Polysaccharides displaying cytotoxic activity have been identified in various species of mushroom, with a broad spectrum of activity against tumour cell lines *in vitro*. Aqueous extracts containing exopolysaccharides from the culture filtrate of *Ganoderma lucidum*, have been found to be inhibit the growth of human hepatoma carcinoma (Hep3B), human stomach cancer (AGS), and human lung carcinoma (A549) cells at a concentration of 0.8-1 mg mL^{-1} (Chung *et al*., 2001). A polysaccharide-protein complex (PSPC) from the culture broth of *Tricholoma lobayense* has been reported to have cytotoxic activity against HL-60, H3B and PU tumour cell lines at doses of 30 μg mL^{-1} and 60 μg mL^{-1} *in vitro* (Liu *et al*., 1996).

Only recently, Yu *et al*. (2008) reported that the broth fractions from *Agaricus blazei,* (a mixture of both mycelium and culture filtrate), composed of approximately 50% polysaccharide (consisting of 1.2% (1,3)-β-D-glucan), demonstrated *in vitro* cytotoxic activity against both androgen-dependant (LNCaP) and androgen-independent (DU145 and PC3) human prostate cancer cell lines. This is first report of cytotoxic activity in fermentative products from *A. blazei*, and the aims of this study were to further examine the spectrum of cytotoxic activity in *A. blazei* fermentation products by testing exopolysaccharide extracts from culture filtrate against tumour cell lines *in vitro*.

1xEPS and 2xEPS extracts precipitated from *A. blazei* culture medium with a 1x and 2x volumes of ethanol, respectively, were tested for cytotoxic activity against two human tumour cell lines, the human cervical carcinoma cell line, HeLa, and a B-cell lymphoma cell line, Raji. Each stock extract solution (10 mg mL^{-1} in PBS or dH_2O) was diluted to concentrations of 12.5, 25, 50, 100, 200, 400, 600, 800 and 1000 μg mL^{-1} in the appropriate medium for the cell line under investigation. The concentration of PBS or dH_2O in the final assay procedure was not toxic to the cell lines tested. PBS and dH_2O

controls were included in every assay, and an example of the lack of cytotoxicity shown by these solutions is given in Figure 8.5.

Cytotoxic activity was determined using the MTT cell viability assay described in Sections 3.5.5.1 and 3.5.5.2 from HeLa and Raji cells respectively. The results given are the average and standard deviation of three replicate extracts obtained from three separate extractions. Cytotoxic activity exhibited by extracts that reduced the cell viability to at least 50% was quantified by the calculation of the cytotoxic concentration 50 (CC_{50}).

The cytotoxicity of *A. blazei* 1xEPS against HeLa cells is shown in Figure 7.5.

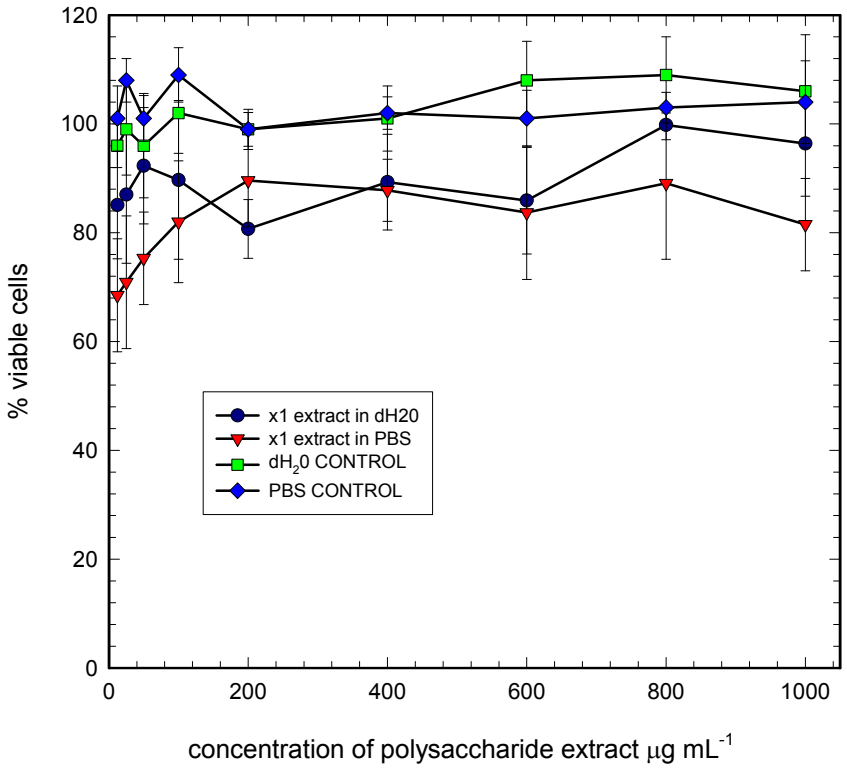


Figure 8.5 *Cytotoxicity against HeLa cells – 1xEPS extract*

The results in Figure 8.5 indicate that the 1xEPS extract precipitated from *A. blazei* CMM culture filtrate does not display cytotoxic activity against HeLa cells. At the highest extract concentration examined (1000 μg mL^{-1}) the viability of the HeLa cells

was 82% and 97% when prepared in dH_2O and PBS, respectively, and not significantly different from the dH_2O and PBS only controls ($p>0.001$).

The cytotoxicity of *A. blazei* 2xEPS against HeLa cells is shown in Figure 8.6.

