Use of Stimulatory Agents to Enhance the Production of Bioactive Mushroom Exopolysaccharide by Submerged Fermentation: A Mechanistic Study

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Abstract

This study aimed at comparing the effectiveness in the use of stimulatory agents with different chemical structures for enhancing the production of mycelial biomass and exopolysaccharide (EPS) by submerged fermentation of an edible mushroom *Pleurotus tuber-regium* (PTR). The chemical characteristics and antitumor activity of the EPS produced with and without the addition of the most effective stimulatory agent (Tween 80 which is a permitted food additive) were also compared. The underlying mechanisms by which Tween 80 could exert its effect on the mushroom mycelium were investigated by using chemical methods and microscopic techniques as well as proteomic analysis.

The effects of different kinds of stimulatory agents including fatty acids, surfactants and organic solvents were compared. The optimum results were achieved when 3.0 g/L Tween 80 was added to the fermentation broth on the 5^{th} day of the fermentation, to give a maximum increase of 51.3 and 41.8% ($p < 0.05$) in the yield of mycelial biomass and EPS production, respectively.

The EPS, a highly branched glucomannan produced by the addition of Tween 80 in the fermentation broth of PTR mycelium had similar carbohydrate and protein content, monosaccharide composition and glycosidic linkages except by having a significantly lower molecular weight when compared to those of the control. Both EPS, with and without addition of Tween 80, could significantly inhibit ($p < 0.05$) the *in vitro* growth of a chronic myelogenous leukemia cells K562 in a dose dependent manner, with an estimated IC_{50} value of 43.7 and 47.6 μg/mL, respectively.

The underlying mechanisms by which Tween 80 could increase the mycelial growth and EPS production in PTR were investigated by three novel approaches including changes in the nutrient uptake by mycelium, the morphology of mycelial pellets, and the fatty acid composition in the mycelial cell membrane. Firstly, the addition of Tween 80 significantly increased the glucose consumption rate by the mycelium, implying that the efficiency of nutrient uptake from the fermentation

broth was enhanced. Secondly the addition of Tween 80 could extend the growth period of the mycelium possibly by maintaining the intact structure of the mycelial pellets and preventing its disintegration caused by shear stress in the fermentation system. Thirdly, the addition of Tween 80 could increase the incorporation of oleic acid which was a constituent of Tween 80 itself into the mycelial cell membrane of PTR, altering its fatty acid composition and increase the cell membrane permeability. The first two results explained the enhancement in the mycelial growth and EPS production while the last one was related to the extracellular transport of EPS to the fermentation broth.

By use of one- and two-dimensional gels in proteomic analysis, some functional mycelial proteins that were differentially expressed by the addition of Tween 80 were identified. The up-regulation of heat shock proteins might help to maintain cellular viability under environmental stress. A down-regulation of YALI0E34793p and an up-regulation of ATP citrate lyase isoform 2 might suppress the activity of TCA cycle and subsequently stimulated the EPS production. Up-regulation of fatty acid synthase alpha subunit FasA might promote the synthesis of long-chain fatty acids and their incorporation into the mycelial cell membranes. Up-regulation of mitogen-activated protein kinase might facilitate the signal transduction in these processes.

All these results advance our understanding on how stimulatory agents can be used to increase the production of useful metabolites by submerged fermentation of mushroom mycelium and indicate its potential biotechnological applications.

摘要

本論文對比研究了具有不同化學結構的刺激劑對液體深層發酵可食用虎奶菇菌絲體生產胞外多糖的影響，并比較了添加以及不添加效果最好的刺激劑（吐溫 80，一種被許可的食物添加劑）的情況下，胞外多糖的化學結構以及抗腫瘤活性是否發生變化。最後，利用一些化學分析，顯微鏡技術和蛋白質組分析的方法，深入研究吐溫 80 對虎奶菇菌絲體生產胞外多糖的作用機理。

本實驗對比了不同刺激劑包括脂肪酸，表面活性劑和有機溶劑的作用，發現當發酵進行到第五天的時候向發酵液中添加 3.0 g/L 吐溫 80，能獲得最優結果，菌絲體和胞外多糖的產量分別提高 51.3% 和 41.8%。

在發酵液中添加吐溫 80 后產生的虎奶菇菌絲體胞外多糖是具有高度分支結構的葡甘露聚糖，與不添加吐溫 80 產生的胞外多糖相比，具有相似的碳水化合物和蛋白質含量，單糖組成以及糖苷鍵鏈接，不同的是胞外多糖的分子量有著顯著的降低。添加或者不添加吐溫 80 得到的胞外多糖對慢性髓性白血病 K562 細胞的體外增殖都有顯著的抑製作用，而且作用呈劑量依賴性，半抑制濃度分別為 43.7 和 47.6 μg/mL。

本論文分別從菌絲體的營養吸收，其顆粒形態，以及細胞膜脂肪酸含量的變化這三個方面研究了吐溫 80 對菌絲體生長以及胞外多糖的生產的作用機制。首先，研究發現隨著吐溫 80 的添加，發酵液中葡萄糖的消耗速率顯著加快，表明吐溫 80 能提高菌絲體對營養物質的吸收效率。其次，添加吐溫 80 能協助維持菌絲體顆粒的結構完整性，抵制由發酵系統的剪切應力引起的解體，從而延長菌絲體的生長期。最後，吐溫 80 的組分之一油酸能被結合到菌絲體細胞膜上，從而能改變細胞膜的脂肪酸組成，提高膜通透性。前兩個結果解釋了菌絲體生長以及胞外多糖產量提高的原因，而后一個結果則跟胞外多糖向發酵液的胞外運輸過程相關聯。

通過一維和二維電泳進行的蛋白質組學分析，本論文鑒定了一些由於添加吐溫 80 而引起的表達水平發生顯著變化的相關蛋白。熱休克蛋白的表達上調能夠協助維持處於不利環境應力下的菌絲體細胞活力。YALI0E34793p 的表達下調連同 ATP 檸檬酸裂合酶的表達上調能夠抑制 TCA 循環的活力，進而促進胞外多糖的生產。脂肪酸合成酶的表達上調能夠促進長鏈脂肪酸的合成以及其結合到細胞膜上的過程。丝裂原活化蛋白激酶的表達上調有助於調節這些過程中的信號傳導。

本研究增進了對如何在液體深層發酵過程中利用添加刺激劑來提高蘑菇菌絲體生產有用代謝產物的理解，而且指明了該方法在生物技術中的應用前景。

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Abbreviations

1D	One-dimensional
2D	Two-dimensional
5-DTAF	5-(4,6-dichlorotriazinyl)aminofluorescein
ABC	ATP-binding cassette
ACN	Acetonitrile
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CLSM	Confocal laser scanning microscopy
CoA	Coenzyme A
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EPS	Exopolysaccharide
EI-MS	Electron impact-mass spectrometry
ESI	Electrospray ionization
FA	Formic acid
FAME	Fatty acid methyl ester
FTICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
HSP	Heat shock protein
IAA	Iodoacetamide
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
MALDI	Matrix-assisted laser desorption ionization
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Mw	Molecular weight
m/z	Mass-to-charge ratios
NCBI	National Center for Biotechnology Information

PDA	Potato dextrose agar
pI	Isoelectric point
PMAA	Partially methylated alditol acetate
PTR	*Pleurotus tuber-regium*
RI	Refractive index
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SmF	Submerged fermentation
SSF	Solid-state fermentation
TCA cycle	Tricarboxylic acid cycle
TEM	Transmission electron microscope
TFA	Trifluoroacetic acid
TOF	Time-of-flight
Triton X-100	Polyoxyethylene octyl phenyl ether
Tween 20	Polyoxyethylene sorbitan monolaurate
Tween80	Polyoxyethylene sorbitan monooleate

1 Introduction

1.1 Mushroom

1.1.1 Mushroom life cycle

Traditionally, mushroom has been defined as a fleshy, aerial umbrella-shaped, fruiting body of macro-fungi, which has been consumed both in the East and the West for over two thousand years due to the pleasant flavor and texture (Miles and Chang, 1997; Wasser, 1997). In the literature, the word "mushroom" is not a botanically term or a taxonomic group but is most commonly defined as the macro-fungus with a distinctive fruiting body that is large enough to be seen by the naked eye and to be picked up by hand (Chang and Miles, 1992). “Fruiting body”, refers to the spore-producing organ (basidiocarps and ascocarps) of a fungus, which is formed by the mycelium and consists of a stipe (stem or stalk) crowned by the pileus (cap). While the majority of the macro-fungi belong to the class of *Basidiomycetes*, there are a few others from the class of *Ascomycetes* (Chang and Miles, 1989; Miles and Chang, 1997).

The mushroom life cycle is shown in Figure 1.1. It all starts when the spores are released from the gills in the fruiting body. Millions of spores are released into the surrounding environment and these spores are dispersed by various methods depending on the kind of mushroom. When the conditions are right, the spores germinate to form tiny threads called hyphae. In order for the hyphae to develop and eventually produce a mushroom, it has to find other hyphae that are compatible. When two compatible hyphae meet, they fuse together to form a network or threads called a mycelium. This mycelium eventually forms what is known as a hyphal knot which grows and develops into a pinhead before developing into a mushroom fruiting body to restart the cycle again.

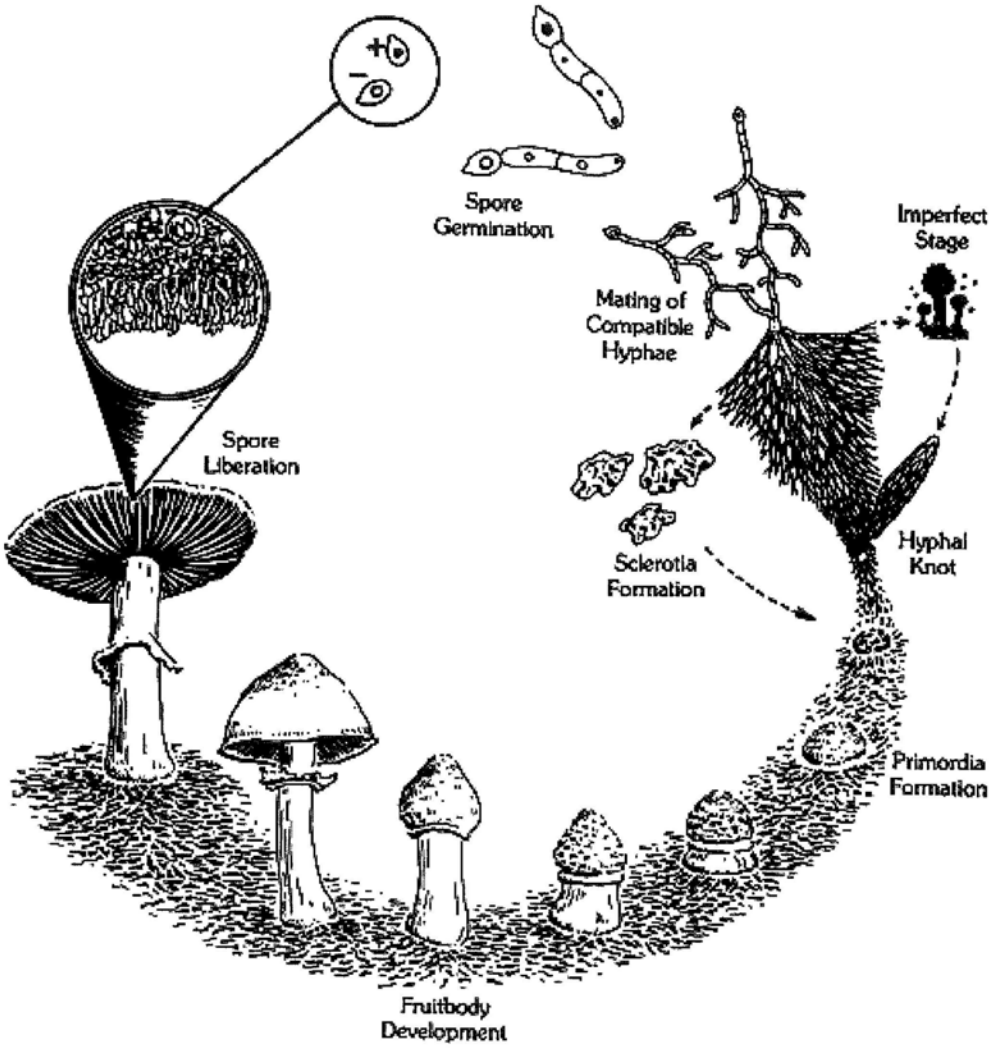


Figure 1.1 A pictorial overview of the mushroom life cycle (Adapted from Fungi Perfecti. Ltd. Co., Olympia, USA.)

1.1.2 Mushroom mycelium

Mycelium which is loosely packed hyphae composed of threadlike cells, is invisible to naked eye unless it bundles to form rhizomorphs (Arora, 1986). For supporting the growth of the mycelium and the mushroom, enzymes are secreted from the tips of mycelial hyphae for digesting the food outside the cells (Arora, 1986). For research purposes, pure culture of mushroom mycelium should be used. It could be obtained by sub-culture (obtained from other pure culture), single-pore culture (derived from the isolation of single sexual spores from the mushroom), multi-spore culture (cultured from the germination of many spores of a mushroom fruiting body) and tissue culture methods (isolated tissue from a mushroom fruiting body) (Chang and Quimio, 1992).

In order to culture mycelium and obtain its metabolites, submerged fermentation method is usually used. According to Eyal (1991), four main fields of industrial applications of mushroom mycelium have been investigated by submerged fermentation.

Firstly, it could be used for human and animal consumption as food and as mushroom-flavoring agents. Dry powder of edible mushroom mycelium is now used in many countries as a valuable and versatile food additive to soups, sauces, vegetable and meat concentrates, sausages and semifinished meat products and even special drinks in Japan. This is because mycelial filaments have various sizes and texture which make them easy to be introduced to different kinds of foods (Feofilova, 1998). Secondly, it could be used for the production of spawn for the cultivation of mushroom fruiting bodies. Thirdly, it could be used for the production of some chemical specialties such as polysaccharides, vitamins, organic acids, nucleotides and enzymes. Lastly, it could be used for the production of therapeutic compounds such as antibiotics, anticancer agents and antiviral agents.

Since mushroom mycelium has comparable nutritive value, medicinal value and industrial value to mushroom fruiting body but having a shorter cultivation period, it is a valuable commodity to be explored.

1.1.3 Nutritional and medicinal values of mushroom

Apart from its delicious taste, the nutritional values of mushroom are very high. Mushrooms are high in protein (about 10-30%), carbohydrate (about 50%), fiber (about 10-20%) but low in fat (lower than 10%) as shown in Table 1.1. Many mushrooms are also good source of essential amino acids which comprised 25-40% of the total amino acid content of mushrooms (Crisan and Sands, 1978).

Table 1.1 Proximate compositions of some edible mushroom fruiting bodies

Species	Crude protein	Crude fat	Carbohydrate	Crude fiber
Agaricus bisporus	23.9-34.8	1.7-8.0	44.0-53.5	8.0-10.4
Agaricus breitscheidri	11.5	2.1	73.5	7.7
Auricularia polytrica	7.7	0.8	73.6	14.0
Flammulina velutipes	17.6	1.9	69.4	3.7
Lentinus edodes	13.4-17.5	4.9-8.0	59.5-70.7	7.3-8.0
Pholiota nameko	20.8	4.2	60.4	6.3
Pleurotus ostreatus	10.5-30.4	1.6-2.2	48.9-74.3	7.5-8.7
Vovariella diplasia	25.9	2.6	40.0	17.4

All the data are in percentage of dry weight and adapted from Crisan and Sands, 1978.

In resent years, the medicinal values of mushrooms have attracted much research attention and the most studied mushrooms include *Ganoderma lucidum*, *Lentinus edodes*, *Tremella faciformis*, *Pleurotus ostreatus*, *Hericium erinaceum*, *Cordyceps sineusis*, *Volvariella volvaceae* and *Poria cocos* (Feofilova, 1998; Hetland et al., 2008; Wasser, 2002; Weng et al., 2010). Many reports have shown that mushrooms posses a number of potent medicinal properties such as anti-tumor, immuno-modulatory, antioxidant, anti-viral, anti-hypertensive, anti-inflammatory, liver protective and antifibrotic activities (Hetland et al., 2008; Ooi and Liu, 1999; Wasser and Weis, 1999a, b; Weng et al., 2010). Different kinds of pharmacological components have been extracted from mushrooms. For example, a potent pharmacological mushroom component is the lectin, which has been investigated largely for their immuno-modulatory and anti-tumor or cytotoxic activities (Wang et

al., 1998). Eritadenine, or so called lentinacin, which is a [2(R), 3(R)-dihydroxy-4-(9-adenyl)-butyric acid], isolated from *Lentinus edodes* has been found to be effective in lowering the blood cholesterol level in rats when consumed as food (Shimada et al., 2003). Currently, another group of bioactive components from mushrooms that has aroused the interest of scientists is polysaccharides. The most potent biological activity of the mushroom polysaccharides is their anti-tumor effects. More detail on mushroom polysaccharides is mentioned in section 1.2.

1.1.4 Mushroom under investigation: *Pleurotus tuber-regium* (PTR)

Pleurotus tuber-regium (PTR) is a saprophytic edible mushroom classified as *Basidiomycete* found in the tropic and subtropical regions of the world (Okhuoya and Okogbo, 1990; Zoberi, 1973). It was found that PTR can grow on the bark of a medium-sized tree of the Guinea Savanna in Nigeria called *Daniella oliveri* and many other hard or soft woods like *Mangifera India* and *Treculia Africana* (Okhuoya and Okogbo, 1990; Isikhuemhen and Nerud, 1999). PTR is different from many other *Pleurotus* species that it forms a large (could be over 50 cm in diameter) subterranean sclerotium which is globose to ovoid in shape with a dark brown outer covering and white inner part (Oso, 1977; Zoberi, 1973). The sclerotium buried in soil could produce fruiting bodies with cinnamon cap up to 10 cm in diameter if it is kept in a warm and humid place (Oso, 1977; Zoberi, 1973).

PTR is used as a nutritious food (as an ingredient in soup) as well as in the treatment of many diseases, such as constipation, stomach pain, fever, and colds (Oso, 1977; Zoberi, 1973). Apart from the potential medicinal use in Africa and southwest China, the non-starch polysaccharides extracted from the sclerotium, mycelium and culture medium of PTR have been reported to have both immuno-modulatory and direct cytocidal anti-tumor activities (Wong et al., 2007; Zhang et al., 2001; Zhang and Cheung, 2011). Commercial cultivation of the fruiting body and sclerotium of PTR is rare and the supply of this mushroom from the wild cannot meet the growing demand (Ilori et al., 1997). Because of the low biomass conversion and long harvest time, there is only limited success when cultivation of the sclerotium of PTR using solid compost in the laboratory (Fasidi and Ekuere, 1993; Huang et al., 1996). Since production of fungal polysaccharides from mushroom mycelium by submerged

fermentation is more efficient than that from cultivation of fruit body and sclerotium, the influence of external factors and optimization of culture conditions in submerged fermentation of PTR has drawn much attention (Wu et al., 2003). However, there were very few studies that were on the EPS production by PTR and they only reported the optimization of the conditions in EPS production in other *Pleurotus* species (Rosado et al., 2003; Wang et al., 2005). More detail on production of polysaccharides from mushroom mycelium by submerged fermentation is discussed in section 1.3.

1.2 Mushroom polysaccharides

1.2.1 Types of microbial polysaccharides: endo- and exo- polysaccharides

Polysaccharides belong to a structurally diverse class of macromolecules, with polymers of monosaccharide residues joined to each other by glycosidic linkages. The biosynthesis of polysaccharide is an energy-intensive, precise regulated process. An understanding of the subtle interaction between the energy conservation and its regulation; the polymer modification and its synthesis; and the external ecological functions is a large area of research. Polysaccharides are synthesized at some stage in the growth of many living cells. During the life-cycle of microorganisms, the polysaccharides may be produced both by intracellular and extracellular enzymes. They may act as energy reserves, structure units, colloids for ions and food transfer, or defensive barriers for the cell, etc. (Suresh, 2009).