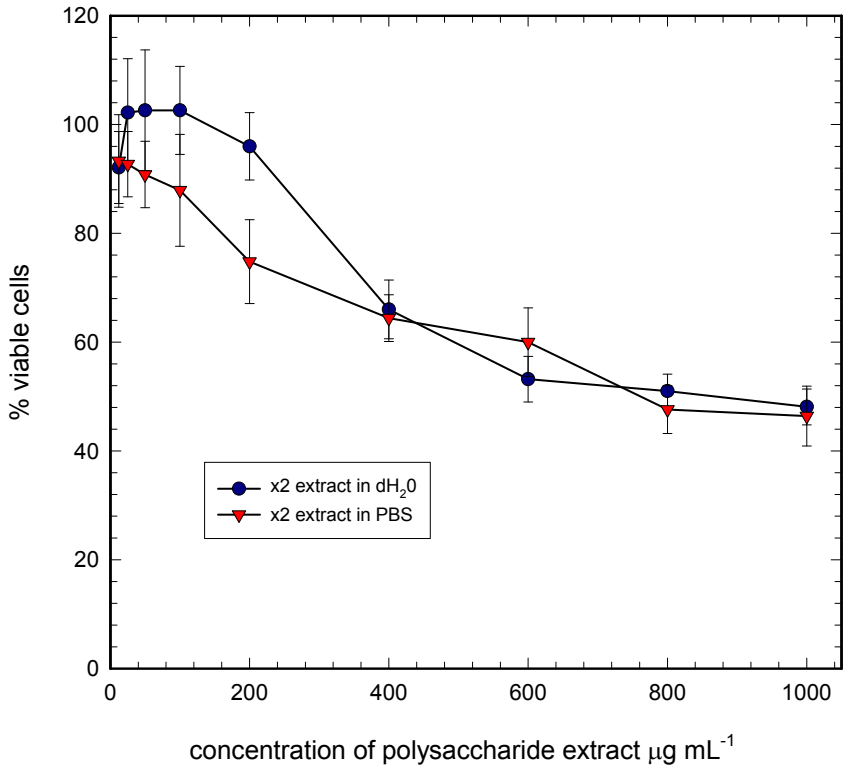


Figure 8.6 *Cytotoxicity against HeLa cells – 2xEPS extract*

In contrast to the 1xEPS, the 2xEPS from *A. blazei* CMM culture filtrate demonstrated cytotoxic activity against HeLa cells in a dose-dependant manner, with a CC_{50} of 603 μg mL^{-1}. The cytotoxic effect appeared to plateau at around 800-1000 μg mL^{-1} and complete inhibition was not observed over the concentration range tested.

Cytotoxicity of 1xEPS and 2xEPS extracts was also examined against the B cell lymphoma cell line, Raji. The cytotoxicity of *A. blazei* 1xEPS extract from CMM culture filtrate against Raji cells is shown in Figures 8.7.

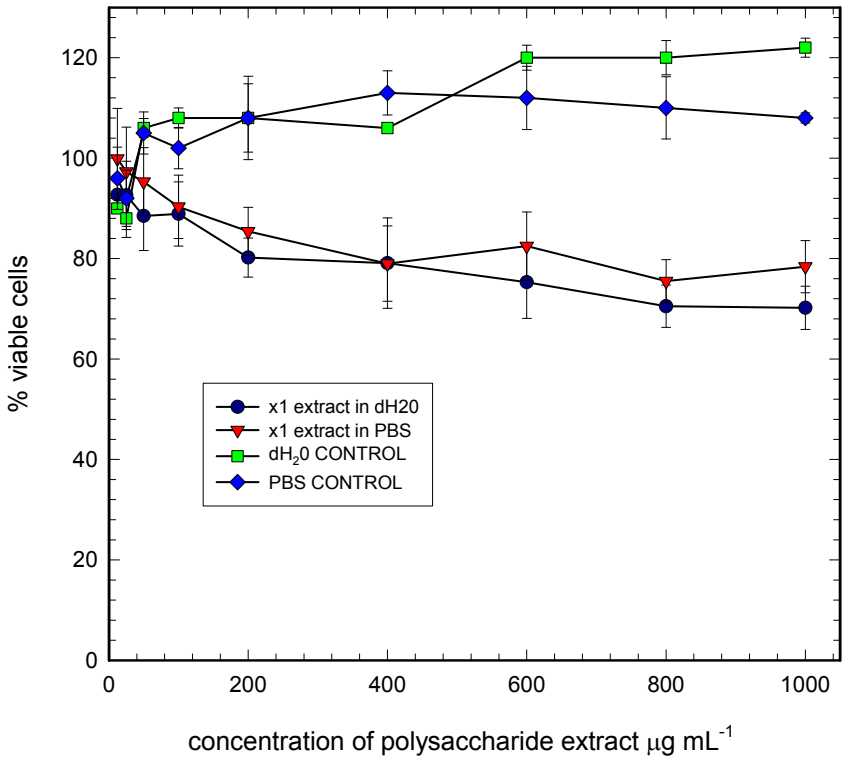


Figure 8.7 *Cytotoxicity against Raji cells – 1xEPS extract*

The results in Figure 8.7 indicate that the 1xEPS extract does not have cytotoxic activity against Raji cells. At 1000 μg mL^{-1}, the viability of the HeLa cells was 70% and 78% when prepared in dH_2O and PBS respectively and not significantly different from the dH_2O and PBS only controls ($p<0.001$).