The microbial polysaccharides can be distinguished into endo- and exo-polysaccharides. Exo-polysaccharides are high-molecular-weight polymers that are synthesized by the microorganism and secreted into the surrounding environment while the endo-polysaccharides are kept in the microorganism.

Endo-polysaccharides possess a number of functions such as acting as structural components (e.g. chitin in the cell wall) or important virulence factors (e.g. poly-N-acetylglucosamine in *S. aureus*) (Sutherland, 2002). Exopolysaccharides (EPS) should have certain kind of biological functions for the microorganisms because bacteria have to utilize both substrate and energy to produce these

metabolites (Dudman, 1977). Some kinds of bacteria use more than 70% of their energy for the EPS production, possibly to gain a superior advantage in their survival environment (Weiner et al. 1995). Moreover, microbial biosynthesis of EPS facilitated the survival of microorganisms in different environments. EPS are apparently not essential for bacteria since physical or enzymatic removal of EPS does not negatively influence the cell growth *in vitro*. In generally, the most likely functions of EPS are of a protective property such as protection against protozoa, macrophages, dehydration, antibiotics, bacteriophages, and toxic compounds (Roberts, 1996, Weiner et al., 1995, Whitfield, 1988). Besides, other proposed functions of EPS include involvement in biofilm formation and adhesion (Roberts, 1996) and sequestering of essential cations (Weiner et al., 1995).

1.2.2 Chemical structures of mushroom polysaccharides

Polysaccharides are polymeric carbohydrate structures, formed of repeating units of different monosaccharide joined together by glycosidic bonds. It is noted that, when compared with other biopolymers such as proteins and nucleic acids, polysaccharides possess the highest capacity of carrying biological information because they have the greatest potential for structural variability. The amino acids in proteins and the nucleotides in nucleic acids can interconnect in only one way whereas the monosaccharide units in polysaccharides can interconnect at several points to form a wide variety of branched or linear structures (Sharon and Lis, 1993). Due to the huge potential variability, polysaccharides offer the necessary flexibility to the subtle regulatory mechanisms of many cell-cell interactions in higher organisms.

Polysaccharides with antitumor function differ greatly in their chemical composition and configuration, as well as their physical properties. Antitumor activity is exhibited by a wide range of glycans extending from homopolymers to highly complex heteropolymers (Ooi and Liu, 1999). Differences in bioactivity can be correlated with the carbohydrate and protein content, molecular weight, monosaccharide composition and glycosidic likages of the polysaccharides. However, until now, it is still very difficult to make it clear about the relationship between the structure and antitumor activity of complex polysaccharides.

In the study of some species of edible mushroom, polysaccharides bound with proteins as a polysaccharide-protein complex have been showed to possess higher potent biological activity (Cui and Chisti, 2003). Molecular weight is another very important parameter of the biological function of mushroom polysaccharides. However, the direct relationship between the molecular weight and biological activity is still not clear and the published results are not consistent.

The primary structure of mushroom polysaccharide is mainly depended on the monosaccharide composition and position of glycosidic linkages, which also have great impacts on the biological activities. The analysis of monosaccharide composition involves cleavage of all glycosidic linkages, fractionation of the resulting monosaccharides, and detection and quantification of each monosaccharide. The glycosidic linkages of polysaccharides could be analyzed by methylation method in which the polysaccharides are converted to partially methylated alditol acetates (PMAAs). The resulting PMAAs are identified by a combination of their retention times and their electron impact-mass spectrometry (EI-MS) fragmentation patterns. By the methylation analysis results, one can suggest the occurrence of branching points, how each monosaccharide is substituted, which residues are the terminal ones and determination of the ring size for each monosaccharide. However, methylation analysis does not provide information on the sequence of constituent residues and the type of anomeric configurations (i.e. α or β).

Mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1→3), (1→6)-β-glucans and (1→3)-α-glucans. Lentinan (extracted from *Lentinus edodes*) is the first polysaccharide found to stimulate macrophages and it is a homoglycan with a (1→3)-β-glucan backbone and (1→6)-β-glucan side chains having a molecular weight of about 1000 kDa (Sasaki and Takasuka, 1976). Non-starch α-glucans are seldom found in mushroom, but there are some exceptions. A linear (1→3)-α-glucan of molecular weight about 560 kDa could be obtained from *Agrocybe cylindracea* (Kiho et al., 1989). Another linear (1→3)-α-glucan could also be found in *Armillariella tabescens* (Ukawa et al., 2000). There are still very few studies on the biological actions of non-starch α-glucans. Apart from the well-known antitumor homoglucans, there are still many other

biologically active heteroglucans and heteroglycans. Immunomodulatory or antitumor polysaccharides with different kinds of chemical structures from a wide range of mushrooms have been investigated (Table 1.2).

Table 1.2 Chemical structures of antitumor or immuno-modulatory mushroom polysaccharides

Polysaccharide	Linkages and types	Mushroom resources	References
Homoglucans	(1→6)-β-; (1→3)-β-glucans	*Lentinus edodes*	Mizuno, 1997
		Schizophyllum commune	Yamamoto et al., 1981
		Grifola frondosa	Zhuang et al., 1994
	(1→3)-β-glucans	*Auricularia auricula*	Ukai et al., 1983
		Lyophyllum decastes	Ukawa et al., 2000
	(1→6)-β-glucans	*Armillariella tabescents*	Kiho et al., 1989
	(1→3)-α-glucans	*Armillariella tabescens*	Ukawa et al., 2000
	(1→4)-α-; (1→6)-β-glucans	*Agaricus blazei*	Mizuno, 1998
Heteroglucans	(1→3)-β-glucurono glucan	*Ganoderma lucidum*	Mizuno, 1998
	Xyloglucan	*Grifola frondosa*	Zhuang et al., 1994
		Polyporus confluens	Sugiyama et al., 1992
	Arabinoglucan	*Ganoderma tsugae*	Wang et al., 1993
	Riboglucan	*Agaricus blazei*	Mizuno, 1998
	Galactomannoglucan	*Leucopaxillus giganteus*	Wasser, 2002
	Galactoxyloglucan	*Hericium erinaceus*	Mizuno, 1998

	Mannoxyloglucan	*Grifolan frondosa*	Zhuang et al., 1994
	Xylogalactoglucan	*Inonotus obliquus*	Kim et al., 2005
Heterogalactan	Glucogalactan	*Ganoderma teugae*	Peng et al., 2005
	Arabinogalactan	*Pleurotus citrinopileatus*	Wang et al., 2005
	Fucogalactan	*Sarcodon aspratus*	Mizuno et al., 2000
	Mannogalactan	*Pleurotus pulmonarius*	Wasser, 2002
	Fucomannogalactan	*Grifola frondosa*	Zhuang et al., 1994
Other heteroglycans	Xylan	*Hericium erinaceus*	Mizuno, 1998
	Mannogalactofucan	*Grifola frondosa*	Zhuang et al., 1994
	Mannoglucoxylan	*Hericium erinaceus*	Mizuno, 1998
	(1→3)-α-mannan	*Dictyophora indusiata*	Hara et al., 1991
	(1→2)-β-; (1→3)-β-glucomannan	*Agaricus blazei*	Mizuno, 1998
Polysaccharide -protein complexes	Polysaccharide-protein complexes	*Coriolus versicolor*	Cui and Chisti, 2003

1.2.3 Biological activity of mushroom polysaccharides

Polysaccharides are the best known and the most potent mushroom-derived substances with immunomodulatory and antitumor properties, which function as host defense potentiators or biological response modifiers for inhibiting tumor growth (Franz, 1989; Jong et al., 1991; Ooi and Liu, 1999).

From clinical practice, it is well known that mushroom polysaccharides are most effective when in conjunction with other types of 'tough' chemotherapy and

surgery, which have many negative side effects and are quite invasive to the normal cells. The most important principal points of antitumor and immunomodulating effects of mushroom polysaccharides are: (1) prevention of oncogenesis by oral consumption of mushrooms or their preparations (cancer-preventing activity); (2) direct antitumor activity to induce the apoptosis of tumor cells (direct tumor inhibitory activity); (3) immunopotentiation activity against tumors and tumor metastasis (immuno-enhancing activity) (Wasser, 2002).

Mushroom polysaccharides do not normally attack cancer cells directly, but generate their antitumor effects through activating different immune responses in the host. These substances are regarded as biological response modifiers (Wasser and Weis 1999a, b). This basically means that: (1) they are not harmful and produce no additional stress to the body; (2) they assist the body to response to different kinds of biological and environmental stresses; and (3) they produce a nonspecific effect on the body, supporting some or all of the major systems, including immune, nervous, and hormonal systems, as well as regulatory functions (Brekhman and Fluder, 1980). It is well known that mushroom polysaccharides could stimulate B-cells, T-cells, natural killer cells, and macrophage-dependent immune system responses.

Some previous studies on the bioactivity of polysaccharides isolated from different parts and the culture medium of mushrooms are listed in Table 1.3. Bioactive polysaccharides can be obtained from mycelium, fruiting body and sclerotium, which represent three different forms of a macrofungi in the life cycle. Among the polysaccharides listed in Table 1.3, some of them such as the schizophyllan, lentinan, grifolan, krestin (polysaccharide-peptide complex) and PSK (polysaccharide-protein complex), have been commercialized for the clinical treatment of patients undergoing anticancer therapy.

Fermentation broth of mycelium could also be good sources of medicinal polysaccharides (Table 1.3). For instance, the polysaccharide from the fermentation broth of mycelium of *Ganoderma lucidum* could have an immunomodulating effect. Therefore, it is worth to study how the culture conditions affect the production of mushroom mycelial polysaccharides in submerged fermentation.

Table 1.3 Source and bioactivity of medicinal polysaccharide from some mushrooms

Species	Polysaccharide Source	Main bioactivity	Reference
Ganoderma lucidum	Fruiting body, fermentation broth	Hyperglycemia, immunomodulating, antitumor	Miyazaki and Nishijima, 1981; Mizuno, 1997
Schizophyllum commune	Mycelium	Antitumor	Yamamoto, 1981
Lentinus edodes	fermentation broth, fruiting body	Immunomodulating, antitumor, antiviral	Chihara et al., 1970; Hobbs, 2000
Sclerotinia sclerotiorum	Sclerotium	Antitumor	Palleschi et al., 2005
Polystictus versicolar	Fruiting body, fermentation broth, mycelium	Immunomodulating, antitumor, antiradiative	Cui & Chisti, 2003
Grifola frondosa	Fruiting body	Immunomodulating, antitumor, antiviral, hepatoprotective	Zhuang et al., 1994
Tremella fuciformis	Fruiting body, mycelium, fermentation broth	Hyperlipidemia, hyperglycemia, immunomodulating, antitumor	Huang, 1982
Pleurotus ostreatus	Fruiting body	Antitumor, hyperglycemia, antioxidant	Solomko, 1992
Pleurotus tuber-regium	Sclerotium, mycelium, fermentation broth	Immunomodulating, antitumor	Wong et al., 2007; Zhang et al., 2001, 2003; Zhang and Cheung, 2011

1.3 Submerged fermentation

1.3.1 Solid-state fermentation (SSF) and submerged fermentation (SmF)

Solid-state fermentation (SSF) is defined as the fermentation involving solid substrate in absence (or near absence) of free water ($a_w \approx 0$); however, substrate must have enough moisture to sustain the growth and metabolism of microorganism (Mitchell and Lonsane, 1992; Pandey, 2003). SSF general utilizes agro-industrial wastes as substrates which are beneficial for filamentous fungi to penetrate into these hard solid substrates, assisted by the existence of turgor pressure on the tip of fungi mycelia (Ramachandran et al., 2004). Moreover, the use of these agro-industrial residues aids in solving environmental pollution problems, which otherwise may need additional disposal (Pandey et al., 1999). SSF stimulates the growth of some common microorganisms in its natural situation on moist solid substrate and has been extensively applied in our daily life for a long time. For instance, the Koji processes which produce *sake*, *miso*, soy sauce, and *tempeh*, are an ancient technology, as is the production of the composts and mushroom growing. And SSF has also been extensively applied in detoxification of agro-industrial wastes, biodegradation and bioremediation of hazardous substances, biopulping, production of some valuable fungal enzymes and secondary metabolites, nutraceuticals and pharmaceuticals (Carlisle et al., 1994; Viniegra-Gonzalez et al., 2003; Mukheriee and Nandi, 2004; Nwe et al., 2002; Ngai and Ng, 2003). There are several important factors, which should be considered in general for the development of any bioprocess in SSF, including selection of suitable micro-organism and substrate, optimization of process parameters and isolation and purification of the product (Pandey, 2003).

Submerged fermentation (SmF) is a method used for the production of microorganisms including mushroom mycelium in synthetic defined medium, complex medium or on a variety of waste substrates without the stage of sporulation of the microorganisms during the fermentation process (Eyal, 1991).

Although SSF was used in almost all ancient fermentation processes, SmF approaches have widely replaced SSF in the production of microbial cells and their

metabolites in western countries after 1940. Consequently, the SSF method has not been used for producing higher value metabolites in developed countries. This may have been because of the enormous impact the large-scale production of penicillin had during World War II, and subsequently, SmF becoming the preferred method of fermentation for commercial applications (Eyal, 1991). Since in the SmF process, the oxygen, mass and heat transfer are much superior, and fermentation homogeneity is usually better than that of SSF, SmF seems intrinsically less problematic and more reproducible and reliable. It is easier, more flexible to monitor and to control key operational parameters in the SmF process.

However, production of macrofungi and its metabolites by SmF only began slowly during the 1950s, applying the principles which commonly used in the lower fungi in fermenters (Eyal, 1991). The adjustment in this approach is apparent. Traditionally, macrofungi were cultured in composts, sawdust, or logs by SSF processes which may take about one to several months before the formation of fruiting bodies. In addition, the composition of fruiting body is highly variable, especially in its content of bioactive components. For example, the traditional approach of SSF makes it challenging in the extraction of polysaccharides from fruiting bodies (Lo et al., 2006). In contrast, SmF process can ensure reliable supply of macrofungi metabolites not influenced by seasonal variations and is also superior to produce these compounds more hygienically and rapidly. Since the first publication on the production of mushroom mycelia of *Agaricus campestris* in a synthetic medium by SmF (Humfeld, 1948), great efforts have been made to develop SmF processes for the macrofungi on a large scale. But mushroom mycelia in SmF grow slower than bacteria, yeast and other filamentous fungi, making the process control and optimization a challenge.

However, SSF or SmF approach is not interchangeable. The respective advantages of SSF and SmF are listed in Table 1.4. Many factors such as product quality, economic considerations, and regulations for disposal of wastes by the government should be overall considered when determine which process to use (Couto and Sanroman, 2006).

Table 1.4 Respective advantages of SSF and SmF *

Advantages of SSF	Advantages of SmF
Higher productivity	Easier on scale-up
Better oxygen circulation	Shorter harvest period
Low-cost media, simple technology, scarce operational problems	Good control of process parameters (pH, heat, moisture, nutrient conditions, . . .)
Reduced energy and cost requirements	No problems with heat build-up
It resembles the natural habitat for several microorganisms	Lower impurity product, decreasing recovery product costs

* From Couto and Sanroman, 2006

1.3.2 Factors affecting the submerged fermentation process

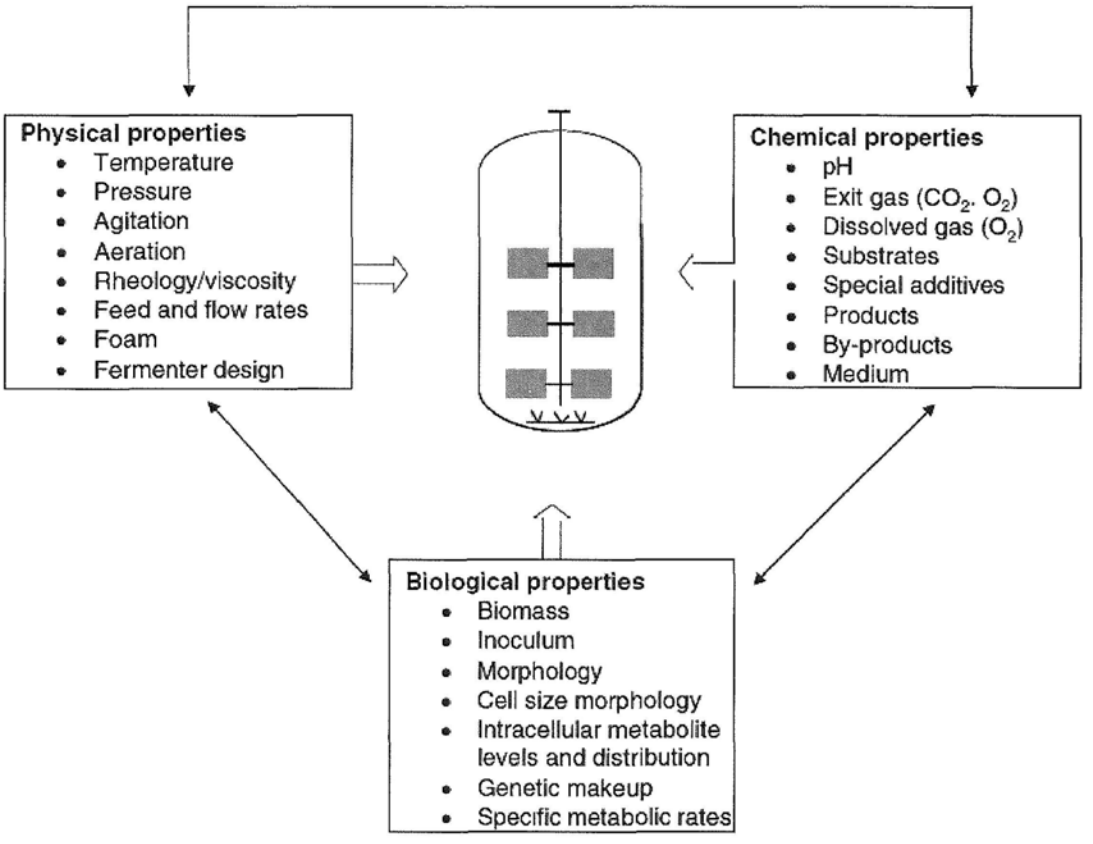


Figure 1.2 Factors that influence the performance of a submerged fermentation bioprocess and the complexity of interactions between them (Adapted from Vaidyanathan et al., 1999)

Factors influencing the performance of a submerged fermentation bioprocess can be categorized into physical, chemical or biological (Figure 1.2). The physical and chemical factors define the environment of the biocatalyst, while the biological factors describe its behavior.

1.3.2.1 Physical factors

Generally, the physical factors such as temperature, agitation and aeration rate could affect the macro-environment of the biocatalyst, its physiological and morphological behavior and in turn influencing the performance of the SmF process (Vaidyanathan et al., 1999).

1.3.2.1.1 Temperature

In SmF of mushroom mycelia, temperature is an important parameter and can be controlled precisely. Even small variations in temperature can greatly affect the productivity. The optimal temperature may be different for mycelial growth and their metabolites. It was reported that optimum temperatures for the cell growth (10 ^{o}C), the production of exopolysaccharide (25 ^{o}C) and endopolysaccharide (10 ^{o}C) were totally different in SmF of *Ganoderma applanatum* (Lee et al., 2007). Similar results have been reported by Yang et al. (2002), in which the mycelium of *Auricularia polytricha* grew best at 20-25 ^{o}C while production of EPS was the best at 30 ^{o}C.

1.3.2.1.2 Agitation

Agitation is using done by orbital vibration in shake-flask in a shaker or by stirring or air-lifting in fermentors. Agitation is required for efficient mixing, heat and mass transfer. However, agitation also creates shear forces which cause morphological changes, variation in the growth of mycelium and product formation, and even damage the cell structure of mycelium (Kim et al., 2003). For example, Lee et al. (2004) reported that EPS production was favored at mild agitation rates (166 rpm) with *G. frondosa* grown in a 5-liter bioreactor but high agitation rates gave lower EPS yield. It was proposed that this might be due to the denaturation of some

key enzymes caused by the shearing effect during high-speed agitation, or due to the cleavage of the mycelia.