The cytotoxicity of *A. blazei* 2xEPS against HeLa cells is shown in Figure 8.8.

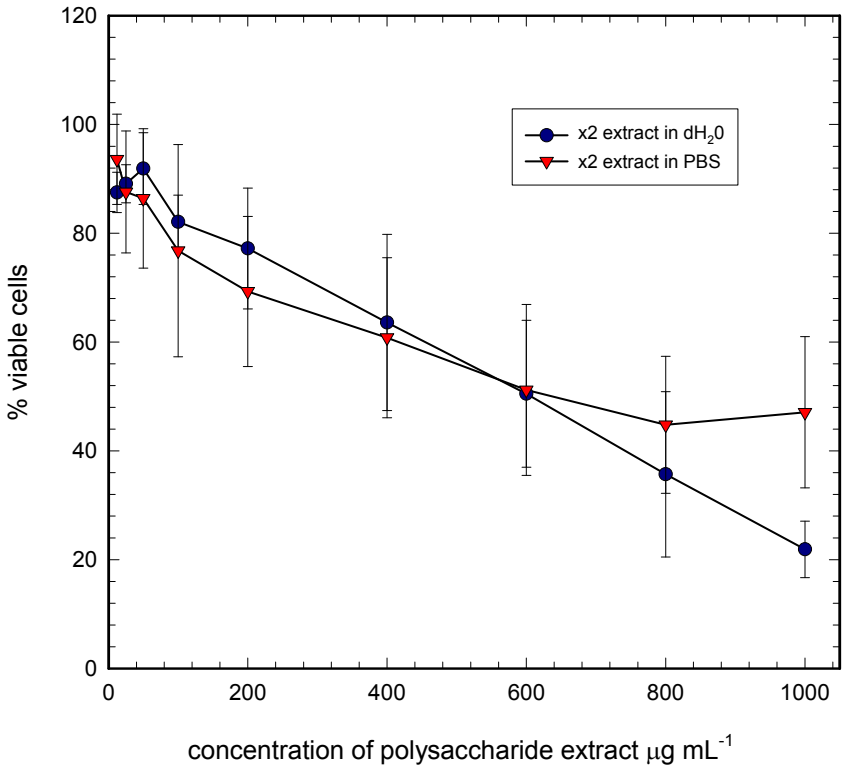


Figure 8.8 *Cytotoxicity against Raji cells – 2xEPS extract*

As was observed for HeLa cell cytotoxicity, the 1xEPS extract did not show a cytotoxic effect, however the 2xEPS extract was active against Raji cells *in vitro*, with a CC_{50} of 600 μg mL^{-1}. The CC_{50} is in the range of cytotoxic activity also observed for *A. blazei* broth against prostate cancer cells *in vitro* (Yu *et al*., 2008) where a dose-dependant inhibitory effect at concentrations of 400 μg mL^{-1} and 800 μg mL^{-1} was observed, where cytotoxicity was the result of induction of apoptosis.

In order to further determine the nature of the polysaccharides in the 2xEPS extract from *Agaricus blazei* responsible for the cytotoxic activity observed against HeLa and Raji cells, the extract was separated into fractions using DEAE-Sepharose and the cytotoxic activity of each fraction was determined against both cell lines.

8.2.4 Fractionation of exopolysaccharide extract

Crude 2xEPS extract from *A. blazei* CMM culture filtrate was fractionated using the anion exchanger DEAE-Sepharose CL-6B as described in Section 3.7.2. Fractions of 2xEPS were eluted from the column via step elution with dH_2O followed by increasing concentrations of NaCl (0.1 M, 0.2 M, 0.5 M and 1 M). The elution of compounds via increasing concentrations of NaCl will separate acidic species of polysaccharides (Reshetnikov *et al*., 2001). Fractions were assayed for polysaccharide content using the phenol-sulphuric acid method described in Section 3.7.3, where the amount of glucose generated indicates the presence of polysaccharide. The chromatogram in Figure 8.9 outlines the amount of polysaccharide obtained with each eluant solution.

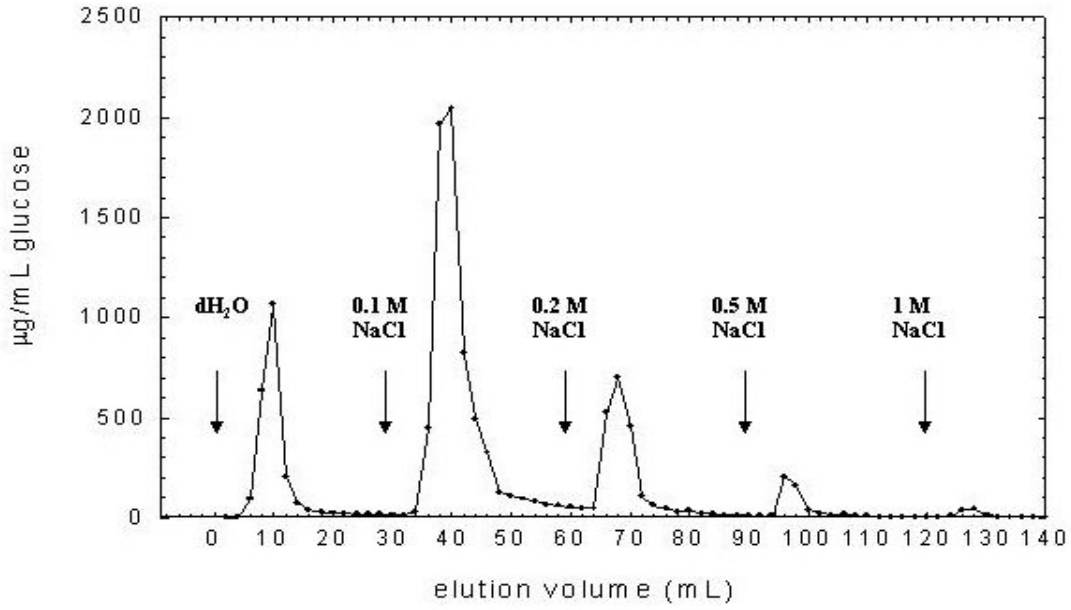


Figure 8.9 *Fractionation of* Agaricus blazei *2xEPS extract on DEAE-Sepharose CL-6B.*

The largest amount of polysaccharide was eluted with 0.1 M NaCl, identified by the largest peak in the chromatogram in Figure 8.9., followed by polysaccharide fractions obtained with dH_2O and 0.2 M NaCl. Only a small fraction of polysaccharide was eluted with 0.5 M NaCl, while the negligible amount eluted with 1 M NaCl was not sufficient for further testing.

Fractions containing polysaccharide for each eluant (dH_2O, 0.1 M NaCl, 0.2 M NaCl, and 0.5 M NaCl) were pooled, extensively dialysed against dH_2O, lyophilised and made up to a concentration of 10 mg mL^{-1} in dH_2O for further screening for cytotoxic activity.

8.2.5 Cytotoxicity testing of 2xEPS fractions

The four 2xEPS extract fractions (Fraction1: dH_2O; Fraction 2: 0.1 M NaCl; Fraction 3: 0.2 M NaCl; Fraction 4: 0.5 M NaCl) were tested individually against HeLa and Raji cells using the method previously described in this chapter (Section 8.2.3).

Each stock extract solution (10 mg mL^{-1} in dH_2O) was diluted to concentrations of 12.5, 25, 50, 100, 200, 400, 600 μg mL^{-1} in the appropriate medium for the cell line under investigation. The cytotoxicity of the four fractions against HeLa cells is shown in Figure 8.10. The results are the average of three replicate extracts for each fraction obtained from three DEAE-Sepharose procedures.

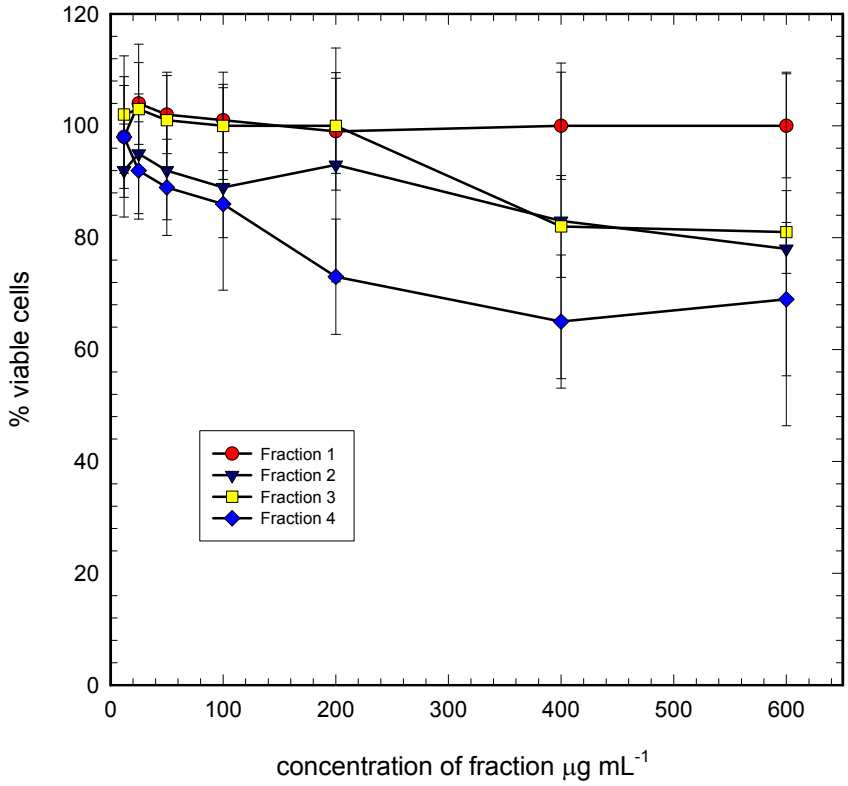


Figure 8.10 *Cytotoxicity of* Agaricus blazei *2xEPS fractions against HeLa cells*

In contrast to the results for the complete 2xEPS extract in Figure 8.6, the results in Figure 8.10 indicate that fractionation leads to a reduction in cytotoxic effect, as the CC_{50} of the extract (603 μg mL^{-1}) was neither replicated by any fraction, nor reduced as may be expected by concentrating different species of polysaccharide from the original extract by fractionation. The average maximum cytotoxic activity was obtained with fraction 4, although the variability between replicate fractions was quite marked for this fraction and the other three fractions tested. This suggests that the cytotoxic activity observed for the complete 2xEPS extract in Figure 8.6 may be due to synergenistic effects of more than one species of polysaccharide.

The results for cytotoxicity testing of the four fractions against Raji cells are summarised in Figure 8.11.