1.3.2.1.3 Aeration

Aeration is a very important factor in SmF because it is one of the primary energy entries to the SmF process. Together with agitation, aeration usually meets oxygen demand of the cultured cells. Dissolved oxygen concentrations change about 10 times faster than the cell mass and other substrate concentrations, making it the most important physiological variable to control and optimize in aerobic fermentations (Gomes and Menawat, 2000). Oxygen supply could influence key enzymes in the bioprocess, and thus may directly affect the production of SmF. Oxygen concentration could also influence the substrate uptake rates, the cell morphology and physiology, and its metabolite biosynthetic rates (Gomes and Menawat, 2000; Tang and Zhong, 2003). The mycelial and EPS yield of *Paecilomyces sinclairii* was significantly enhanced by increasing the aeration rate from 1.5 to 3.5 $vol \cdot vol^{-1} \cdot min^{-1}$ (vvm) (Kim et al., 2003). The morphology of the mycelium of *P. sinclairii* was different in different aeration rate in which the hyphal length was the longest at an aeration rate of 0.5 vvm while the hyphal density was highest at an aeration rate of 3.5 vvm (Kim et al., 2003).

1.3.2.2 Chemical factors

1.3.2.2.1 pH

Initial pH of the fermentation broth is a vital factor that governs cell biomass and metabolite formation. The pH value of the fermentation broth may influence cell morphology, cell membrane structure and function, the uptake rate of various nutrients, ionic state of the dissolved substrates, the production of bicarbonate from dissolved CO_2, and overall affect the cell growth and metabolite production (Fang and Zhong, 2002). When pH is not automatically controlled in a SmF process, pH will probably change during the cell growth due to substrate consumption or metabolite excretion which in turn affecting the cell biomass and product formation (Papagianni, 2004). Many mushrooms have different optimum pH for biomass

growth and EPS production. For example, mycelia growth requires a low optimum pH (pH 4.0), while EPS production needs a higher optimum pH (pH 6.0) (Jonathan and Fasidi, 2003; Kim et al., 2005). It was reported that a bi-stage control method increased EPS production in SmF of *G. lucidum* mycelia, in which the pH for maximum mycelial biomass (3.0) was shifted to another value more favorable for EPS production (6.0) (Lee et al., 1999). However, it should be noted that these pH requirements were not applicable to all the situations (Fang and Zhong, 2002b; Xu and Yun, 2003). The optimum pH for SmF process will depend on various factors, including the strain of the organism used (Xu and Yun, 2003).

1.3.2.2.2 Carbon sources

Carbon sources provide the structural substances and energy requirements for the growth of mushrooms. Mushrooms can utilize a variety of carbon sources including various monosaccharides, disaccharides, polysaccharides, organic acids, amino acids, certain alcohols, polycyclic compounds and natural products such as lignin (Chang and Miles, 1989). The growth rate of mushroom mycelium may be affected by the availability of different carbon sources. Different fungal species adapted distinct patterns of use of carbon sources (Hwang et al., 2004). Carbon source and its concentration may have complex influence on the SmF process (Kim et al., 2005b; Lin et al., 2006). It has been widely studied about the effects of various carbon sources and their concentrations on cell biomass and production of metabolites (Wymelenberg et al., 2006). It was suggested that disaccharides such as lactose, sucrose, maltose are normally more suitable than monosaccharides such as glucose, galactose, fructose when applied for EPS production (Shih et al., 2006), probably due to their relative ease of polymerization (Fan et al., 2007). In general, higher concentrations of the carbon source (>35 g/L) lead to greater production of EPS, implying a high C: N ratio is important. However, the best carbon source for the production of mushroom mycelium is not necessary to be the best carbon source for the production of EPS. The production of mushroom mycelium and EPS may both be affected by the source of carbon but may not have any direct relationship (Kim et al., 2005b; Lin et al., 2006; Shih et al., 2006; Xiao et al., 2006). In addition, it was reported that under carbon limited conditions, some macrofungi released β-glucanases that could degrade any preformed β-glucan, resulting in a concomitant

decrease of viscosity of the fermentation broth (Rau, 2004). These phenomena generally occur during the end of a batch fermentation process. Hence, if the target is to obtain good quality and the maximal production of EPS, these situations should be avoided by careful control of harvest time or by use of appropriate feeding of carbon source.

1.3.2.2.3 Nitrogen sources

Nitrogen is needed for producing proteins, purines, pyrimidines and chitin in mushrooms (Chang and Miles, 1989). Ammonium ion, nitrate and organic nitrogen can be the nitrogen sources for the growth of mycelium. However, many studies have found that organic nitrogen including yeast extract, peptone and corn steep powder should be a better nitrogen source for the growth of mushroom mycelium due to the addition of growth factors in trace amounts. The mycelial yield of *Cordyceps militaris* C738 was higher than 10 g dry wt/L in medium containing organic nitrogen including corn steep powder, meat peptone, polypeptone, soypeptone and yeast extract. On the other hand, the mycelial yield of *C. militaris* was as low as 2.42 to 4.48 g dry wt/L in medium containing various ammonium salts (Kim et al., 2003b). The mycelium of *Paecilomyces japonica* also grew better in organic nitrogen including yeast extract, meat peptone, trytone, polypeptone and bacto-peptone than inorganic nitrogen sources including various ammonium salts and nitrates (Bae et al., 2000). It was demonstrated that the kinds and concentrations of nitrogen sources greatly affected the mycelial biomass and yield of polysaccharide in *Tremella mesenterica*, and combined use of corn-steep liquor and yeast extract enhanced production of EPS (Wasser et al., 2003).

1.3.2.2.4 Minerals

Minerals are essential for mushroom mycelial growth in SmF, and are usually provided as salts at low concentration (about 10^{-3} M). Potassium regulates osmotic potential and the turgor pressure necessary for hyphal tip extension; magnesium is activator of many enzymes, cofactor in many enzymatic reactions, stabilizes the plasma membrane; sulphur is used for production of sulphur-containing amino acids, thiamine, biotin and some other metabolites; and phosphorus is used for production

of ATP, nucleic acids and phospholipids of membranes (Chang and Miles, 1989). In order to provide the above four essential minerals, many studies had used potassium phosphate to provide potassium and phosphorus, and magnesium sulphate to give magnesium and sulphur for the SmF of mycelium. Requirements for minerals and their effects on the SmF depend on the mushroom strain and medium used. It is not always necessary to supply minerals to the fermentation broth, especially if complex medium is being used.

1.3.2.3 Biological factors

1.3.2.3.1 Inoculum

It is well known that concentration and form of inoculum exerts a major influence on the fungal SmF process. Normally, the age, amount, viability and type of the inoculum all may influence the morphological state of the cells, especially in pellet production and the type of pellets produced (Gibbs et al., 2000). However, very few studies on the effects of inoculum on SmF of macrofungi have been reported.

Because sexual spores of most macrofungi are only produced when the fruiting body has matured, it is not practical to readily use spores as an inoculum like other filamentous fungi (Wagner et al., 2003). Hence, mycelium-based inoculum should be used, which makes the standardization difficult. On one hand, most experiments use small pieces of mycelium that are still attached to the agar on which the fungi were grown, and inoculate them directly into the fermentation broth (especially for shake flasks). On the other hand, submerged seed culture is often used to inoculate a bioreactor. In the former, the mycelium has to adapt from a solid to a liquid environment, resulting in long lag phases and low inoculum densities. In the latter, the environmental and nutritional conditions used to prepare the seed culture should be the same as those in the bioreactor to avoid lag phases. Moreover, to ensure the inoculum standardization, the size of the agar pieces plus mycelium should be standardized and removed at the same radial distance from the colony center, to ensure that all have the same amount of mycelium at the same stage of growth (Stanbury et al., 1995). An active inoculum will help to decrease the lag phase in

subsequent fermentation process and the main aim of using a standardized inoculum is to achieve process reproducibility. It is essential that the inoculum is transferred at an appropriate time (i.e., when it is in the correct physiological state, which can be determined experimentally).

1.3.2.3.2 Morphology

The morphology of macrofungi in SmF is affected by the operating conditions in the fermenter. In order to obtain maximal cell biomass and production of polysaccharides, it is important to investigate the relationship between the macrofungi morphology and the operating conditions (Olsvik and Kristiansen, 1992). A detailed structural analysis of fungal morphology is therefore desirable if a better understanding of the relationships between morphology and target metabolite production by mycelium is being sought. Hence, application of some advanced techniques such as image analysis would make it more feasible to investigate the morphology of fungal mycelium (Riley et al., 2000).

Macrofungal may grow as discrete pellets about 1–20 mm in diameter (pellet growth) or as a pulpy filamentous form in SmF process. Intermediate forms may also be recognized. It was reported that macrofungi were usually characterized as large pellets form in the SmF process (Eyal, 1991). However, the interrelations between fungal morphology and target metabolite production are still controversial. Most of basidiomycetes exhibit two typical morphologies in submerged fermentation: pellet and free filamentous form. These two growth forms are determined by many factors including genotype, pH of the culture medium, constituents of the culture medium, inoculums types and other fermentation conditions (Hwang et al., 2004). "Pellet" form seems to be preferred in most cases, and especially for the production of EPS (Gehrig et al., 1998; Hwang et al., 2004; Kim et al., 2003; Lee et al., 1999; Sinha et al., 2001).

1.3.3 Production of EPS in submerged fermentation of mushroom mycelia

Fruiting body of mushroom contains polysaccharides as major structural cell wall components. Mushroom mycelia could also yield large amounts of EPS, under

proper fermentation conditions. However, this is not always the case because the fermentation conditions essential for the production of EPS have not been clearly understood and elucidated (Kues and Liu, 2000).

The advantages of growing mushroom mycelia in defined culture medium in SmF instead of using their fruiting bodies as the source of polysaccharides have been stated before, but are worth repeating. Large-scale production facilities and pure cultures grown under carefully monitored and controlled conditions guarantee a more reliable product of known composition. Therefore, it is possible to manipulate the physicochemical characteristics of the polysaccharides to enhance their bioactivities and further improve the productivity. Furthermore, the purification and recovery of the biosynthetic EPS is much easier. Compared to the long harvest time needed for the fruiting body, growing mushroom mycelia in SmF has been found to be a simple, fast and efficient alternative method for producing EPS that have similar bioactivities.

1.4 Stimulatory agents

1.4.1 Types of stimulatory agents and their mechanism of action

Many factors may affect the production of mushroom mycelial EPS by SmF and a lot of investigations have been conducted on the optimization of SmF conditions (Chen et al., 2008; Cho et al., 2006; Fan et al., 2007; Kim et al., 2005; Pokhrel and Ohga, 2007). However, one of the problems of the production of mushroom mycelial EPS by SmF is the low productivity. Hence, there is an increasing interest in search of chemical agents to stimulate mushroom mycelial EPS production by SmF. These stimulatory agents include plant oils, fatty acids, organic solvents and surfactants. Many of them have been proved to be effective stimulatory agents in the production of useful metabolites in bacteria, fungi and medicinal mushrooms.

1.4.1.1 Plant oils

Plant oils, which are frequently used as an antifoam agent in fermentation, have been reported to be favorable to mycelial growth in several medicinal mushrooms and to increase the production of bioactive metabolite (Chang et al., 2006; Huang et al., 2009; Park et al., 2002).

Enhanced production of β-glucan by plant oil in the fungus *Acremonium persicinum* was firstly reported by Stasinopoulos and Seviour (1990). When olive oil or sunflower oil was added to the fermentation broth, a 2-fold increment in EPS production was observed. After that, many researches have been carried out on this area. For example, it was found that the addition of safflower oil markedly increased the mycelial yield in *Ganoderma lucidum*, while olive oil resulted in a high polysaccharide production (Chang et al., 2006). However, it was also reported that soybean oil decreased the production of EPS in SmF of *Ganoderma lucidum* (Yang et al., 2000). By the results of Huang et al. (2009), with addition of 2% (v/v) corn oil at the beginning of fermentation, the mycelial biomass and EPS productions of *G. lucidum* reached a maximum of 12.9 and 1.04 g/L, respectively, after a 13-day submerged fermentation. Hsieh et al. (2006) reported that the mycelial biomass and EPS productions could be greatly enhanced by addition of 1% olive oil in *Grifola frondosa* submerged fermentation.

Yang et al. (2000) and Park et al. (2002) proposed that such stimulation arises from incorporation of their fatty acids into the cell membrane, rendering them more permeable thereby facilitating both nutrient assimilation and metabolite excretion. The expression of enhancement or suppression might be determined by the types of fatty acids present in oil. However, no explanations supported by any experimental data were provided.

1.4.1.2 Fatty acids

Plant oils have been proved to be an effective stimulatory agent. However, plant oils are composed of different kinds of fatty acids in different concentrations. For example, palm oil contains different kinds of unsaturated and saturated fatty acids in the composition of oleate (39%, monounsaturated), linolenate (0.3%, polyunsaturated), linoleate (10%, polyunsaturated), palmitate (44%, saturated), stearate (5%, saturated), myristate (1%, saturated) and glyceryl laurate (0.1%, saturated). The extent of enhancement or suppression of plants oils used as stimulatory agent depends on the types and composition of fatty acids present in them. So it is hard to explain stimulatory effects of the plant oils which constitute a mixture of individual fatty acids. Therefore, it has been paid more and more attention to study the effect of pure fatty acids as potential stimulatory agents in recent years.

Fatty acids were added into the fermentation broth to investigate their effects on the mycelial biomass and EPS formation by *Ganoderma lucidum* (Yang et al., 2000). It was found that oleic acid (0.15 g/100 ml) could significantly increase the mycelial biomass and palmitic acid greatly enhanced the production of EPS. In contrast, addition of linoleic acid (0.1 g/100 ml) drastically decreased both mycelial growth and EPS formation (Yang et al., 2000). Another similar study shown that addition of 2% oleic acid and palmitic acid markedly stimulated the EPS production (enhancement factors were 6 and 5.5, respectively), whereas linoleic acid significantly suppressed both mycelial growth and EPS production in *Cordyceps militaris* (Park et al., 2002). The proposed mechanisms of the stimulatory effect of fatty acids are that they modify the cell membrane structure and increase permeability, or directly affect the level of synthesis of the enzymes involved in EPS production (Park et al., 2002).

However, these studies could not demonstrate any clear relationship between the length of the carbon chain or the level of unsaturation of the different fatty acids and the lipids composition of the mushroom cell membrane. Again, there have been no explanations supported by any experimental data yet.

1.4.1.3 Organic solvents

In order to enhance the production of extracellular metabolites, the method of reorganizing the cell membrane is usually used by reagents that can change the cell permeability. It is well known that the use of organic solvents is a relatively effective approach for cell permeabilization, since organic solvents are less expensive than other stimulatory agents and could be eliminated by simple methods like evaporation (Choi et al., 2004; Smet et al., 1978). The representative organic solvents that are commonly used to increase the cell membrane permeability are toluene, methanol, ethanol, chloroform, dimethyl sulfoxide (DMSO) and diethyl ether (De Leon et al., 2003). However, there are relative fewer studies or the use of organic solvents to enhance the production of mycelial biomass and EPS in mushroom when than studies on bacterial and yeast cells for improving the production of other specific metabolites.

Alcohols have been successfully used for enhancing the production of fugal extracellular metabolites. For example, 1.0% (v/v) methanol was found to have significant enhancing effect (1.96 fold higher than the control) on the citric acid production by fermentation of *Aspergillus niger* GCB-47 (Haq et al., 2003). It was proposed that methanol increased the permeability of cell membrane, resulting in better excretion of citric acid from the mycelial cells. It could also be explained in terms of pellet size controlled by means of air or oxygen. Methanol was responsible for the proper pellet size which could increase the active surface of mycelial cells and allowed better oxygen availability, which is a critical point in citrate synthesis by *A. niger* (Haq et al., 2003). In another similar study by Yang et al. (2004), four kinds of aliphatic alcohols were used to examine their effects on the mycelial growth and EPS production in SmF of *Ganoderma lucidum*. Ethanol at 1.5% (v/v) was the most effective for increasing the biomass production, while the maximal EPS concentration was obtained with 2% (v/v) ethanol in the fermentation broth. Moreover, ethanol did not appear to act as a carbon source and remained in the fermentation broth, almost at the original concentration throughout growth and EPS production. And there was no new polysaccharide composition found by the addition of ethanol confirming that the EPS biosynthetic way had not been changed (Yang et

al., 2004). In contrast, another study shown that five kinds of alcohols all expressed negative effect on the mycelial growth and EPS production in SmF of *Grifola umbellata* (Chen et al., 2010).

Apart from alcohols, other types of organic solvents (e.g. toluene, chloroform, acetone and heptane) were investigated for their stimulatory effects on the mycelial growth and EPS production in SmF of *Collybia maculata* TG-1 (Lim and Yun, 2006). Among the solvents examined, 0.3% (v/v) toluene significantly enhanced EPS production by 86% when supplemented at the late growth phase of the SmF of *C. maculata* TG-1, although mycelial growth was slightly inhibited by toluene treatment. The microscopic observations showed that an obvious morphological difference existed between the mycelial cells of *C. maculata* TG-1 before and after toluene addition. The altered outer mycelial cell structure, as observed by the transmission electron microscope (TEM) seems to be the most likely reason for the increased EPS production in the SmF with toluene addition (Lim and Yun, 2006). The organic solvent addition strategy may be worth attempting in other mushroom SmF processes for enhancing EPS production, particularly those with commercial or medical values.

1.4.1.4 Surfactants

Surfactants are very useful in biology and biochemistry and have been widely applied in biotechnology for improving the production of a number of extracellular enzymes by SmF of microorganisms. For example, the addition of the surfactants (Triton X-100, CHAPS, Tween-80 and sodium taurocholate) to SmF of *Clostridium thermosulfurogenes* SV2 all resulted in significant increase in the production of thermostable β-amylase and pullulanase (Reddy et al., 1999). With addition of 1.0 mM Triton X-100, *C. thermosulfurogenes* SV2 produced 2.4 fold of β-amylase and 2.1 fold of pullulanase compared with the control, respectively. These surfactants also further enhanced the stability of the enzymes. However, all the surfactants tested showed a little inhibitory effect on the growth of the bacterium (Reddy et al., 1999). Similarly, there were other reports showed that the yields of ligninases and cellulases were markedly enhanced by addition of surfactants in SmF of some microorganisms

(Asther et al., 1987; Stutzenberger et al., 1987). The mechanisms by which surfactants enhance extracellular enzyme production are not completely clear; however, increased cell membrane permeability, change in lipid metabolism, and stimulation of the release of enzymes are among the possible modes of action (Galindo and Salcedo, 1996).

Since this strategy was proved to be successful in the production of extracellular enzymes, addition of surfactants into the fermentation broth may be an effective strategy to increase the yields of EPS in SmF. It was reported that surfactants improved the yield and quality of EPS in cultures of *Xanthomonas campestris*, with 0.1 g/L Triton X-100 having a 1.5-fold increase in the production of the xanthan gum (Galindo and Salcedo, 1996). It was suggested that one mode of the action of the surfactant is by altering oxygen transfer in this study. Another report showed that surfactant (Tween 80) could significantly increase the production of botryosphaeran, an EPS of the (1→3; 1→6)-β-D-glucan produced by the fungus *Botryosphaeria rhodina* MAMB-05 (Silva et al., 2007). More detailed study of the stimulatory effect of Tween 80 on the EPS production in SmF of mushroom mycelia will be discussed in section 1.4.2.

1.4.2 Tween 80

Tween 80, a trade name representing polyoxyethylene sorbitan monooleate, is a non-ionic surfactant that is derived from polyethoxylated sorbitan containing 21.6% by weight of a monounsaturated fatty acid (mainly oleic acid). Tween 80 has been successfully applied as a vehicle for enhancing the production of microbial extracellular metabolites including EPS and enzymes in some bacteria and fungi.

It was reported that Tween 80 improved the yield and quality of a well-known bacterial EPS (xanthan) in cultures of *Xanthomonas campestris* (Galindo and Salcedo, 1996). In another study, an extracellular peroxidase (ligninase) production by *Phanerochaete chrysosporium* INA-12 was markedly enhanced in the presence of Tween 80 (Asther et al., 1987). Addition of 0.1% Tween 80 to a culture medium of

Aspergillus niger increased the amount of pectolytic enzymes excreted by 70% (Nemec and Jernejc, 2002).