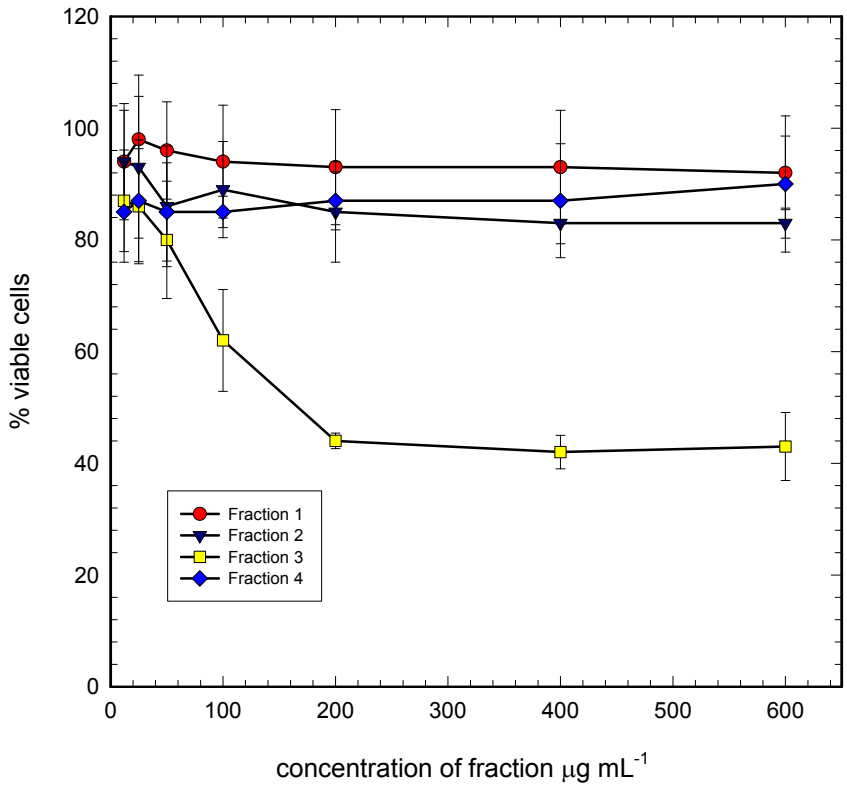


Figure 8.11 *Cytotoxicity of* Agaricus blazei *2xEPS fractions against Raji cells*

The results presented in Figure 8.11 clearly indicate that the cytotoxicity observed in the complete 2xEPS extract (Figure 8.8) was contained in Fraction 3. No cytotoxic activity was observed for the other fractions, while the CC_{50} for Fraction 3 of the extract against Raji cells was 168 μg mL^{-1}. As expected, due to the fact that the active species were concentrated following fractionation, Fraction 3 had more potent cytotoxic activity than the original 2xEPS extract, which had a CC_{50} of 600 μg mL^{-1} against Raji cells.

Previous results regarding the antiviral activity of the exopolysaccharide extracts (Section 8.2.2), indicated that x2EPS extract was not toxic to the non-cancerous monkey kidney cells (MA104 cells) used for antiviral screening. The CC_{50} for the x2EPS against MA104 cells was >1000 μg mL^{-1}, with 54% of cells still viable at 1000 μg mL^{-1} (Figure 8.4), indicating that the cytotoxic activity of the extract against Raji cells showed some specificity for tumour cells, with a selectivity index of >5.9 (CC_{50} Raji lymphoma cells/CC_{50} normal MA104 cells).

While cytotoxic activity was observed for *A. blazei* culture broth fraction against prostate cancer cell lines (Yu *et al*. 2008), this is the first report of cytotoxic activity against of culture filtrate extracts against a lymphoma cell line *in vitro*. Due to time constraints, no further attempt at the isolation of the active species in the 2xEPS extract Fraction 3 could be undertaken in this study, although should be pursued. Further fractionation using gel filtration to separate molecules on the basis of molecular weight, and identification of the structure of the active compound in the extract, would identify if the compound was similar to cytotoxic polysaccharides from *A. blazei* fruiting bodies that have been reported to be active against human leukemic and lymphoma cell lines (Shen *et al*., 2001; Chu & Chen 2006; Chen *et al*., 2007) and, in particular, of similar structure to reported cytotoxic (1→6)-β-D-glucan-protein complexes (Ito & Itoh, 2000). Other extracts from *A. blazei* fruiting bodies have been found to be active against leukemic cell lines, although their structure has not been elucidated (Jin *et al*., 2007; Cai *et al*., 2008; Kim *et al.,* 2009).

It would also be interesting to determine whether the basis of the cytotoxic effect observed for the 2xEPS extract against Raji cells was via the induction of apoptosis. The majority of research regarding activity of *A. blazei* extracts and polysaccharides

from fruiting bodies, liquid cultured mycelium and culture broth against a range of tumour cell lines including human liver carcinoma cells (Jiang & Gu, 2005; Liu *et al*. 2006), human prostate cancer cells (Yu *et al*. 2008), and leukemic cell lines (Shen *et al*. 2001; Chu & Chen, 2006; Chen *et al*. 2007; Jin *et al*. 2007; Cai *et al*. 2008; Kim *et al*. 2009) suggests induction apoptosis as the mechanism of cytotoxicity.