Compared to the wide application in the bacteria and other fungi, the use of surfactants such as Tween 80 to increase mushroom mycelial EPS production by submerged fermentation is a relatively hot spot in recent studies. However, the effect of Tween 80 on mushroom EPS production was inconsistent. For example, addition of 0.6% (v/v) Tween 80 enhanced EPS production by 47.9% in submerged fermentation of *Schizophyllum commune* (Hao et al., 2010). In contrast, addition of Tween 80 had shown an increase in the mycelial growth of *Grifola frondosa* but a decrease in EPS production was found at the same time (Hsieh et al., 2008). Addition of Tween 80 in the submerged fermentation of *Collybia maculate* TG-1 did not affect both mycelial growth and EPS production compared to the control (Lim and Yun, 2006).

Although results on the use of stimulatory chemical agents to increase the yield of extracellular fungal metabolites are accumulating, the underlying mechanisms of their actions are poorly understood and rarely reported. Researchers who studied the use of Tween 80 to enhance the growth and the production of extracellular metabolites from microorganisms had only proposed some mechanisms but without any substantial proof. Some generic factors such as the choice of carbon source, increase in cell membrane permeability, change in microbial physiology and membrane-associated enzymatic functions have been suggested as the possible modes of action of Tween 80 (Galindo and Salcedo, 1996; Nemec and Jernejc, 2002; Taoka et al., 2011). The underlying mechanisms of the stimulatory effect of Tween 80 on extracellular fungal metabolites are worth investigating.

1.5 Proteomics

It is well known that nearly all cellular functions are determined by the activity of proteins and proteins act as catalysts, receptors or structural components that are required for the life of a cell. Cellular processes are performed by complexes of many different proteins. Therefore, an understanding of cellular functions at the molecular level requires knowledge of the patterns of expression of all of the component proteins (Graves and Haystead, 2002).

Proteomics offer a holistic and integrated view of the entire protein complement expressed by a genome, a cell or a tissue, namely the proteome (Graves and Haystead, 2002). Unlike genome, proteome is more dynamic and complex as chemistry of proteins is more heterogeneous as compared with nucleic acids. Many techniques have been described for separation of cell proteins, for instance, centrifugation, membrane dialysis, immunoprecipitation, electrophoresis and chromatography (Pitarch et al., 2003). The most common approach for handling large number of proteins in proteomics analysis involved one-dimensional (1D) or two-dimensional (2D) gel electrophoresis for separation, visualization and quantification of thousands of proteins and the subsequent identification and characterization by mass spectrometry (MS). Hence, proteomics can be used to determine the complement of proteins that are expressed in a fungal cell and how they are changed under different conditions. Although the development of proteomics is rapid, investigations to find out the protein expression changes in mushroom mycelia under different culture conditions are very few at present. Such researches can give us a better understanding of the relationship between the chemical conditions of the mycelial fermentation and fungal metabolism in terms of cellular and molecular mechanisms.

1.5.1 One-dimensional (1D) and two-dimensional (2D) gel electrophoresis

In general, protein purification and separation start with a whole cell-lysate and end with gel-separated protein band or spot by using 1D or 2D gel electrophoresis.

One-dimensional gel electrophoresis commonly refer to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, which separates proteins based on molecular mass. When it is coupled with tandem mass spectrometry (MS/MS), this technique can be used to identify proteins in moderately complex mixtures. For more complex protein mixtures which are not sufficiently resolved in a 1D separation, a multidimensional separation may be necessary (Delahunty and Yates, 2005). Multidimensional separations exploit two or more independent physical properties of the proteins to achieve a higher level of resolution and higher loading capacity than that can be achieved in a 1D separation. Separation strategies can be chosen so that protein components not separated in the first dimension could be separated in the second. 2D gels are the most common multidimensional separation technique used to separate proteins in very complex mixtures. In the first dimension, proteins are resolved according to their isoelectric points (pI) by isoelectric focusing (IEF). Proteins are separated within an immobilized pH gradient (IPG) strip, in which all individual proteins migrate until they reach their corresponding pI, at which their net charge is. After IEF, the IPG strips are subjected to a second dimension separation, which separate proteins on the basis of their corresponding molecular mass through SDS-PAGE. The position occupied by a protein spot on a 2D gel is an indication of its approximate pI and molecular mass.

1.5.2 Mass spectrometric analysis

According to the definition given in the International Union of Pure and Applied Chemistry (IUPAC), mass spectroscopy (MS) is "the study of systems by the formation of gaseous ions, with or without fragmentation, which are then characterized by their mass-to-charge ratios (m/z) and relative abundances" (Todd, 1995).

Because of the low abundance of many of the proteins and the high degree of complexity of cellular proteomes, there are great demands for the highly sensitive analytical techniques. MS has become a vital enabling technology in the field of proteomics (deHoog and Mann, 2004), which involves protein identification in very complex mixtures such as cell lysates, tissues, or other biological samples, as well as

the identification of interacting partners, quantification of protein expression levels, characterization of modifications, and studies of non-covalent protein complexes (Deshaies et al., 2002). In most cases, proteomics involves an initial separation step, usually done by 1D or 2D gel electrophoresis, followed by the proteolysis with a site-specific protease (e.g. trypsin) to generate peptides, together with further separation/enrichment steps if necessary, and then finally the MS analysis of the peptide mixtures.

MS analyses are carried out in the gas phase on ionized analytes. Generally, a mass spectrometer encompasses a sample introduction system, an ion source, a mass analyzer which measures the m/z of the ionized analytes, and an ion detector that provide a quantitative measure of the number of ions at each m/z value. Because their functions are frequently interconnected, they are shown in Figure 1.3 as a series of interrelated boxes rather than as separate components.

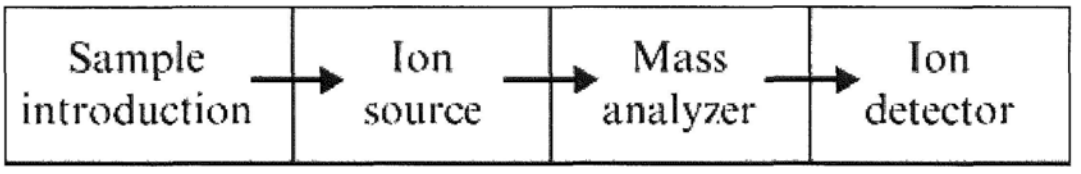


Figure 1.3 The basic components of a mass spectrometer

Two ionization techniques developed in the late 1980s, namely electrospray ionization (ESI) (Fenn et al., 1989) and matrix-assisted laser desorption ionization (MALDI) (Karas and Hillenkamp, 1988) have become the most commonly used tecniques in MS to volatize and ionize the proteins or peptides even at present.

ESI ionizes the samples out of an analyte solution and is readily coupled to liquid-based (e.g. chromatographic and electrophoretic) separation tools. It involves spraying a continuous stream of analyte solution from a needle held at a high potential into a chamber at atmospheric pressure. Formation of droplets and evaporation of the solvent produces a continuous stream of ions which are applied to mass analyzers (Figure 1.4).

In contrast, MALDI is a solid state sputtering/desorption method that sublimates and ionizes the samples out of a dry, crystalline matrix via laser pulses. When carried out in high vacuum and at high accelerating voltage, the pulsed nature of laser radiation produces ions in pulses (Figure 1.5).

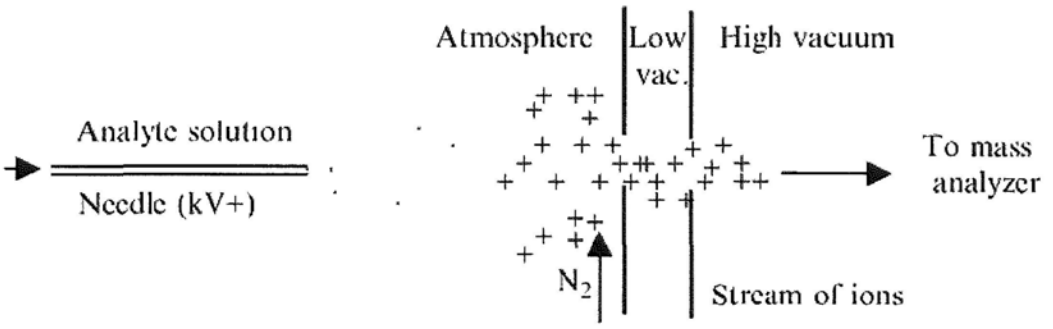


Figure 1.4 Principle of electrospray ionization (ESI)

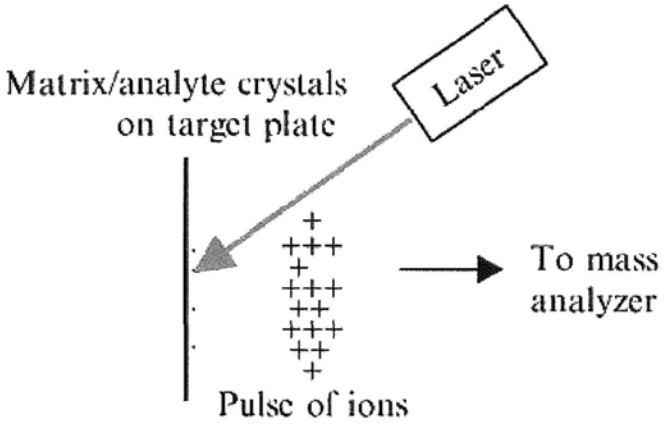


Figure 1.5 Principle of matrix-assisted laser desorption ionization (MALDI)

The mass analyzer is central to the MS analysis. For proteomics, its key parameters are resolution, sensitivity, mass accuracy and the ability to develop information-rich ion mass spectra from peptide fragments. There are four basic types of mass analyzer currently used in proteomics research. These are time-of-flight (TOF), quadrupole, ion trap and Fourier transform ion cyclotron (FT-MS) analyzers. They are distinct in design and performance, corresponding with their own strengths and weaknesses. These analyzers can be applied alone or, in some cases, put together in tandem to make use of their advantages (Mann et al., 2001).

Due to the different characteristics of each method, the choice of ionization technique largely decides the nature of the mass analyzer. ESI is usually coupled to triple quadrupole and ion traps analyzers and used to produce fragment ion spectra (collision-induced (CID) spectra) of selected precursor ions whereas MALDI has mostly been coupled to TOF analyzers that measure the mass of intact peptides (Aebersold and Goodlett, 2001). MALDI-MS is usually applied to analyze relatively simple peptide mixtures, while integrated liquid-chromatography ESI-MS systems (LC-MS) are preferred for the analysis of more complex protein samples.

1.5.3 Proteomic analysis of microbial cells under different environmental conditions

Proteomics has been widely used to study the effect of culture conditions on protein expression. Basically, it compares protein patterns of a given microorganism strain which is subjected to different environmental conditions. Whole cell protein content is separated by 1D or 2D gel electrophoresis and the changes in protein expression are identified by mass spectrometric analysis. This method is probably the most straightforward and most commonly used one nowadays in the world for many types of microorganisms.

For example, studies of the adaptive responses to acid in several lactic acid bacteria (LAB) by 2D gel electrophoresis had revealed the induction of a large number of proteins. The responses to acid induced 33 proteins in *L. lactis* (Hartke et al., 1996), 64 in *S. mutans* (Svensater et al., 2000) and 29 in *S. oralis* (Wilkins et al., 2001) during the exponential growth or stationary phase, respectively. For all the LAB studies, 2D gel electrophoresis confirmed the induction of some subunits of the F_0F_1 ATPase (also called H^+-ATPase) which could expel protons from the bacteria at low pH environment.

Another study reported how a common yeast *Saccharomyces cerevisiae* adapted its metabolism during the exponential growth on three different concentrations of glucose (Francesca et al., 2010). It was noticed that differential

expressions of groups of proteins appeared at different concentrations of glucose. In both glucose restriction and in glucose abundance, an over-expression of a protein (Peroxiredoxin) involved in protection against the oxidative stress damage was found. The molecular basis of environmental variations such as fermentation in different glucose concentrations could be investigated by using the proteomics (Francesca et al., 2010).

Oda et al. (2006) reported a comparative proteomic analysis of extracellular proteins from a filamentous fungi *Aspergillus oryzae* grown in submerged and solid-state fermentation conditions. From the proteomic analysis and Northern blot analysis, it was suggested that the secretion of α-amylase and β-glucosidase was regulated by trapping these proteins in the cell wall in submerged fermentation and that the secretion of glucoamylase A and xylanase G2 was regulated at the post-transcriptional level in the solid-state fermentation (Oda et al., 2006).

Therefore, proteomic techniques can be used to better understand the molecular mechanism of microbial/fungal metabolic changes and provide great insight which would have been difficult to obtain by conventional metabolic studies.

1.6 Objectives

Polysaccharides are the best known and most potent mushroom-derived substances with a number of medicinal properties. The non-starch polysaccharides extracted from the sclerotium, mycelium and culture medium of *Pleurotus tuber-regium* (PTR) have been demonstrated to have both immunomodulatory and direct cytocidal antitumor activities (Wong et al., 2007; Zhang et al., 2001, 2003). Growing the mycelium of PTR in a defined medium by submerged fermentation has been found to be a simple, fast and efficient alternative method for producing exopolysaccharides (EPS) that have similar bioactivities as the polysaccharides isolated from this edible mushroom (Wong et al., 2007). Although the influence of conventional factors such as carbon and nitrogen source in the optimization of culture conditions in submerged fermentation of PTR have been reported previously (Wu et al., 2003), addition of some chemical agents to the fermentation broth to stimulate mushroom mycelial EPS production have attracted an increasing interest recently. Moreover, reports on how the culturing conditions influence fungal metabolism in the production of mycelial EPS in terms of cellular and molecular mechanisms are very rare. The underlying mechanisms of these biological activities still remain largely unknown.

In this project, stimulatory agents with different chemical nature were applied in submerged fermentation of PTR to study their effects on mycelial growth and EPS production. Moreover, this project aims at finding out the underlying mechanisms of how the addition of stimulatory agents can affect the production of mushroom mycelia and EPS. The mechanisms of these biochemical processes will be investigated by using physical and chemical methods as well as molecular techniques such as the proteomic analysis.

This project will provide significant insights into the relationship between the stimulating agents and mushroom mycelial biomass and EPS production by finding out the molecular mechanisms on how the expression changes of related proteins under different conditions control and adjust the secretion of metabolites, especially EPS. The correlation between the appropriate use of stimulating agents and maximal

accumulation of mushroom mycelial EPS will facilitate the development of the production of bioactive mushroom polysaccharides in the food and pharmaceutical industries. This project will also expand our knowledge of using stimulating agents for the enhancement of mushroom mycelial biomass and EPS production with potential application to the production of other mushroom metabolites with commercial interest. Moreover, the use of molecular techniques such as the proteomics analysis in this area will facilitate a deeper understanding on the effect of stimulant on the development of mushroom mycelia at the molecular level.

In general, the main objectives of this project were as follows:

a) To investigate the effects of different kinds of stimulatory agents such as fatty acids, surfactants and organic solvents on the production of mycelial biomass and EPS by submerged fermentation of PTR mycelia;

b) To characterize the changes in the structural composition of mushroom mycelial EPS before and after addition of stimulatory agents, including the total carbohydrate content, protein content, monosaccharide composition, molecular weight and glycosidic linkages;

c) To investigate the changes in bioactivity of mushroom mycelial EPS before and after addition of stimulatory agents by studying their cytotoxic effects against some tumor cell lines;

d) To investigate the underlying mechanisms of the influence of stimulatory agents on fungal metabolism in the production of mycelial EPS by using physical and chemical methods as well as molecular techniques such as the proteomic analysis.

2 Materials and methods

2.1 Inoculum and culture conditions

The slant culture of PTR was obtained from Fungi Perfecti. Ltd. Co (Olympia, USA). Stock culture was maintained on potato dextrose agar (PDA) plate and sub-cultured periodically.

The inoculum was prepared by transferring 25 pieces of 10-day-old mycelia each with an area of 1 cm^2 cut from the PDA plate with a sterilized cutter to 50 mL of defined culture medium in a 250 mL conical flask and incubated for 4 days. Shake-flask fermentation experiments were carried out in 250 mL conical flasks containing 100 mL of defined culture medium after inoculating with 10% (v/v) of the inoculum. The defined medium contained: 30 g/L glucose, 4 g/L yeast extract, 1g/L KH_2PO_4, 0.6 g/L $Mg_2SO_4 \cdot 7H_2O$. The cultures were incubated at 30 °C in a rotary shaker incubator at 200 rpm. The overall process of inoculating the mycelial culture of PTR is shown in Figure 2.1.

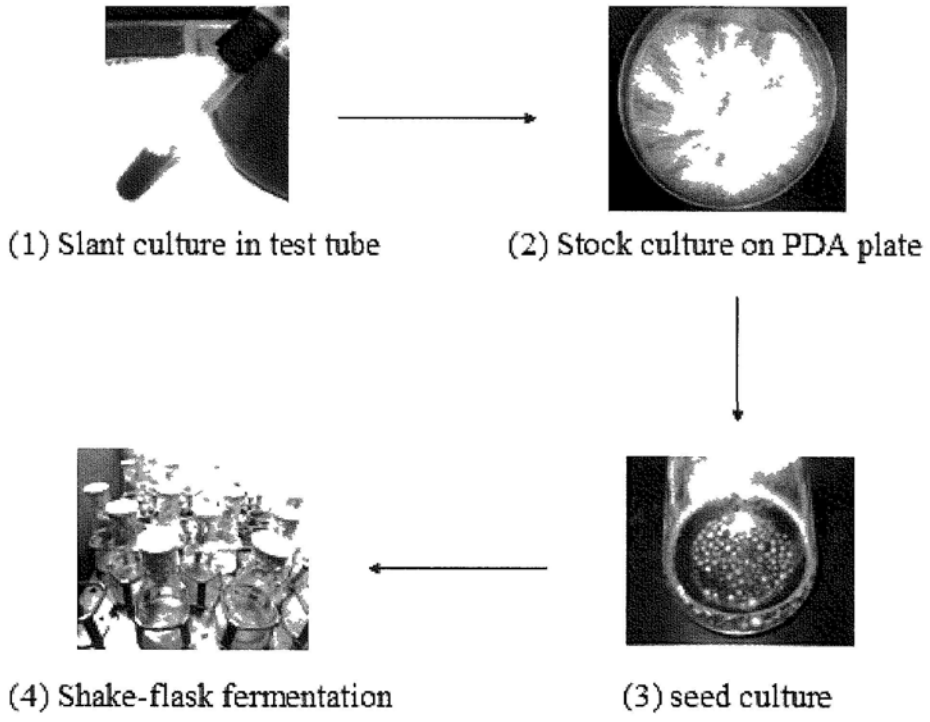


Figure 2.1 Inoculation of the mycelial culture of PTR

2.2 Chemical agents used for study their stimulatory effects

A total of 13 chemical agents in 3 categories were chosen based on their applications in previous studies (Chen et al., 2010; Galindo and Salcedo, 1996; Lim and Yun, 2006; Nemec and Jernejc, 2002; Park et al., 2002; Yang et al., 2000) and they were added into the shake-flask fermentation broth to investigate their potential stimulatory effects. They include:

1) Fatty acids: linoleic acid, oleic acid, palmitic acid, stearic acid;

2) Organic solvents: methanol, ethanol, hexane, chloroform, toluene;

3) Surfactants: Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 80 (polyoxyethylene sorbitan monooleate), CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), Triton X-100 (polyoxyethylene octyl phenyl ether).

All the chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2.1 Effect of fatty acids

The effect of fatty acids on mycelial biomass and EPS productions was studied by applying different fatty acids including linoleic acid, oleic acid, palmitic acid and stearic acid at a concentration of 1.0 g/L (w/v) with reference to previous study (Yang et al., 2000) into the fermentation broth on the 1st day (24 hours) of fermentation. Each culture was carried out in triplicate at 30 ^{o}C for 7 days.

2.2.2 Effect of organic solvents

Five common organic solvents including methanol, ethanol, hexane, chloroform and toluene were chosen for the investigation of their effects on mycelial

growth and EPS production in PTR. Each of these solvents was applied at a concentration of 0.1% (v/v) with reference to previous study (Lim and Yun, 2006) individually into fermentation broth on the 1st day (24 hours) of fermentation. Each culture was carried out in triplicate at 30 °C for 7 days.

2.2.3 Effect of surfactants

Four common surfactants including Tween 20, Tween 80, CHAPS and Triton X-100) were selected for the investigation of their effects on mycelial growth and EPS production in PTR. Individual solvents were applied at a concentration of 1.0 g/L (w/v) with reference to previous study (Nemec and Jernejc, 2002) into the fermentation broth on the 1st day (24 hours) of fermentation. Each culture was carried out in triplicate at 30 °C for 7 days.

2.2.4 Effect of concentration of Tween 80

The effect of the concentration of Tween 80 on mycelial biomass and EPS productions of PTR was studied by applying Tween 80 at different concentrations (0.5, 1.0, 3.0, 5.0 g/L, w/v) into the fermentation broth on the 1st day (24 hours) of fermentation. Each culture was carried out in triplicate at 30 °C for 7 days.

2.2.5 Effect of addition time of Tween 80

The effect of the addition time of Tween 80 on the mycelial growth and EPS production of PTR was studied by applying 3.0 g/L (w/v) Tween 80 on the 1st day (24 hours), 3rd day (72 hours) and 5th day (120 hours) of fermentation, which represented the initial, intermediate and late stage of exponential growth phase of the mycelia. Each culture was carried out in triplicate at 30 °C for 7 days.

2.3 Determination of the yield of Mycelia and EPS

At the end of the fermentation period, the mycelia were separated from the fermentation broth by filtering through a Whatman #4 filter paper. The mycelia obtained were washed twice with distilled water and then the yield of mycelial

biomass was determined gravimetrically after lyophilization. Four volumes of 95% ethanol were added to the mycelium-free fermentation broth to precipitate the EPS overnight. The precipitated EPS were separated by centrifugation at 3838 g for 15 minutes and the supernatant was discarded. The yield of EPS was determined gravimetrically after lyophilization.

2.4 Chemical structure of EPS

2.4.1 Carbohydrate content

The carbohydrate content of the EPS was estimated by the phenol-sulphuric acid method (Dubois et al., 1956). Half a milliliter of phenol solution (5% w/v in water) was added into 0.5 mL of glucose standards (12.5 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL in 2 M sulfuric acid), blank solution (2 M sulfuric acid) and sample solution (prepared by dissolving about 5 mg sample into 5 mL of 2 M sulfuric acid and then further dilute 20 times), respectively. Two and a half milliliter of 18 M sulfuric acid was then added rapidly into the solution and vortex-mixed. After 30 minutes reaction, the absorbance of the solution was determined at 490 nm by an UV-VIS spectrophotometer (Genesys5, Spectronic Instruments, USA). A standard curve passing through the origin was plotted by absorbance at 490 nm (OD_{490}) against concentration of glucose standard. And then the carbohydrate concentration of samples [carbohydrate] was determined by reading against the standard curve ($OD_{490sample}$). Carbohydrate content expressed as percentage by weight was calculated by the following equation:

$$\text{Carbohydrate content} = \frac{\text{concentration of carbohydrate (mg/mL)} \times 20 \times 5\text{ (mL)}}{\text{sample weight (mg)}} \times 100\%$$

2.4.2 Protein content

The protein content of the EPS was estimated using protein assay kit (Sigma, St. Louis, MO, USA) which was based on the modified Lowry method described by Peterson (1977). Sample with a concentration of 500 μg/mL was prepared by dissolving about 5 mg (sample weight) into 10 mL distilled water for the protein

determination. Lowry reagent (1.0 mL) was added into 1.0 mL of sample, blank (distilled water) and protein standards (BSA solution, 25 μg/mL, 50 μg/mL, 100 μg/mL and 150 μg/mL), respectively. The mixture was vortex-mixed and allowed to stand at room temperature for 20 minutes. Half a milliliter of Folin and Ciocalteu's phenol reagent was added into the sample, blank and standard, respectively, with rapid mixing and incubated at room temperature for 30 minutes. The absorbance of the final solution was determined at 750 nm. A standard curve was plotted by using absorbance at 750 nm against concentration of BSA. The protein concentration of sample [protein] was determined by reading against the calibration curve. Protein content expressed as percentage by weight was calculated by the following equation:

$$\text{Protein content} = \frac{\text{concentration of protein (mg/mL)} \times 2 \times 5\ \text{(mL)}}{\text{sample weight (mg)}} \times 100\%$$

2.4.3 Molecular weight (M_w) determination

Size exclusion chromatography (SEC) is one type of HPLC which is widely used to separate large molecules such as polysaccharide and protein. During the separation, smaller molecules in the samples will be trapped in the pores of the column and take longer time to be eluted out than the larger molecules which are too large to enter the pores. The elution time is inversely correlated to the molecular weight of the sample. Eluent from the column is analyzed by one or more detectors such as refractive index (RI) and ultraviolet radiation (UV) which provide retention time for samples (Brummer and Cui, 2005).

TSK-Gel PW columns are commonly used in high performance size exclusion separation of industrial water-soluble polymers and oligosaccharides. The hydrophilic polymer matrix in these columns has excellent chemical and mechanical stability. Although commonly used with aqueous solvents, the polymer matrix is compatible with up to 50% organic solvent. PW-type resin is less hydrophilic than polysaccharide gels. Thus, addition of organic modifier or a decrease in salt concentration sometimes is needed to reduce hydrophobic interaction. TSK-Gel PW resins have a small residual negative charge (Supelco, 2000).

A TSK gel G5000 PW size exclusion column (SEC) (30 cm x 7.5 mm i.d., Cat. # 8-05764, Supelco) with a PWH Guard column (7.5 cm x 1.5 mm i.d., Cat. # 8-06762, Supelco) was used to determine the M_w of EPS according to the SEC profile provided by a range of pullulan standards (6, 12, 24, 48, 112, 212, 404, 788 kDa; Shodex Standard P-82, Showa Denko). The flow rate of the eluent (0.2 M sodium chloride) was 0.8 mL/min and the temperature of the column was controlled externally at 25 °C. The samples and standards were dissolved in 0.2 M sodium chloride, filtered by the 0.45 μm Millipore filter, passed through the size exclusion column, and then were detected by a Waters 2414 refractive index (RI) detector. A calibration curve was plotted by using the retention time of pullulan standards against the log value of their molecular weights. M_w of EPS was estimated from the calibration curve.

2.4.4 Monosaccharide composition analysis

The EPS of PTR was first acid hydrolyzed (2.4.4.1) and then was derivatized to alditol acetates according to the method described by Blakeney et al. (1983) (2.4.4.2). The derivatives of EPS were separated and analyzed by gas chromatography (GC) (2.4.4.3).

2.4.4.1 Acid hydrolysis

About 15 mg samples was hydrolyzed with sulfuric acid (0.7 mL, 12 M) and kept in a water bath at 35 °C for 60 minutes with vortex-mixing in every 15-minute time interval. Afterwards, the mixture was diluted to 2 M sulfuric acid by an addition of 3.5 mL distilled water and kept in a water bath at 95 °C for another 60 minutes with vortex-mixing in every 15-minute time interval.

2.4.4.2 Sugar derivatization

Three milliters of the above acid hydrolyzate and 3 mL sugar standard (prepared by dissolving 0.1 g of L-rhamnose (Rha, Sigma), D-fucose (Fuc, Sigma), D-ribose (Rib, Sigma), L-arabinose (Ara, Sigma), D-xylose (Xyl, Sigma), D-galactose (Gal, Sigma), D-glucose (Glc, Supelco), D-glucosamine (GlcNAc,

Sigma) and D-galactosamine (GalNAc, Sigma) in 100 mL 50% saturated benzoic acid). One milliliter of D-allose (Sigma, 1 mg/mL in 50% saturated benzoic acid) was then added in both the acid hydolysate and sugar standard. Afterwards, the pH of the solutions was made alkaline by drop-wise addition of 12 M ammonia solution to acid hydrolyzate and 2 M ammonia solution to sugar standard, accordingly. The monosaccharide in these mixtures were reduced by keeping at 40 °C for 30 minutes after the addition of 5 μl octan-1-ol and freshly prepared sodium borohydride (0.2 mL, 200 mg/mL in 2 M ammonia). Afterwards, 0.4 mL glacial acetic acid was added to degrade the excess sodium borohydride. An aliquot (0.2 mL) of the reduced monosaccharides was acetylated by vortex-mixing with 2.0 mL acetic anhydride with 0.3 mL 1-methylimidazole as catalyst at room temperature for 10 minutes. Five milliliters of distilled water was then added to degrade the excess acetic anhydride and the mixture was cooled under tap water at room temperature. Extraction of the alditol acetate derivatives was done by vortex-mixing the mixture with 1 mL dichloromethane. After standing for 10 minutes for phase separation, the upper phase was carefully removed and the lower phase was washed twice with 1 mL of distilled water. The lower phase was dried with anhydrous sodium sulfate (Sigma) and then stored in vial at -20 °C before GC analysis.

2.4.4.3 Determination of sugar derivatives by GC analysis

The alditol acetates derivatives of the monosaccharides were quantified by an HP6890 Series II gas chromatography, using an Alltech DB-225 capillary column (15 m×0.25 mm id., 0.25 μm film) with the following oven temperature program: initial temperature, 170 °C; temperature rise at 2 °C/min to 220 °C and final hold for 15 minutes. The temperature of the injector and detector were set at 220 °C. The carrier gas was helium and detection was made by flame ionization. Individual sugars were corrected for losses during hydrolysis and derivatization, and for their different responses to the flame ionization detector.

Calibration was made with the ten monosaccharides mentioned above. N-acetylglucosamine (an amino sugar component of chitin) was detected as glucosamine due to the fact that the N-acetyl group at C-2 would be deacetylated during the sulfuric acid hydrolysis. The amount of sugar standards detected was

compared with the internal standard to monitor recovery because of the losses during acid hydrolysis and derivatization as well as to correct for the different response factors (R_f) of the flame ionization detector to individual monosaccharides. The values for monosaccharides were expressed as polysaccharide residues (anhydro-sugars) by multiplying the amounts of pentoses, hexoses and deoxypentoses with a coefficient factor of 0.88, 0.90 and 0.89, respectively.

The amount of the monosaccharides was calculated as below:

$$\text{Amount of individual monosaccharide (mg)} = \frac{\text{Peak area}_{\text{monosaccharide}} \text{ in sample} \times \text{Amount of allose (mg)}}{\text{Peak area}_{\text{allose}} \text{ in sample}}$$

After determining the amount of individual monosaccharides, their corresponding percentages by weight in the sample were calculated as follows:

$$\text{Monosaccharide \% by weight} = \frac{R_f \times \text{coefficient} \times \text{Amount of monosaccharide (mg)} \times 100\%}{\text{Sample weight (mg)}}$$

R_f – response factor obtained by the equation below:

$$R_f = \frac{\text{monosaccharide concentration in standard (mg/mL)} \times 1.5\ \text{mL} \times \text{area of allose in standard}}{\text{area of monosaccharde in standard} \times 1\ \text{mg/mL} \times 1\ \text{mL}}$$

The relative amount (normalized) of monosaccharide as % of total monosaccharide in the sample was calculated as follows.

$$\text{Normalized monosaccharide \%} = \frac{\text{\% of individual monosaccharide} \times 100\%}{\text{Total \% monosaccharide in the sample}}$$

2.4.5 Glycosidic linkages

The sugar linkages of the EPS were analyzed by methylation using the method described by Anumula and Taylor (1992) followed by GC-MS of the partially methylated alditol acetate (PMAA) derivatives.

2.4.5.1 Preparation of dry dimethyl sulfoxide (DMSO)

Dry DMSO was prepared by distilling 500 mL reagent grade DMSO (Sigma) under nitrogen at reduced pressure for 5 hours. The DMSO was stirred with excess powdered calcium hydride (CaH) for 3 hours at 65 °C in a three-necked round-bottom flask before distillation. The distilled dry DMSO was transferred into a 500 mL brown round bottle with the flushing of nitrogen and was stored over molecular sieve (Acros, 4 Å, pre-dried at 550 °C for 3 hours) at 4 °C.

2.4.5.2 Preparation of methylsulfinyl methyl sodium ($CH_3SOCH_2^-Na^+$)

About 2 g sodium hydride (NaH) powder was prepared by washing appropriate amount of NaH suspended in oil with 30 mL hexane twice to remove the oil. The NaH was dried with argon after the hexane was removed by centrifugation before used. Thirty milliliters of dry DMSO from 2.4.5.1 were added to 2 g NaH in 3-necked flask under moisture-free condition. After leaving the mixture for 30 minutes at room temperature, the color of the final solution should change to grayish green. Otherwise, the mixture was sonicated at 25 °C for 1 hour until the color of the final solution turned grayish green. The final solution ($CH_3SOCH_2^-Na^+$) was then transferred to 5 mL serum bottles and flushed with nitrogen before capped and stored at -20 °C before use.

2.4.5.3 Methylation

EPS (about 4 mg) dried over phosphorus pentoxide overnight under vacuum was dissolved in 1 mL dry DMSO by vortex-mixing and sonication at room temperature for 30-50 minutes. If the polysaccharide remained insoluble in DMSO

after this treatment, premethylation was done by addition of 20 μL $CH_3SOCH_2^-Na^+$ to the mixture which was kept in an ice-bath until frozen, followed by addition of 5 μL pre-cooled methyl iodide (MeI). The premethylation steps were repeated except 60 μL $CH_3SOCH_2^-Na^+$ and 15 μL MeI was used instead. The solution was then sonicated for 60 minutes at room temperature after the addition of 0.4 mL $CH_3SOCH_2^-Na^+$ followed by adding 0.3 mL pre-cooled MeI with sonication for another 15-20 minutes at room temperature. The resulting solution was kept at room temperature overnight.

2.4.5.4 Extraction of methylated polysaccharide

The methylated polysaccharide in the solution from 2.4.5.3 was extracted by adding 4 mL chloroform ($CHCl_3$) after adding 0.3 mL distilled water to neutralize the excess $CH_3SOCH_2^-Na^+$. The chloroform layer was washed 3 times with 3 mL distilled water which was removed by centrifuging at 3000 rpm for 5 minutes. The organic solvent was evaporated under a stream of nitrogen at 40 °C after removing the remaining water by adding 3 mL 2,2-dimethoxypropane and 40 μL glacial acetic acid.

2.4.5.5 Acid depolymerization and preparation of aditol acetate derivatives

The residue from 2.4.5.4 was depolymerized by heating with 0.6 mL 2 M trifluoroacetic acid (TFA) at 121 °C for 1 hour with the acid being removed by drying a stream of nitrogen at 40 °C. The residue was then made alkaline by adding 0.5 mL 12 M ammonia solution. The partially methylated monosaccharide in the solution was reduced and acetylated according to the method described in 2.4.4.2 using 0.2 mL allose (5 mg/mL of 50% saturated benzoic acid) as the internal standard.

2.4.5.6 Determination of partially methylated alditol acetates (PMAAs) by gas chromatography-mass spectrometry (GC-MS)

The alditol acetates derivatives of the partially methylated monosaccharide were analyzed by a gas chromatography (Agilent Technology, 6890N)-mass spectrometry (Agilent Technology, 5973N). The GC conditions were as follows:

Alltech DB-225 capillary column (15 m×0.25 mm id., 0.25 μm film); helium as carrier gas at a flow rate of 1.0 mL/min; initial oven temperature at 160 °C, followed by 4 °C /min rise to 220 °C and final hold for 15 minutes; injector temperature at 280 °C; interface temperature at 280 °C. The MS conditions were as follows: ion source temperature at 250 °C, ionization energy at 70 eV, detector voltage at 1.5 kV, and mass range from 50 to 350. Each PMAA was identified by matching its mass spectrum with the NIST/EPA/NIT data base in the computer.

2.5 Biological activity

2.5.1 Cell lines and their subculture

Cell lines including human hepatocellular carcinoma cells, HepG2 (HB-8065, ATCC), human breast cancer cells, MCF7 (HTB-22, ATCC), human chronic myelogenous leukemia cells, K562 (CCL-243, ATCC), monkey normal kidney cells, Vero (CCL-81, ATCC) and human normal foreskin cells, Hs68 (CRL-1635, ATCC) were grown in tissue culture flasks (NUNC) in different media and conditions as shown below.

HepG2 cells were maintained in Minimum Essential Medium (MEM, GIBCO) while MCF7 and K562 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO). The normal Vero and Hs68 cells were maintained in Roswell Park Memorial Institute (RPMI, Sigma) medium. All media were adjusted to pH 7.4 with the addition of 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% antibiotics (penicillin-streptomycin, Cat. # SV30010, HyClone).

All of the above cell cultures were seeded at a concentration ranged from 1×10^5 to 1×10^6 cells/mL and incubated at 37 °C in a humidified atmosphere of 5% of CO_2. Cells with a density of at least 2.5×10^5 cells were cultured in a 25 cm^2 in tissue culture flask.

They were subcultured about every three days according to the ATCC guidelines. There were a few differences between the procedure of subculture for suspended and adherent cell lines. For the suspended cells such as K562, a part of the cells was transferred into a new tissue culture flask with the addition of 7 mL of fresh medium during subculture. For the adherent cells such as HepG2, MCF7, Vero and Hs68, the medium in the tissue culture flask was first removed and washed with phosphate buffered saline (PBS, pH 7.4) to remove the remaining medium that contained trypsin inhibitor. The cells were trypsinized with 1 mL of trypsin-EDTA solution (2% EDTA and 2.5% trypsin solution were diluted by sterile PBS) for about 2 minutes until they were all detached from the bottom of the tissue culture flask. Together with 4 mL of the corresponding medium, they were subject to centrifugation at 1000 rpm for 5 minutes. Cell pellets were resuspended in fresh medium and part of the cell suspension was transferred to a new tissue culture flask containing 7 mL of fresh medium.

2.5.2 Detection of endotoxin

The presence of endotoxin in EPS of PTR was detected by *Limulus* Amebocyte lysate (LAL) test kit (Cat. #ET0200, Sigma) according to manufacture's instructions. Briefly, 0.1 mL of each sample, 0.1 mL of endotoxin-free LAL reagent water, 0.1 mL of control standard endotoxin at concentrations of 4 EU/mL, 0.5 EU/mL, 0.25 EU/mL, 0.125 EU/mL, 0.06 EU/mL, 0.03 EU/mL and 0.015 EU/mL were added into the single test vials, shaken gently for 10 seconds, and then incubated in a 37 ^{o}C water bath for 1 hour. The test vials were then removed and inverted in one smooth motion. A positive test result was the formation of a hard gel that permits complete inversion of the vial without disruption of the gel.

2.5.3 *In vitro* antitumor activity

Trypan blue dye exclusion assay and Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay were used for testing the cell viability and cell proliferation, respectively. The *in vitro* assays were performed in five replicates.

2.5.3.1 Trypan blue dye exclusion assay

Trypan blue dye exclusion method was applied for suspension cells (K562 cancer cells). Cell viability is measured according to the ability of viable cells to exclude the dye. Cells (2.5×10^3 cells/well) were added to a round-bottomed 96-well plate (Cat. # 83.1835.500, Sarstedt) and incubated for 24 hours. Different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL) of EPS from PTR were added to the cell suspensions and mixed with equal volume of trypan blue dye solution (0.4% in PBS), i.e. 100 μL of cell suspension was mixed with 100 μL of trypan blue dye solution, followed by a 72-hour incubation. And then the living cells that excluded the trypan blue dye were counted using a hemacytometer. The number of living cells (not being stained) in the treatment groups (with addition of EPS) were counted and compared with that in the control group (without addition of EPS) to calculate the inhibition ratio by the formula below:

$$\text{Inhibition ratio (\%)} = \frac{\text{Living cell number (control)}-\text{Living cell number (treatment)}}{\text{Living cell number (control)}} \times 100\%$$

2.5.3.2 MTT assay

MTT (Cat. # M5655, Sigma) assay that reflects the cell viability indirectly was applied for adherent cells including HepG2, MCF7, Vero and Hs68. Reduction of MTT salt by mitochondrial succinate dehydrogenase gives a purplish-blue formazan product, of which color absorbance is measured at 570 nm (Mosmann, 1983). Cells (2.5×10^3 cells/well) were seeded on a flat-bottomed 96-well plate (Cat. # 83.1835, Sarstedt) and incubated for 24 hours. Different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL) of EPS from PTR were added and the mixture was incubated for 72 hours. At the termination of the experiments (72 hours later), MTT (5 mg/mL) were added to the wells and incubated for 5 hours at 37 °C. After the MTT solution was removed by aspiration, the blue formazan reaction product was extracted with 150 μL DMSO and was mixed well using a multi-channel pipette.