Overall, the results reported in this chapter indicate that ethanol an be used to isolate cytotoxic compounds from *A. blazei* culture filtrates. This is a well regarded method and has been used extensively for the last 30-40 years for extraction of antitumour polysaccharides from aqueous extracts of mushroom fruiting bodies and mycelium (Chihara *et al*., 1970; Fujii *et al*., 1978; Kawagishi *et al*., 1989; Wang *et al*., 1996; Mizuno *et al*., 1999; Chen *et al*., 2006; Gonzaga *et al*., 2009). It has also been used for extracting antitumour polysaccharides from liquid culture fluid (Mizuno, 1995), although this is the first report of the isolation of cytotoxic substances using this method. The use of different volumes of ethanol for precipitation as examined in this study highlighted that different masses of crude polysaccharide extract were obtained with different composition, and all extracts crude extracts from *A. blazei* culture filtrate had a protein component as well as a polysaccharide component. Extracts with the highest proportion of polysaccharide and protein were extracted with smaller volumes of ethanol (1x to 3x volumes) and increasing the ethanol concentration tended to yield extracts with a high percentage of uncharacterised substances. Extracts obtained with a 1x and 2 x volumes of ethanol, designated 1xEPS and 2xEPS, respectively, were further examined for antiviral activity against rotavirus and cytotoxicity against a human cervical cancer cell line (HeLa) and B-cell lymphoma cell line (Raji).

The results from antiviral screening against rotavirus indicated that while 2xEPS extract did not show antiviral activity, the 1xEPS extract showed a slight antiviral effect although testing at a concentration higher than 600 μg mL^{-1} or re-testing following fractionation of the extract will clarify whether a true antiviral effect exists.

In contrast, in the cytotoxicity testing, the 1xEPS extract showed no cytotoxic effect against HeLa and Raji cells, while the 2xEPS extract showed activity against both cell lines. The CC_{50} values for the 2xEPS extract against HeLa and Raji cells were 603 μg

mL^{-1} and 600 μg mL^{-1}, respectively. Four fractions of the 2xEPS extract, obtained using separation by DEAE-Sepharose, were tested individually against both cell lines. The cytotoxic effect against HeLa cells was lost following fractionation and was not observed for any individual fractions suggesting the effect seen in the original extract was due to a synergenistic effect of multiple compounds. Against Raji cells, it was clear that the cytotoxic activity was present in the fraction eluted with 0.2 M NaCl (Fraction 3), as the CC_{50} for this fraction was 168 μg mL^{-1}, while no cytotoxic activity was evident in the other three fractions. This activity was also specific for tumour cells with a specificity index for activity against tumour cells compared to that of MA104 cells of >5.9.

Further fractionation of this extract via gel filtration or further chemical extraction would be required to identify the active species present in the fraction. Given that the mode of action for the majority of cytotoxic polysaccharides from *A. blazei* fruiting bodies and mycelium has been shown to be through the induction of apoptosis (Shen *et al*. 2001; Jiang & Gu, 2005; Liu *et al*., 2006; Chu & Chen, 2006; Yu *et al*., 2008; Kim *et al*., 2009), it should be determined whether the effect seen in Raji cells in this study is also due to this mechanism.

Chapter Nine

Conclusion

9.1 Growth and storage of *Agaricus blazei* on solid media

The ATCC recommended yeast malt agar (YMA) for the growth of the particular *A. blazei* strain utilised in this study. While it proved to be one of the more suitable growth media, five other commercially available agar media we found to also support the growth of *A. blazei* mycelium, indicating that this fungus is not particularly fastidious with regard to the nutritional requirements for growth on solid agar. The optimal temperature and pH ranges for growth of *A. blazei* mycelium were found to be 28-29°C and pH 5-6, respectively (Section 4.2.1.1). These growth conditions were found to be different from the other fungal species examined as part of this study, i.e. *Agaricus bisporus*, *Lentinus edodes*, and *Pycnoporus coccineus*. The optimum temperature for growth of *A. blazei* identified in this study was slightly higher than that previously reported by Eguchi *et al*. (1994) (26°C) and of that advised by the ATCC (24°C). This current research highlighted the importance of performing a thorough investigation of the physiological requirements of a particular fungal species, given that it is not possible to have one culture medium or a single set of conditions suitable for maximal growth of different basidiomycetes (Bilay *et al*., 2000).

Effective short-term and long-term storage of *A. blazei* mycelium was achieved by freezing healthy mycelial plugs in 10% (v/v) glycerol at -80°C (Sections 4.2.2.2 and 4.2.2.3). This method of storage was used for the maintenance of all working cultures of *A. blazei* throughout the course of this research.

9.2 Biomass and exo-polysaccharide production by *Agaricus blazei* in liquid culture

The production of biomass by *A. blazei* grown in YMB was examined as a function of temperature and pH (Section 5.2.1.1). The optimum temperature for biomass production was found to be 30°C. While no single optimum growth pH was identified, the highest

level of biomass was produced over the pH range 4-8, which is consistent with the data reported by Shu *et al.* (2004), where a pH range of 4-7 was reported for the growth of *A. blazei* in liquid culture. Other research groups have reported an optimal pH within the range of 5.0-6.0 for biomass production (Zou *et al*., 2002; Kim *et al*., 2004; Fan *et al*., 2003; Lin & Yang, 2006; Hamedi *et al*., 2007).

The production of biomass by *A. blazei* over a 30 day period was also measured using YMB and PDB growth media (Section 5.2.1.2). While the characteristic fungal growth phases were identified (a lag phase followed by exponential growth or logarithmic phase growth, followed by a plateau or senescence of growth), the duration of these phases varied depending on the culture media formulation used.