Then, the absorbance was measured at a wavelength of 570 nm by a microplate reader (SpectraMAX 250). The inhibition ratio was calculated by the formula below:

$$\text{Inhibition ratio (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100\%$$

2.6 Determination of glucose concentration

Glucose concentration was determined by an enzymatic kit (Biosystems, S.A., Barcelona, Spain) based on the coupled reaction of glucose with glucose oxidase and peroxidase to form a colored complex of which absorbance was measured at 500 nm by an UV-VIS spectrophotometer (Genesys5, Spectronic Instruments, USA).

In brief, 10 μL glucose standard and sample were added into 1.0 mL Reagent A, separately. The mixture was mixed thoroughly and incubated for 10 minutes at room temperature (16-25 °C) or for 5 minutes at 37 °C in water bath. The absorbance (A) of the glucose standard and the sample was measured at 500 nm against the blank (Reagent A).

The glucose concentration in the sample was calculated using the following general formula:

Concentration of sample = Concentration of standard × (Absorbance of sample / Absorbance of standard)

2.7 Mycelial morphology observation by stereo microscope

The morphology of the PTR mycelia was observed by a stereo microscope (SZX16, Olympus, Japan) equipped with a CCD camera (DP71, Olympus, Japan). The mycelial pellet samples were washed thoroughly using ultra pure water and then transferred to a slide before microscopic observation.

2.8 Determination of pH value

The pH value of the fermentation broth was measured by a Beckman 240 pH meter (Beckman coulter, Inc., CA, USA).

2.9 Concentration of Tween 80

The concentration of Tween 80 was determined by measuring its absorbance at 223 nm according to Lestan et al. (1993) with the UV-VIS spectrophotometer. Substrate without Tween 80 was used as blank.

2.10 Analysis of fatty acid composition of PTR mycelia

2.10.1 Fatty acid extraction and esterification

The fatty acid extraction and esterification procedures were modified from the Folch procedures (Christie, 2003).

Fifty microliters of internal standard (IS), heptadecanoic acid (C17:0, 1.013 mg/mL, Sigma, USA) was added into about 10 mg of freeze-dried PTR mycelia sample, followed by 1 mL Reagent 1 (45 g NaOH + 150 mL methanol + 150 mL deionized distilled water). The mixture was sonicated for 15 minutes, vortex-mixed for 10 seconds and kept in a water bath at 80 °C for 30 minutes (Shaking Water Bath SBS30, UK). Two milliliters of Reagent 2 (6 N HCl / methanol, 13:11, v/v) was then added and the mixture was vortex-mixed for 10 seconds and kept at 80 °C for 10 minutes. Afterwards, two milliliters of Reagent 3 (hexane / methyl tert-butyl ether, 1:1, v/v) was added and the sample tube was placed in a laboratory rotator (Roto-Shake Genie, Scientific Industries Inc., USA) and gently mixed end-over-end for 10 minutes. The aqueous phase was removed and discarded. Three milliliters of Reagent 4 (10.8 g NaOH + 900 ml deionized distilled water) was then added to the sample tube and it was gently rotated end-over-end for 5 minutes. Finally, the sample

was centrifuged at 3500 *g* for 10 minutes and all the top phase was collected and evaporated to dryness by flushing with nitrogen. One milliliter of hexane was added to dissolve the residue and the dissolved sample was transferred into GC vial and stored at -20 °C before analysis.

2.10.2 GC analysis of fatty acid methyl esters

Fatty acid methyl esters (FAMEs) were quantified by a HP6890 Series II GC, using an Alltech 007 FFAP capillary column (30 m × 0.25 mm, 0.25 μm film thickness, USA). The injection port and flame-ionization detector were held at 250 °C. The initial oven temperature was set at 80 °C, held for 1 minute and then increased to 200 °C at a rate of 10 °C/min. This temperature was kept for 2 minutes and then increased to 220 °C at a rate of 5 °C/min and held for 20 minutes. Helium was used as the carried gas at a constant flow rate of 1 ml/min. Individual fatty acids were identified by comparing their retention times with those of the fatty acid standards (Sigma, USA). All the GC samples had included C17:0 (heptadecanoic acid, Sigma, USA) as an internal standard and the content of individual fatty acids was calculated from their peak areas relative to that of the internal standard in the GC chromatogram.

2.11 Proteomic analysis

2.11.1 Extraction of total protein

The freeze-dried PTR mycelia (about 500 mg) were ground into fine powder in liquid nitrogen using a pre-chilled pestle and mortar. The mycelial powders of PTR were transferred into the eppendorf tube and used for the following proteomics analysis. Total protein of PTR mycelia was extracted by using phenol extraction protocol with some modifications (Hurkman and Tanaka, 1986; Horie et al., 2008).

The total protein was extracted from the fine powder by addition of 5 mL of extraction media [0.1 M Tris (pH 8.8), 10 mM EDTA, 0.9 M sucrose and 0.4% (v/v) 2-mercaptoethanol] and 5 mL of Tris (pH 8.8) buffered phenol, followed by gently

inverted mixing at room temperature for 30 minutes. The suspensions were centrifuged at 3838 *g* for 20 minutes at 4 °C. After centrifugation, the top phenol phase was transferred to a new eppendorf tube. The remaining aqueous phase at the bottom was re-extracted once. The top phenol phase from the second extraction was combined with the first extraction and vortexed. Soluble proteins in this phenolic extract were precipitated by addition of five volumes of 0.1 M ammonium acetate in 100% methanol, follwed by vortex-mixing and standing at -20 °C overnight. The preciptitated protein was collected as pellet by centrifugation at 3838 *g* for 20 minutes at 4 °C. The pellet was washed twice with 1 mL of 0.1 M ammonium acetate in 100% methanol, then with 1 mL of 80% ice-cold acetone, and finally once with 1 mL of ice-cold 70% ethanol. Following centrifugation (at 3838 *g* for 20 minutes at 4 °C), the supernatant was discarded and the pellet was dried at 37 °C for 10–15 minutes. Proteins were redissolved in 1 mL of rehydration solution [8 M urea, 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue and 2% (v/v) IPG buffer], followed by centrifugation at 3838 *g* for 20 minutes at 4 °C. The clear supernatant containing the soluble proteins was used for protein quantification by the PlusOne 2D Quant Kit (GE Healthcare) and stored in aliquots at -80 °C.

2.11.2 Protein quantification

Each protein concentration was measured using the PlusOne 2D Quant Kit (GE Healthcare). A standard curve was prepared for calibration using the 2 mg/mL bovine serum albumin (BSA) standard solution provided in the kit. The calibration range was from 0.5 to 50 μg. Five hundred microliters of the protein sample solution from section 2.11.1 were added in each tube, including the BSA standard ones. The tubes were vortexed briefly and then incubated at room temperature for 3 minutes. Then 500 μL of co-precipitant was added to each tube, mixed by inversion and the tubes were centrifuged at 10000 *g* for 5 minutes. A small pellet of protein should then be visible. In order to avoid resuspension, the supernatant was decanted rapidly. The tubes were repositioned in the centrifuge with the cap-hinge and the pellet pointing outward. The tubes were then centrifuged briefly again in order to bring the remaining fluid to the bottom of the tubes. The remaining supernatant should be completely removed. Then, 100 μL copper solution and 400 μL distilled water was added to each tube and the precipitated protein should be totally dissolved. One

milliliter of the working reagent, which was prepared by mixing 100 parts of color reagent A with 1 part of color reagent B, was added to each tube. The tubes was mixed by inversion and incubated at room temperature for 15~20 minutes. Finally, the absorbance of all the tubes including the BSA standard tubes was measured at 480 nm. A calibration curve of the absorbance of the BSA standards against the amount of protein was plotted and the protein concentration of the samples was determined using the standard curve.

2.11.3 One-dimensional gel electrophoresis and mass spectrometry analysis

2.11.3.1 One-dimensional gel electrophoresis

Forty micrograms of proteins in rehydration solution were mixed with 1/3 volume of loading buffer [16% (w/v) SDS, 48% (v/v) glycerol, 2.4% (w/v) Tris base, 8% (v/v) β-mercaptoethanol and 0.1% (w/v) bromophenol blue] and boiled for 4 minutes at 94 ^{o}C. The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using gels (4% stacking gel and 12% separating gel) on a vertical electrophoresis unit at constant voltage of 110 V. The running buffer was composed of 25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS. About five microliters of the commercially available Broad range SDS-PAGE standards (prestained, Bio-Rad, Hercules, CA) was loaded at the same time in the well next to the samples.

After the SDS-PAGE run, the gel was washed with Milli-Q water and then stained with 50 mL Coomassie staining solution which contains 45% (v/v) methanol, 10% (v/v) acetic acid and 0.15% (w/v) Coomassie Brilliant Blue R350 for 1 hour. The gels were finally destained overnight using 100 mL destaining solution containing 45% (v/v) methanol, 10% (v/v) acetic acid in Milli-Q water.

2.11.3.2 In-gel digestion and peptide extraction

After distaining, each lane of sample was sliced into four pieces and further cut into about 1 mm cubes using a clean razor blade on a clean glass surface. The

cubes were transferred into Eppendorf tube. About 25~35 μL acetonitrile (ACN) was added to each tube to cover the gel pieces and incubated for 10 minutes at room temperature to dehydrate and shrink the gel pieces. After vaccum-drying using Speed-Vac (LABCONCO), protein gel pieces were swelled by 150 μL 10 mM Dithiothreitol (DTT) in 100 mM ammonium bicarbonate (NH_4HCO_3) and incubated for 1 hour at 56 ^{o}C. The mixture was cooled to room temperature. Then the DTT solution was replaced by 150 μL 55 mM iodoacetamide (IAA) in 100 mM NH_4HCO_3 and incubated for 45 minutes at room temperature in the dark with occasional vortex. The solution was removed and 150 μL ACN was added to dehydrate gel pieces followed by 10-minutes incubation. After vaccum-drying using Speed-Vac, the gel pieces were incubated in 25~35 μL digestion buffer [12.5 ng/μL trypsin (sequencing grade modified trypin, Promega) in 50 mM NH_4HCO_3] for 45 minutes in an ice water bath. The trypsin-containing buffer was removed. Ten microliters of 50 mM NH_4HCO_3 was added to keep gel pieces wet during cleavage and the mixture was incubated over night at 37 ^{o}C. The mixture was centrifuged at 10000 *g* and the supernatant was stored in a new Eppendorf tube. Twenty microliters of 20 mM NH_4HCO_3 was added to the gel pieces, followed by incubation for 10 minutes and the supernatant was removed and combined with the previous extracts. About 25 μL extraction buffer (50% ACN, 5% formic acid) was added to the gel pieces and incubated for 20 minutes. This supernatant was combined with the previous ones. Finally, the extracted sample in the pooled supernatants was completely dried by Speed-Vac and store at -80 ^{o}C until MS analysis.

2.11.3.3 nESI-LC-MS/MS Analysis

Just before MS analysis, the trypsin-digested peptides were desalted by using the $ZipTip_{u\text{-}C18}$ (Millipore) treatment. Then the desalted peptides were separated by a C18 reverse-phase column and analyzed on a nano-electrospray ionization mass spectrometer (nESI-LC-MS/MS). A Ultimate 3000 (Dionex, USA) nanoLC system, combined with the Ultimate 3000 auto-sampler were used. The samples were loaded onto a pre-column (5 mm × 300 μm i.d.; Acclaim@PepMap100 C18, Dionex, USA) and an analytical column (15 cm × 75 μm i.d.; Acclaim@PepMap100 C18, Dionex, USA). The nanoflow rate of the loading solvent was 300 nL/minute and the samples were subjected to a 60 minutes gradient elution program which was shown in Table

2.1. The column outlet was coupled directly to the high voltage ESI source, which was interfaced to the Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Apex Ultra 7.0, Bruker, USA). The nanospray voltage was typically 1.7 kV in the nESI-LC-MS/MS mode. The *m/z* range was 200~2200 Da.

Acquired data were searched against the National Center for Biotechnology Information (NCBI) non-redundant protein database (fungi) using the MASCOT software package (Version 2.3, Matrix Science, UK; www.matrixscience.com). The peptide mass and MS/MS tolerance were both 0.05 Da. The peptides have the allowance of one tryptic missed cleavage and also with Carbamidomethyl (C) fixed modification and with variable modification by oxidation (M). The peptides could be charged at (1+, 2+ and 3+). The protein hit with a confidence level higher than 95% ($p < 0.05$) was considered as an identified protein.

Table 2.1 The gradient elution program of loading solvents

Time (minute)	Solvent A (%)	Solvent B (%)
0	100	0
8	100	0
38	45	55
39	20	80
44	20	80
45	100	0
60	100	0

Solvent A was 2% acetonitrile with 0.1% formic acid while solvent B was 80% acetonitrile with 0.1% formic acid.

2.11.4 Two-dimensional gel electrophoresis and mass spectrometry analysis

2.11.4.1 Two-dimensional gel electrophoresis: IEF and SDS-PAGE

Isoelectric focusing (IEF) was performed using a Ettan IPGphor III isoelectric focusing system (GE Healthcare). The sample was quantified by PlusOne 2D Quant Kit as mentioned in the Protein quantification section and normalized to

600 μg. Rehydration solution (8 M urea, 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue and 2% (v/v) IPG buffer of corresponding pH range) was added to make up to a volume of 250 μL. Just prior to focusing, 18 mM DTT was added to the sample. The prepared sample was applied to the Immobilized gradient (IPG) strips (13 cm, nonlinear pH 3~10, GE Healthcare).

In the presence of sample, rehydration was first carried out at 0 V for 8 hours. To facilitate the entry of high-molecular-weight proteins into IPG strips, low voltage (30 V for 6 hours) was applied afterwards. IEF was then performed with 500 V for 1 hour, 1000 V for 1 hour, 4000 V for 4000 Vh and finally stabilized at 8000 V, for a total 80000 Vh. Before the second dimension, strips were either stored at -80 °C or directly undergone reduction at room temperature for 15 minutes in the SDS equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% bromophenol blue] containing DTT (1%, w/v). The strip was then washed with Milli-Q water (Millipore) briefly and further underwent alkylation with IAA in SDS equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% bromophenol blue] at room temperature for another 15 minutes.

After equilibration, the IPG strip was washed with Milli-Q water (Millipore) briefly again and transferred to 1 mm gel cassettes with separating gels (12.5%). The strip was sealed with about 1 mL agarose sealing solution with SDS electrophoresis buffer containing 25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS, 0.5% agarose and 0.002% (w/v) bromophenol blue. Separation in the second dimension was then performed in the SE600 Ruby electrophoresis systems (GE). By running with the SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), separation was performed at 90 V for 1 hour and then 250 V for about 3 hours until the run was completed.

2.11.4.2 Visualizing and image analysis of protein spots

After the second dimension run, the gel was washed with Milli-Q water and then stained with 100 mL Coomassie staining solution which contains 45% (v/v) methanol, 10% (v/v) acetic acid and 0.15% (w/v) Coomassie Brilliant Blue R350 for

1 hour. The gels were finally destained overnight using 100 mL destaining solution containing 45% (v/v) methanol, 10% (v/v) acetic acid in Milli-Q water.

The gel was stained and destained and then scanned with an Image Scanner (Amersham Biosciences). Analysis was performed using the ImageMaster 2D Platinum 5.0 software (Amersham Biosciences). Protein spots were detected automatically while manual spot editing and deleting was performed if necessary. Distinct spots were selected throughout the gel for alignment and matching. Gels were studied in duplicates and the gel with the protein spots from the PTR mycelia cultured without addition of Tween 80 was used as a reference gel. Normalized volume ratio was used to quantify and compare the gel spots. The spots with at least 2.5-fold increase and at least 0.4-fold decrease in the normalized volume ratio were considered as differentially expressed in two comparative gels (proteins from PTR mycelial cells with and without the addition of Tween 80) and were chosen for further analysis.

2.11.4.3 In-gel digestion and peptide extraction

The differentially expressed protein spots were excised for digestion. They were transferred to 1.5 mL microcentrifuge tubes and cut into smaller pieces with surgical needle. The protein samples were destained with 50% (v/v) methanol in 50 mM NH_4HCO_3 and dehydrated with ACN. After vaccum-drying using Speed-Vac (LABCONCO), protein gel samples were rehydrated with 10 μL (40 ng/μL) sequencing grade modified trypsin (Promega), covered by 30 μL 25 mM NH_4HCO_3 and incubated at 30 ^{o}C for overnight. The digested sample was sonicated for 10 minutes and the supernatant was removed to a new microcentrifuge tube. Ten microliters of extraction buffer (60% ACN, 2.5% TFA) was added to the gel sample, followed by sonicated for 10 minutes. The supernatant was removed and combined with the previous extracts. About 0.5 μL of the digested sample solution in the microcentrifuge tube was spotted onto a MALDI plate twice, and then 0.5 μL matrix solution (4 mg/mL α-cyano-4-hydroxycinnamic acid in 35 % ACN and 1% TFA) was spotted. The samples were ready for MALDI-ToF MS analysis.

2.11.4.4 MALDI-TOF/TOF MS Analysis

MALDI-TOF MS analysis was performed using a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, USA). Mass data acquisitions were piloted by the 4000 Series Explorer™ Software v3.0 using batched-processing and automatic switching between MS and MS/MS modes. All MS survey scans were acquired over the mass range 800-3500 *m/z* in the reflection positive-ion mode and accumulated from 2000 laser shots with an acceleration of 20 kV. The MS spectra were internally calibrated using porcine trypsin autolytic products (*m/z* 842.509, *m/z* 1045.564, *m/z* 1940.935 and *m/z* 2211.104) resulted in mass errors of less than 30 ppm. The detection of MS peaks (MH^+) was based on a minimum S/N ratio ≥20 and a cluster area S/N threshold ≥25 without smoothing and raw spectrum filtering. Peptide precursor ions corresponding to contaminants including keratin and the trypsin autolytic products were excluded in a mass tolerance of ± 0.2 Da. The filtered precursor ions with a user-defined threshold (S/N ratio ≥50) were selected for the MS/MS scan. Fragmentation of precursor ions was performed using MS-MS 1 kV positive mode with CID on and argon as the collision gas. MS/MS spectra were accumulated from 3000 laser shots using default calibration with Glu-Fibrinopeptide B from 4700 Calibration Mixture (Applied Biosystems, USA). The dectection of MS/MS peaks were based on a minimum S/N ratio ≥3 and a cluster area S/N threshold ≥15 with smoothing.

The acquired data were searched against the National Center for Biotechnology Information (NCBI) MS (Peptide Mass Fingerprint, PMF) database using the MASCOT software package (Version 2.3, Matrix Science, UK; www.matrixscience.com). The following search parameters were used: monoisotopic peptide mass (MH^+); allowance of one missed cleavage per peptide; enzyme (trypsin); taxonomy (fungi); peptide mass tolerance of 0.1 Da; variable modifications (oxidation for methionine). The protein hit with an expectation value lower than 0.05 was considered as a possible identified protein. The closer the expectation value is to 0, the more likely the protein is correctly identified. In this study, identified proteins were those that had their protein expectation values were lower than 0.05 and sequence coverage greater than 10%.

2.12 Confocal Laser Scanning Microscopy (CLSM) observation

2.12.1 Fluorescent label of Tween 80

The dye 5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF) (Molecular Probes, Eugene, OR, USA) was used in this study for labeling Tween 80. 5-DTAF could bind covalently to the hydroxyl group at the sorbitan part of Tween 80.

About 0.5 mL 500 μM 5-DTAF in sodium carbonate buffer was mixed with 0.5 g Tween 80 and incubated for 12 hours at room temperature in the dark for the labeling process. Afterward, the labeled Tween 80 was separated from the mixed solution by reaching its clouding point at 93 ^{o}C in a water bath for removal of the unlabeled dye. Then the labeled Tween 80 was added to the fermentation broth on the 5th day (120 hours) of fermentation. The culture was carried out at 30 ^{o}C for 7 days. After fermentation, the mycelia samples were viewed by the CLSM.

2.12.2 CLSM imaging

The excitation and emission wavelength of 5-DTAF were 492 and 516 nm, respectively. The images were captured by the spectral confocal station FV 1000 installed on an inverted microscope IX-81 (Olympus, Tokyo, Japan) and acquired with 60× water-immersion objective lens. Images were processed with FV10-ASW software (Olympus).

2.13 Statistical Analysis

All statistical analyses were performed by using the software SPSS Statistics 17.0 (SPSS, Chicago, Illinois). All the data obtained were analyzed by one-way ANOVA and tests of significant differences were determined by using Tukey multiple comparison or Student's t-test at $p < 0.05$.

3 Results and discussion

3.1 Time course study of submerged fermentation

A time course study of mycelial biomass and EPS production in the shake-flask submerged fermentation of PTR was shown in Figure 3.1.

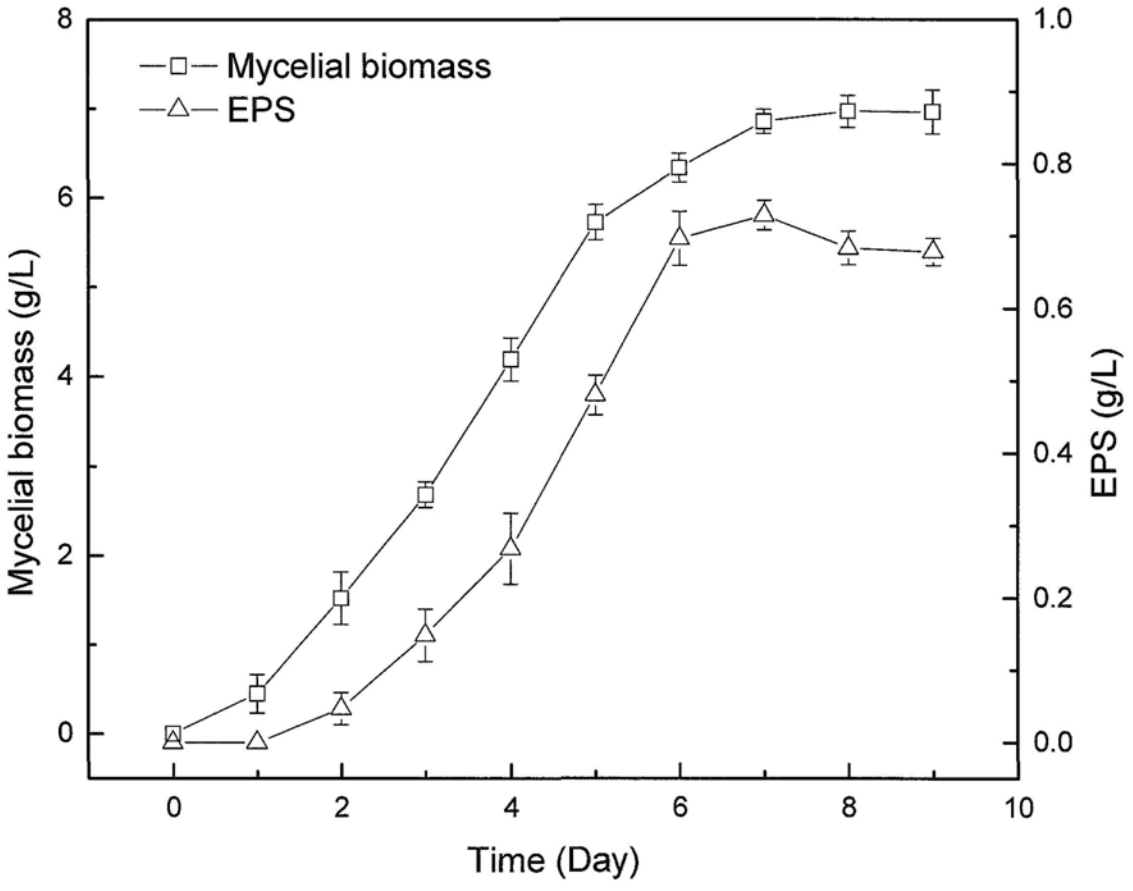


Figure 3.1 Time profiles of mycelial biomass and EPS productions in submerged fermentation of PTR. (Values are mean ± standard deviation, n=3)

There was almost no lag period because the mycelial cells were activated during the 4-day pre-inoculation seed culture period. After inoculation, a rapid increase of EPS concentration was observed from day 1 to day 6, which was in line with the exponential growth of mycelial cells (Figure 3.1). The EPS production

increased with the mycelial cell growth and reached a maximum level of 0.73 g/L after 7 days of fermentation, while the maximum mycelial biomass production occurred at 6.86 g/L before entering the stationary phase. Based on these observations, all subsequent submerged fermentation experiments on the effect of different stimulatory agents were carried out for 7 days.

3.2 Effect of different stimulatory agents

In order to stimulate the mushroom mycelial biomass and EPS production by submerged fermentation of PTR, three kinds of chemical additives including fatty acids, organic solvents and surfactants were added to the fermentation broth to investigate their effects.

3.2.1 Fatty acids

To accelerate mycelial growth of some medicinal mushrooms such as *Ganoderma* species, plant oils have been proved to be an effective stimulatory agent (Huang et al., 2009; Yang et al., 2000). However, it is hard to explain stimulatory effects of the plant oils which constitute a mixture of individual fatty acids. Hence, pure fatty acids were used as potential stimulatory agent in the present study.

The effect of fatty acids on mycelial biomass and EPS productions was studied by applying various fatty acids (linoleic acid, oleic acid, palmitic acid and stearic acid) at 1.0 g/L into the fermentation broth (Figure 3.2). Among all the fatty acids studied, addition of palmitic acid and stearic acid did not have any significant effect on mycelial growth compared to the control. Oleic acid showed a strong stimulatory effect on mycelial biomass with a significant ($p < 0.05$) increase of 20.0%. In contrast, linoleic acid significantly ($p < 0.05$) inhibited the mycelial growth (Figure 3.2). The present results were consistent with the previous results of Park et al. (2002), in which mycelial growth of a medicinal mushroom *Cordyceps militaris* was remarkably increased by oleic acid and drastically suppressed by linoleic acid.

All the fatty acids used in the present study were the major components in the lipid of the mycelium of other *Pleurotus* species, *P. ostreatus* produced by submerged fermentation (palmitic acid 35.0%; stearic acid 5.5%; oleic acid 22.0%; linoleic acid 37.5%) (Hadar and Cohen-Arazi, 1986). Therefore, the stimulatory/inhibitory effect of these fatty acids on mycelial growth, might be due to the partially incorporation into the fungal cell membrane thereby increasing/decreasing the uptake efficiency of nutrients from the fermentation broth. The relationship between the effect of these fatty acids on mycelial growth and the lipid composition of the mycelium of PTR would be worthy for further study.

On the other hand, the effect of fatty acids on EPS production in the submerged fermentation of PTR had no correlation with the effect on mycelial growth. Oleic acid, which increased the mycelial biomass by 20.0%, had no stimulatory effect on EPS production (Figure 3.2). In contrast, palmitic acid improved EPS production by 11.1% although it had no significant influence on mycelial growth. Linoleic acid suppress both mycelial growth and EPS production in PTR and this was in agreement with the results of Yang et al. (2000), in which EPS production by submerged fermentation of a medicinal mushroom *Ganoderma lucidum* was inhibited by addition of linoleic acid (1.0 g/L). Another similar study shown that addition of 2% oleic acid and palmitic acid markedly stimulated the EPS production (enhancement factors were 6 and 5.5, respectively), whereas linoleic acid significantly suppressed both mycelial growth and EPS production in *Cordyceps militaris* (Park et al., 2002).

The proposed mechanisms of the stimulatory effect of fatty acids are that they modify the cell membrane structure and increase permeability, or directly affect the level of synthesis of the enzymes involved in EPS production (Park et al., 2002).

However, the relationship between the length of the carbon chain or the level of unsaturation of the different fatty acids and the lipids composition of the mushroom cell membrane are still not clear understood and therefore, worthy for further study.

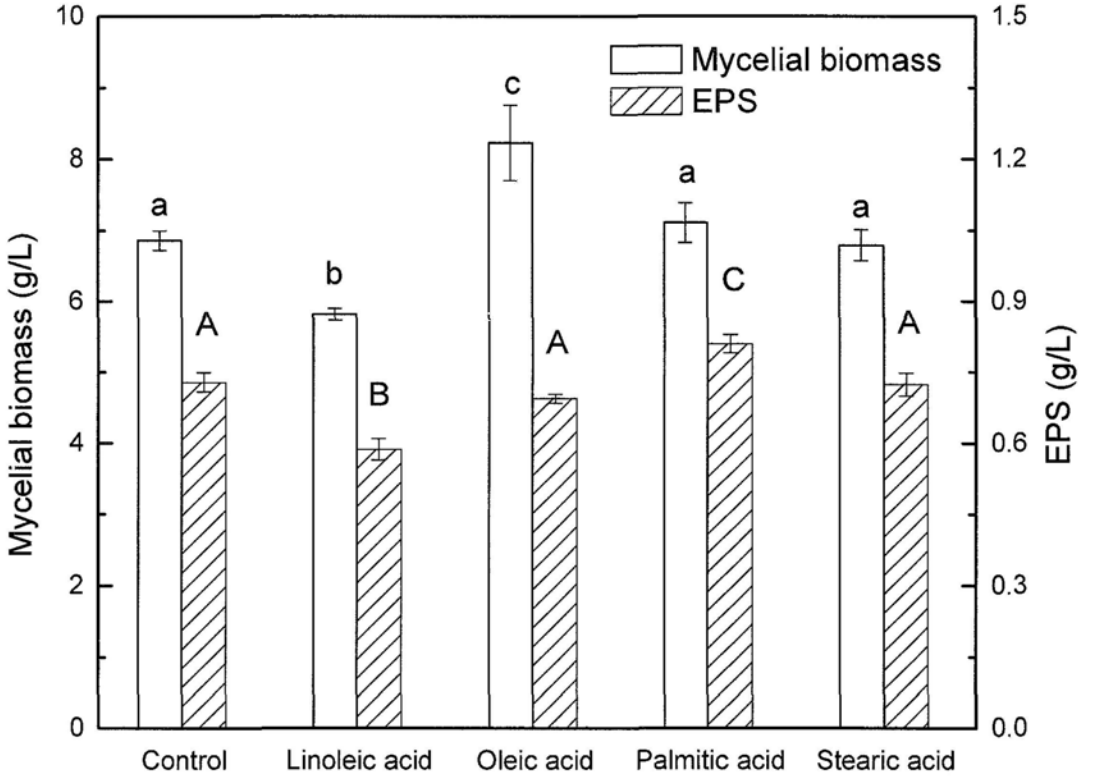


Figure 3.2 Effect of various fatty acids on mycelial biomass and EPS productions in submerged fermentation of PTR. The concentration of fatty acids was 1.0 g/L. Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, n=3) having different superscripts in lowercase and uppercase letters, respectively have significant difference (ANOVA Tukey's test; $p < 0.05$).

3.2.2 Organic solvents

The use of organic solvents is known to be a relatively effective method to increase cell permeability, which may enhance the release of EPS from the mycelial cells (Haq et al., 2003; Lim and Yun, 2006). Hence, in this study, five typical different organic solvents (methanol, ethanol, hexane, chloroform and toluene) were chosen to investigate their effects on mycelial growth and EPS production in PTR by applying 0.1% (v/v) of individual solvents into fermentation broth.

It was found that all the organic solvents, particularly chloroform and toluene, inhibited mycelial growth (Figure 3.3). When chloroform and toluene were added into the fermentation broth, the concentration of the corresponding mycelial biomass was only 53.1% and 62.4%, respectively, compared to that of the control. This result indicated that addition of chloroform and toluene had a detrimental effect on cell growth, thereby significantly reducing the concentration of mycelial biomass.

The use of chloroform and toluene had not only a detrimental effect on the mycelial growth but also on EPS production. This result was not consistent with the previous ones, in which these two organic solvents increased EPS production even though mycelial growth of *Collybia maculate* TG-1 was reduced (Lim and Yun, 2006). This result suggests that the choice of cell-permeabilizing agent is species-specific and related to the composition of the cell wall and its membrane. In contrast, it is noted the addition of methanol and hexane significantly ($p < 0.05$) increased EPS production by 17.7% and 15.5%, respectively, although they decreased mycelial biomass (Figure 3.3). The stimulatory effect of methanol addition on mushroom EPS production was similar to that of a previous study by Haq et al. (2003), in which methanol increased citric acid production by submerged culture of *Aspergillus niger* GCB-47. It was proposed that methanol increased the permeability of cell membrane, resulting in better excretion of citric acid from the mycelial cells. It could also be explained in terms of appropriate pellet size controlled by means of air or oxygen. Methanol was responsible for the proper pellet size which could increase the active surface of mycelial cells and allowed better oxygen availability, which is a critical control point in citrate synthesis by *A. niger* (Haq et al., 2003).

Based on the present results and those previously reported, it is thought that the increased production of mycelial EPS is a secretory function of organic solvent, which acts by modifying the outer cell wall structure, thereby facilitating the release of EPS from the inside of mycelial cells.

However, use of organic solvents as stimulating agent for EPS production should be carefully controlled as cells may be subjected to lysis or destruction of the intracellular components by non-polar solvents like chloroform and toluene. In general, the toxicity of chloroform and toluene on mycelial cells of PTR may be

much greater than that of methanol, ethanol and hexane, resulting in an apparent drop in the mycelial biomass and EPS production. Further studies on using methanol and hexane which both had shown stimulatory effect for EPS production in submerged fermentation of PTR are required.

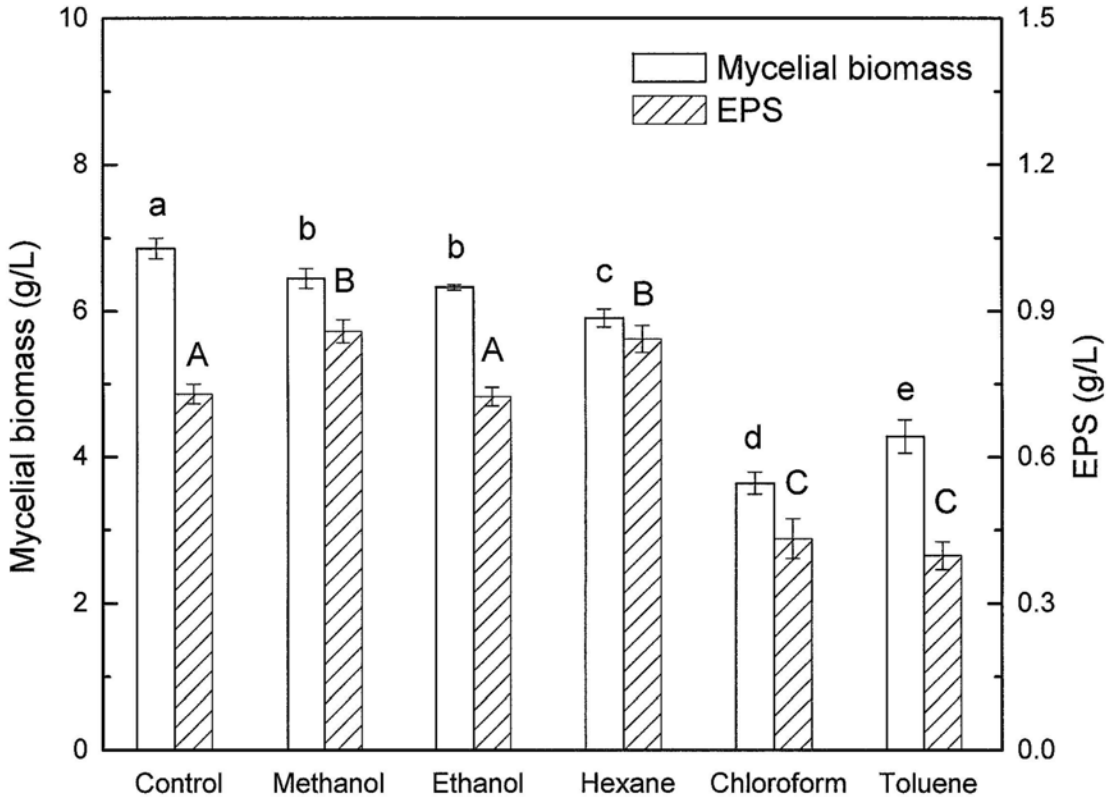


Figure 3.3 Effect of various organic solvents on mycelial biomass and EPS productions in submerged fermentation of PTR. The concentration of organic solvents was 0.1% (v/v). Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, n=3) having different superscripts in lowercase and uppercase letters, respectively have significant difference (ANOVA Tukey's test; $p < 0.05$).

3.2.3 Surfactants

Surfactants are very useful in biology and biochemistry and have been widely applied in biotechnology for improving the production of a number of valuable extracellular metabolites by submerged fermentation of microorganisms (Galindo and Salcedo, 1996; Reddy et al., 1999; Silva et al., 2007).

Therefore, to study the influences of surfactants in submerged fermentation of PTR, four typical surfactants (Tween 20, Tween 80, CHAPS and Triton X-100) at the concentration of 1.0 g/L were added to the fermentation broth and their effects on the cell growth and EPS production are shown in Figure 3.4.

It is interesting to find that with the addition of two surfactants of the Tween series, Tween 80 showed a strong stimulating effect on both cell growth and EPS production of PTR while Tween 20 did not exhibit any favorable or detrimental effect. This result was similar with a previous study which had shown that the shorter carbon chain (12 carbon side chain in Tween 20 vs. 18 carbon side chain in Tween 80) of surfactants exhibited higher diffusion rate in cell wall and thus might damage the cell membrane or interact with other cellular biomolecules, decreasing cell vitality (Hsieh et al., 2008). The maximum mycelial biomass and EPS production obtained with addition of 1.0 g/L Tween 80 reached 8.41 and 0.84 g/L, respectively, which accounted a significant enhancement ($p < 0.05$) of 22.6% and 15.0% in biomass and EPS, respectively (Figure 3.4). A similar report showed that surfactant (Tween 80) could significantly increase the production of botryosphaeran, an EPS of the (1→3; 1→6)-β-D-glucan produced by the fungus *Botryosphaeria rhodina* MAMB-05 (Silva et al., 2007).

It is assumed that this stimulatory effect is likely due to the action between surfactant and cell membrane. In theory, surfactants are amphiphilic, containing both hydrophobic groups and hydrophilic groups. The fungal cell membrane also consists primarily of a layer of amphiphilic phospholipids. Hence, the surfactants might partially be incorporated into the fungal cell membrane thereby increasing the uptake efficiency of nutrients from the fermentation broth. By this way, nutrient absorption to cell surface can be greatly enhanced through the aid of surfactant (Chen et al., 2010). It was also suggested that another mode of the mechanism of the surfactant is by affecting mass transfer either by changing the surface film resistance or the hydrodynamics in the cell membrane (Galindo and Salcedo, 1996).

In contrast, addition of CHAPS and Triton X-100 showed significant inhibition ($p < 0.05$) on the cell growth and EPS production of PTR (Figure 3.4). After the addition of CHAPS, the concentration of mycelial biomass and EPS production

decreased by 22.7% and 21.7%, respectively, compared to that of the control. Similarly, the addition of Triton X-100 had a detrimental effect, which significantly reduced the mycelial biomass and EPS production by 32.8% and 34.0%, respectively. The present results were not consistent with that of a previous report in whichTriton X-100 (0.1 g/L) having a 1.5-fold increase in the production of the xanthan gum by submerged fermentation of *Xanthomonas campestris* (Galindo and Salcedo, 1996).

The present results suggested that CHAPS and Triton X-100 displayed higher toxicity and lower biocompatibility to the mycelial cells than Tween 20 and Tween 80. They were consistent with those reported by Nascimento et al. (1997), in which the addition of 1.0% (v/v) Tween 80 to a *Candida lipolytica* culture medium increased cellular viability, with maximum stimulation of protease secretion.