Exo-polysaccharide (EPS) production by *A. blazei* at different growth temperatures and pH levels (Section 5.2.2.1) was found to be directly proportional to the amount of biomass produced. The effect of different carbon sources for mycelial growth and production of EPS, however, indicated that the amount of EPS produced was not proportional to the amount of biomass produced (Section 5.2.2.2). Whilst glucose as a carbon source yielded the most biomass, the sucrose medium resulted in a greater level of EPS production, an observation consistent with the work reported by Fan *et al*. (2003) and Zou *et al*. (2006). EPS produced by *A. blazei* under different conditions were all found to be mannan-protein complexes consisting of 2-3 % protein, except for those produced with sucrose and mannose as a carbon source with 0.7% and 5.3% protein respectively (Section5.2.2.3).

9.3 Bioactivity of *Agaricus blazei* solvent extracts

Agaricus blazei mycelium samples grown in six different growth media were subjected to extraction using four solvents of increasing polarity - hexane, dichloromethane, ethyl acetate and methanol - using a Soxhlet apparatus. The yields, colour and consistency of the extracts obtained from each mycelial sample were examined and compared (Section 6.2.1), and then examined for antibacterial activity and cytotoxic activity.

9.3.1 Antibacterial activity

Antibacterial activity of the solvent extracts was determined using the disc-diffusion assay against a range of Gram positive and Gram negative bacteria (Section 6.2.2). Interestingly, all extracts showed antibacterial activity against the Gram negative bacterium *Branhamella catarrhalis*. No antibacterial activity was observed against any of the other Gram-negative bacteria examined.

Regarding the activity observed against Gram positive bacteria, all dichloromethane and ethyl acetate extracts were found to be active against *Bacillus cereus*. Activity against *Staphylococcus aureus* was observed in dichloromethane extracts from mycelium grown in all media examined, except sucrose media. The greatest activity against this bacterium, however, was observed in the ethyl acetate extract from PMP media. Dichloromethane and ethyl acetate extracts from mycelium grown in YMB, CMM, PMP and GLUC media all showed inhibition against *Streptococcus pyogenes*. None of the hexane extracts, and only one methanol extract showed antibacterial activity against the Gram positive bacteria examined.

Further investigation of the particular solvent extracts exhibiting antibacterial activity by activity-guided fractionation would be required in order to characterise and identify the active principles. While all extracts were active against *Branhamella catarrhalis*, of particular interest are the dichloromethane and ethyl acetate extracts, which were the most active against this bacterium, and also showed the broadest spectrum of antibacterial activity. Thorough isolation and characterisation of the active compounds, and an assessment of their potency, would allow an assessment of their suitability for use in antibacterial therapy applications.

Previous studies have demonstrated that *A. blazei* fruiting body extracts exhibit antibacterial activity (Osaki *et al*., 1994; Ye & Lin, 2001; Bernardshaw *et al*., 2005), however this study is the first account of antibacterial activity being obtained from liquid-cultured *A. blazei* mycelium extracts.

9.3.2 Cytotoxic activity

The solvent extracts tested for antibacterial activity were also screened for cytotoxic activity against tumour cell lines (Section 6.2.3). Two tumour cell lines were employed for the screening assays, the human cervical cancer cell line HeLa, and the B-cell lymphoma cell line Raji (Sections 6.2.3.1 and 6.2.3.2 respectively).

At the maximum concentration tested (200 μg mL^{-1}), the majority of extracts were not cytotoxic to HeLa cells, although the more polar extracts (hexane and dichloromethane), obtained from MEB, GLUCOSE and SUCROSE media mycelium displayed some activity, with a CC_{50}s between 60-160 μg mL^{-1} being observed. Raji cells were found to be more susceptible to *A. blazei* solvent extracts, with the hexane and dichloromethane extracts giving CC_{50} values between 29 98 μg mL^{-1}, depending on the media used for cultivation of the mycelium. Extracts from methanol, the most polar solvent employed in this study, appear to have no activity and did not show any appreciable cytotoxic activity against the two cell lines tested.

Given that a previous report of cytotoxic steroids isolated from *A. blazei* fruiting bodies showed complete inhibition of HeLa cells at 8-63 μg mL^{-1} (Kawagishi *et al*., 1988), and CC_{50} values of 2-4 μg mL^{-1} have been reported for isolated steroids from *A. blazei* liquid cultured mycelia against human hepatoma cells (Su *et al*. 2008), the degree of cytotoxicity observed for crude extracts against Raji cells in this study warrants further investigation. Isolated compounds would be expected to show increased cytotoxic activity compared to that of the crude extract, therefore the potential of any specific compounds as candidates for future therapy against cancer could be elucidated. It would also be important to characterise the selectivity of the cytotoxic activity by carrying out cytotoxicity assays on non-cancerous cell lines.

This study has expanded the knowledge of the spectrum of cytotoxic activity of the extracts present in liquid cultured mycelia of *A. blazei*. In addition to cytotoxic activity against hepatoma cells *in vitro* (Su *et al*., 2008), this study has shown that solvent extracts from *A. blazei* liquid-cultured mycelia also have cytotoxic activity against B cell lymphoma cells and cervical cancer cells.

9.4 Bioactivity of *Agaricus blazei* protein extracts

Protein extracts from *Agaricus blazei* do not appear to have been previously examined. In the course of this study, the yield and distribution of proteins of different molecular weights were investigated and compared for mycelium grown in different culture media (Section 7.2.1). The yield and distribution of protein species differed depending on the growth media used for propagation of the mycelium, with the extract from complete mushroom medium (CMM) media displaying the highest yield and diversity of protein species of any other the media investigated in this study. This extract, which was composed of 67% protein, was selected for screening for antiviral activity against simian rotavirus SA11 (Section 7.2.2), and for its cytotoxicity against HeLa and Raji tumour cell lines (Section 7.2.3).

The protein extract from *A. blazei* mycelium grown in CMM media appeared to have an antiviral effect on simian rotavirus SA11 with an EC_{50} of 145 μg mL^{-1}, although the mechanism of action for the extract could not be elucidated. It was hypothesized that the extract may contain a trypsin inhibitor that was causing the antiviral effect reported in Section 7.2.2.3, although further work would be required to determine if this is the case.

The CMM protein extract exhibited cytotoxic activity against the leukemic cell line, Raji, with CC_{50} values for the extract dissolved in PBS and dH_2O of 100 μg mL^{-1} and 300 μg mL^{-1}, respectively (Section 7.2.3). This is the first report of a cytotoxic protein extract from *A. blazei*. No marked cytotoxic effect was observed against the cervical cancer cell line HeLa. Given that proteins from other species of mushroom have been investigated and reported to display bioactive properties such as antifungal activity (Lee *et al.*, 1999; Lam & Ng, 2001a; Chu *et al*., 2005; Wang & Ng, 2006a), and immunomodulation (Kino *et al*., 1989; Ko *et al*., 1995; Hsu *et al*., 1997), it would be beneficial to examine proteins isolated from *A. blazei* more extensively in order to identify other potential medicinal properties, such as those mentioned above, in addition to potential antibacterial and antitumour activity. It would also be interesting to determine whether boiling of the protein extracts results in a loss of activity.

9.5 Bioactivity of *Agaricus blazei* exopolysaccharide extracts

The range of medicinal properties exhibited by *A. blazei* exopolysaccharides has not been extensively examined, and previous reports have been limited to their antitumour properties (Mizuno, 1995; Fan *et al*., 2003). Recently, Yu *et al*. (2008), reported that the broth fractions obtained from *A. blazei,* (a mixture of both mycelium and culture filtrate) demonstrated *in vitro* cytotoxic activity against human prostate cancer cell lines. The aim of this study was to initially examine the ethanol extraction of exopolysaccharides from liquid culture filtrate, and explore other bioactive properties such as cytotoxicity and antiviral activity.

Ethanol was found to effectively extract polysaccharides from *A. blazei* CMM culture filtrate, and it was found that different volumes of ethanol to precipitate the polysaccharides yielded different masses of extract of different composition (Section 8.2.1). It was found that using smaller volumes of ethanol to precipitate the polysaccharides yielded extracts containing a higher proportion of polysaccharides, however larger volumes resulted in extracts with higher yields but a higher amount uncharacterised substances.

Extracts obtained with a 1x and 2 x volumes of ethanol, 1xEPS and 2xEPS respectively, were selected for screening activity against rotavirus and for cytotoxicity against the human cervical cancer cell line, HeLa, and the B-cell lymphoma cell line, Raji.

The results from antiviral screening against rotavirus indicated that the 1xEPS extract showed very minimal antiviral effect up to 1000 μg mL^{-1} while no antiviral activity against rotavirus was evident for the 2xEPS extract (Section 8.2.2).

Conversely, the 1xEPS extract showed no cytotoxic effect against HeLa and Raji cells, while the 2xEPS extract showed activity against both cell lines (Section 8.2.3). The CC_{50} values for the 2xEPS crude extract against HeLa and Raji cells were 603 μg mL^{-1} and 600 μg mL^{-1}, respectively.

Four fractions of the 2xEPS extract were obtained using DEAE-Sepaharose by elution with increasing amounts of NaCl (Section 8.2.4) and re-tested individually against both cell lines (Section 8.2.5). The cytotoxic effect against HeLa cells was lost following fractionation and was not observed for any of the individual fractions, suggesting the effect seen in the original extract was due to a synergenistic effect of multiple species of compounds. It was clear that the cytotoxic activity against Raji cells was present in the fraction eluted with 0.2 M NaCl (Fraction 3), as the CC_{50} for this fraction was 168 μg mL^{-1}, while no cytotoxic activity was evident in the other three fractions. The cytotoxic effect of the 2xEPS extract was shown to be specific to tumour cells, as no cytotoxicity effect was observed against MA104 cells (CC_{50} >1000 μg mL^{-1}). This is the first report of *in vitro* cytotoxic activity of *A. blazei* culture filtrate extracts. Further fractionation of this extract *via* gel filtration or further chemical extraction would be required in order to determine the active species present in the fraction.

Though not undertaken as part of this study, the next step for this course of research would involve further activity guided fractionation for the purification and identification of the active components in the various extracts.

Further separation of solvent extracts shown to have antibacterial and cytotoxic activity, could be achieved using methods such as thin layer chromatography, silica gel chromatography or ion-exchange chromatography. Nuclear magnetic resonance spectroscopy (NMR) is commonly used for the elucidation of chemical structures, and would be a useful tool for identification the active species in the solvent extracts.

In order to characterise the species present in protein and polysaccharide extracts responsible for the cytotoxic activity observed, numerous methods are available for the separation/purification, determination of molecular weight, and composition and of the active species. The most appropriate method to employ will depend on the nature of the extracts, and may include gel filtration (size-exclusion) chromatography, ion-exchange chromatography, affinity chromatography, high-performance liquid chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy.

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