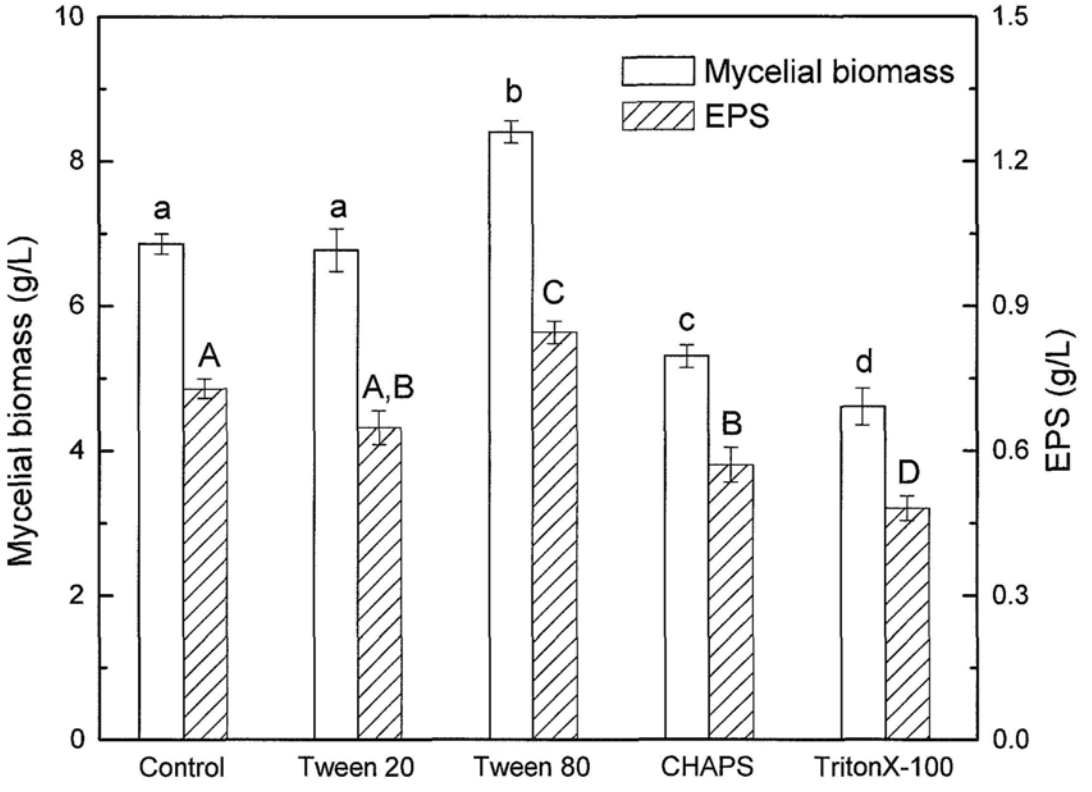


Figure 3.4 Effect of various surfactants on mycelial biomass and EPS production in submerged fermentation of PTR. The concentration of surfactants was 1.0 g/L. Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, n=3) having different superscripts in lowercase and uppercase letters, respectively have significant difference (ANOVA Tukey's test; $p < 0.05$).

Based on the above results, use of palmitic acid, methanol, hexane and Tween 80 as additives could stimulate the EPS production in PTR. Among these, Tween 80 which is a permitted food additive (E433) is safer than the other three chemical agents and more suitable when used in food application. Therefore, Tween 80 was selected as the stimulatory agent in the subsequent experiments.

3.2.4 Effect of concentration and addition time of Tween 80

3.2.4.1 Effect of concentration of Tween 80

The concentrations of additive could exert significant difference on the cell growth and production of extracellular metabolites. Hence, the effect of different concentrations of Tween 80 on mycelial biomass and EPS production in submerged culture of PTR is worthy for study and the results were shown in Table 3.1.

Production of mycelial biomass and EPS increased significantly ($p < 0.05$) as the concentration of Tween 80 increased from 0.5 to 3.0 g/L, beyond which no significant increase was observed. Higher concentration (5.0 g/L) of Tween 80 resulted in the generation of excessive foam, which has detrimental effect not only on sterile environment but also on mass and heat transfer process in submerged culture (Vardar-Sukan, 1998). It was also reported that the production of β-amylase and pullulanase by *C.thermosulfurogenes* SV2 fluctuated with different concentrations of Tween 80 added into the culture medium (Reddy et al., 1999). The yield of β-amylase and pullulanase enhanced with the increase of concentration of Tween 80 and gave the maximal results at 0.1 mM. However, further increase of concentration of Tween 80 had adverse effect on the production of extracellular enzymes (Reddy et al., 1999).

Accordingly, the maximum amount of mycelial biomass (9.26 g/L) and EPS (0.93 g/L) were obtained when 3.0 g/L Tween 80 was added to the medium (Table 3.1). This also corresponded to a significant increase ($p < 0.05$) of 35.0 and 27.4% in the production of mycelial biomass and EPS, respectively, when compared with the control.

Table 3.1 Effect of concentration of Tween 80 on mycelial biomass and EPS production in submerged fermentation of PTR

Concentration of Tween 80 (g/L)	Mycelial biomass (g/L)	EPS (g/L)
0	6.86 ± 0.14^{a}	0.73 ± 0.020^{A}
0.5	7.24 ± 0.16^{b}	0.80 ± 0.017^{B}
1.0	8.41 ± 0.15^{c}	0.84 ± 0.024^{B}
3.0	9.26 ± 0.12^{d}	0.93 ± 0.016^{C}
5.0	8.95 ± 0.09^{d}	0.91 ± 0.014^{C}

Each culture was carried out in triplicate at 30 °C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, n=3) having different superscripts in lowercase and uppercase letters, respectively have significant difference (ANOVA Tukey's test; $p < 0.05$).

3.2.4.2 Effect of addition time of Tween 80

It is important to study the effect of time of addition Tween 80 on this submerged fermentation process. To better understand the effect of Tween 80 on the mycelial growth and EPS production of PTR, 3.0 g/L Tween 80 were added on the 1st, 3rd and 5th day of cultivation, which represented the initial, intermediate and late stage of exponential growth phase of the mycelia, respectively.

Addition of Tween 80 at the late stage of exponential growth phase had resulted in a significant increase in both mycelial growth and EPS production compared with the early growth stages (Table 3.2). This result was consistent with that of the study by Lim and Yun (2006), in which the influence of addition time of toluene on the EPS production was investigated in submerged fermentation of an edible mushroom *Collybia maculata* TG-1. It was shown that the late growth phase was preferred for the increased EPS production when compared with the early growth phases. The maximal EPS yield was achieved when the toluene was added to the fermentation broth at 108 h after the start of fermentation (Lim and Yun, 2006). Nascimento et al. (1997) had reported the addition of Tween 80 to a culture medium of *Candida lipolytica* would increase cellular viability. Accordingly, it is reasonable

to propose that Tween 80 did not only prolong the viability of mushroom mycelia but also resulted in a higher EPS production. The optimum results were achieved when 3.0 g/L Tween 80 was added to the culture medium on the 5th day of the fermentation, corresponding to 51.3 and 41.8% significant increase ($p < 0.05$) in mycelial biomass and EPS production, respectively (Table 3.2).

Table 3.2 Effect of addition time of Tween 80 on mycelial biomass and EPS production in submerged fermentation of PTR

Addition time	Mycelial biomass (g/L)	EPS (g/L)
Control	6.86 ± 0.14^{a}	0.73 ± 0.020^{A}
1st day (24 h)	9.26 ± 0.12^{b}	0.93 ± 0.016^{B}
3rd day (72 h)	9.91 ± 0.13^{c}	0.94 ± 0.018^{B}
5th day (120 h)	10.38 ± 0.25^{d}	1.03 ± 0.018^{C}

The concentration of Tween 80 was 3.0 g/L. Each culture was carried out in triplicate at 30 ^{o}C for 7 days. Mycelial biomass and EPS production (mean value ± standard deviation, n=3) having different superscripts in lowercase and uppercase letters, respectively have significant difference (ANOVA Tukey's test; $p < 0.05$).

3.3 Chemical structure and biological activity of the EPS produced with and without the addition of Tween 80

Polysaccharides with biological activity differ greatly in their chemical structure. Differences in biological activity of polysaccharides can be correlated with carbohydrate and protein content, molecular weight, monosaccharide composition and glycosidic likages of the polysaccharides (Cui and Chisti, 2003; Gao et al., 1996; Mizuno et al., 1996; Ooi and Liu 1999). Hence, in this section, the carbohydrate and protein content, molecular weight, monosaccharide composition and glycosidic likages of EPS produced with and without the addition of Tween 80 were comparatively studied and their impacts on the biological activities were also investigated.

3.3.1 Carbohydrate and protein content

The total carbohydrate and protein content of EPS from PTR grown with and without addition of Tween 80 were shown in Table 3.3. The total carbohydrate content was determined by phenol-sulfuric acid assay using glucose as a standard for constructing a calibration curve. The EPS from submerged fermentation with addition of Tween 80 had 82.2±2.0% carbohydrate content while there was 81.5±2.4% total carbohydrate in the control (Table 3.3). There was no significant difference ($p > 0.05$) between carbohydrate content of EPS from cultures with and without addition of Tween 80. The protein content of EPS was determined by modified Lowry method using BSA as a standard for creating a calibration curve. The EPS with addition of Tween 80 had 12.5±0.2% protein content while there was 12.0±0.3% protein content in the control (Table 3.3). There was also no significant difference ($p > 0.05$) between protein content of EPS from submerged fermentation with and without addition of Tween 80. These results were comparable to that of a previous study in which the carbohydrate and protein content of the PTR sclerotia were 90.5±0.49% and 6.71±0.23%, respectively (Wong et al., 2003). It indicated that the carbohydrate and protein content of polysaccharides separated from different origins of the same *Pleurotus* species were similar.

The protein detected may be co-precipitated with the EPS during ethanol precipitation or it might be a part of the EPS. Polysaccharide-protein complex is a major kind of polysaccharide found in mushrooms. For example, a polysaccharide-protein complex isolated from the culture medium of an edible mushroom *Tricholoma lobayense*, was found to be an antitumor agent for inhibiting the growth of several tumor cell lines including HL-60, H3B and PU5-1.8 and cytotoxic to HL-60 cells by apoptosis (Liu et al., 1996). In the study by Lee et al. (2003), the EPS from submerged fermentation of *Grifola frondosa* had 87% carbohydrate content and 13% protein content, respectively. And it also showed that the ratio of protein to carbohydrate was an important factor to the potency of the biological activity of the mushroom polysaccharides, by which the percentage of superoxide radical inhibition could be enhanced by a higher protein to carbohydrate ratio of polysaccharide (Lee et al., 2003). In other *Pleurotus* species, polysaccharides are bound with proteins or peptides as a polysaccharide–protein or –peptide complex which showed potent antitumor activity. For example, *Pleurotus sajor-caju*, produced protein-containing polysaccharides (76% carbohydrate, 24% protein) which showed 90.8% tumor inhibition of Sarcoma 180 in mice (Zhuang et al., 1993). However, in the present study, the addition of Tween 80 (3.0 g/L added on the 5th day) did not have significant effect on the carbohydrate and protein content of EPS. The biological activity of EPS was studied at section 3.3.5.

From the results in Table 3.3, the EPS from submerged fermentation of PTR mainly consisted of carbohydrate (about 82%) and was subsequently used for the structural analysis without further purification.

Table 3.3 Total carbohydrate and protein content of EPS with and without the addition of Tween 80 (3.0 g/L added on the 5th day)

	Control	With addition of Tween 80
Total Carbohydrate content (%)	81.5±2.4 ^{a}	82.2±2.0 ^{a}
Protein content (%)	12.0±0.3 ^{b}	12.5±0.2 ^{b}

Carbohydrate and protein content (mean value ± standard deviation, n=3) within different column having the same superscripts have no significant difference (Student's t-test; $p > 0.05$).

3.3.2 Molecular weight

Eight pullulan standards were used to construct a calibration curve for determining the M_w profile of EPS with and without addition of Tween 80, which was estimated from the calibration curve obtained from the retention time of pullulan standards against the log value of their M_w. The M_w of this set of pullulan standards are 0.59 × 10^4 Da (P5), 1.18 × 10^4 Da (P10), 2.28 × 10^4 Da (P20), 4.73 × 10^4 Da (P50), 11.2 × 10^4 Da (P100), 21.2 × 10^4 Da (P200), 40.4 × 10^4 Da (P400) and 78.8 × 10^4 Da (P800). Sodium chloride solution (0.2 M) was used to dissolve and elute EPS in this study as to give an ionic strength that prevented aggregation of the polysaccharides and provided better resolution than using water alone as solvent. The eight pullulan standards were well separated by the TSK gel G5000 PW SEC (Figure 3.5).

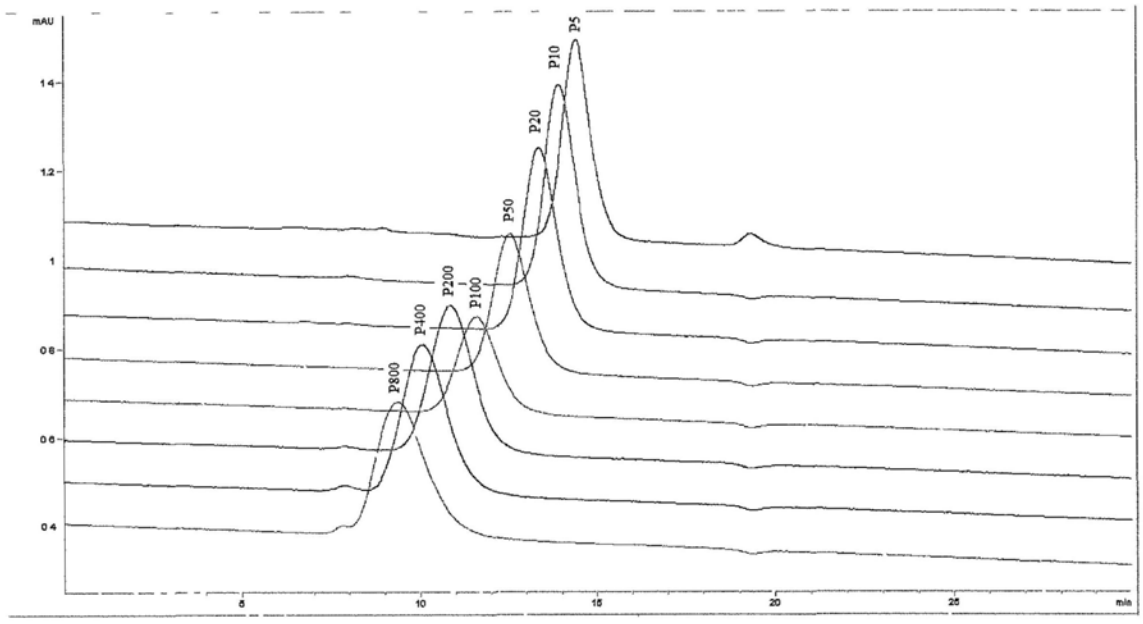


Figure 3.5 The SEC chromatograms of pullulan standards by a G5000 PW column with RI detection. The eluent was 0.2 M sodium chloride at a flow rate of 0.8 mL/min and the column was kept at 25 °C.

From the SEC results, PTR produced a large M_w EPS with high homogeneity (Figure 3.6). Moreover, the addition of Tween 80 in fermentation broth affected the M_w of EPS (Figure 3.6 and Table 3.4). The EPS produced by PTR with

addition of Tween 80 had a signifcantly ($p < 0.05$) lower M_w ($3.18\pm0.09 \times 10^6$) than that of produced without addition of Tween 80 ($4.30\pm0.12 \times 10^6$). It has been suggested that M_w is very important to the bioactivity of the mushroom polysaccharides. Polysaccharides having high M_w usually exert biological responses by receptors presented on cell surface (Zhang et al., 2004). Fungal polysaccharides having M_w higher than 2×10^5 are shown to have potent antitumor activity (Bohn and Bemiller, 1995). Therefore, the biological activities of high M_w EPS of PTR produced with and without addition of Tween 80 were studied in the following section and further investigated if the change of M_w by addition of Tween 80 has any influence on the biological activities of the EPS.

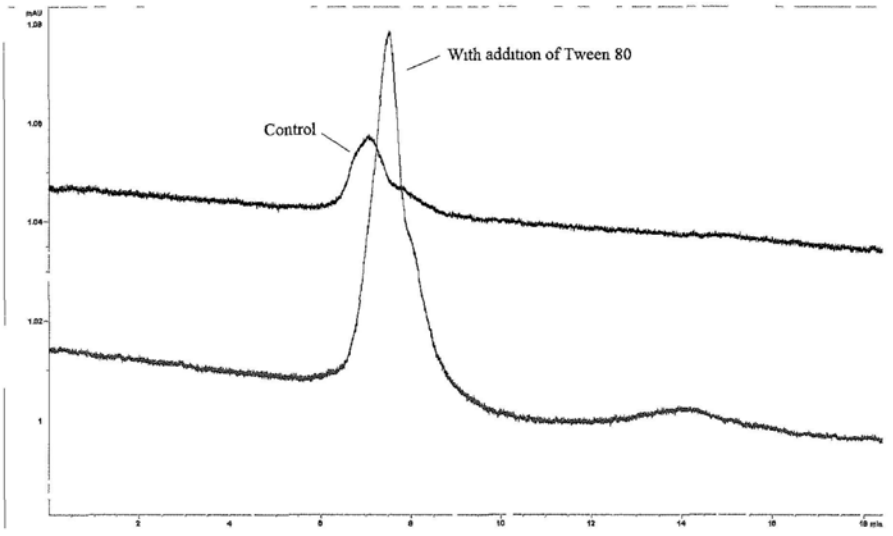


Figure 3.6 The SEC chromatograms of EPS with and without the addition of Tween 80 by a G5000 PW column with RI detection. The eluent was 0.2 M sodium chloride at a flow rate of 0.8 mL/min and the column was kept at 25 °C

Table 3.4 Molecular weight of EPS with and without the addition of Tween 80 (3.0 g/L added on the 5th day)

	Molecular weight (Da × 10^6)
Control	4.30 ± 0.12^{a}
With addition of Tween 80	3.18 ± 0.09^{b}

Data (mean value ± standard deviation, n=3) having different superscripts has significant difference (Student's t-test; $p < 0.05$).

3.3.3 Monosaccharide composition

Monosaccharide composition of PTR were determined by GC analysis and identified by comparing their retention times with those of sugar standards (Figure 3.7 and 3.8).

As showed in Figure 3.8 and Table 3.5, the EPS of PTR with addition of Tween 80 consisted of mainly mannose (57.5±1.7%) and glucose (42.5±1.7%). Similarly, the monosaccharide composition of EPS in control also consisted of mainly mannose (56.6±1.2%) and glucose (43.4±1.2%). The statistic analysis indicated that the EPS composition did not significantly change whether or not Tween 80 was added (Table 3.5). The EPS obtained from the fermentation broth of PTR were consisted of mainly mannose with a substantial amount of glucose, implying the presence of water-soluble glucomannan.

A previous study on submerged fermentation of PTR found that the principal monosaccharide in the mycelia of PTR was glucose (66.3–84.5%), which indicated the presence of glucans, followed by glucosamine (5.96–17.2%), and mannose (4.36–8.66%), implying the presence of chitin and mannans (Wu et al., 2004). Compared to the present results, the EPS of PTR contained mainly mannose and glucose which indicated that the monosaccharide composition and structure of polysaccharides isolated from different stages of the life cycle of the same *Pleurotus* species could be very different.

The monosaccharide composition of the EPS of PTR was different from that of two mushrooms of the same genus, *Pleurotus ostreatoroseus* and *Pleurotus ostreatus* "floride". The EPS produced by submerged fermentation of *P. ostreatoroseus* and *P. ostreatus* "florida" contained 95.5 and 87.7% glucose, respectively with small amount of galactose, mannose, xylose and arabinose (Rosado et al., 2003) while that of PTR in the present study contained both mannose and glucose as the major sugars. It showed that the monosaccharide composition and structure of EPS among different *Pleurotus* species could be very different.

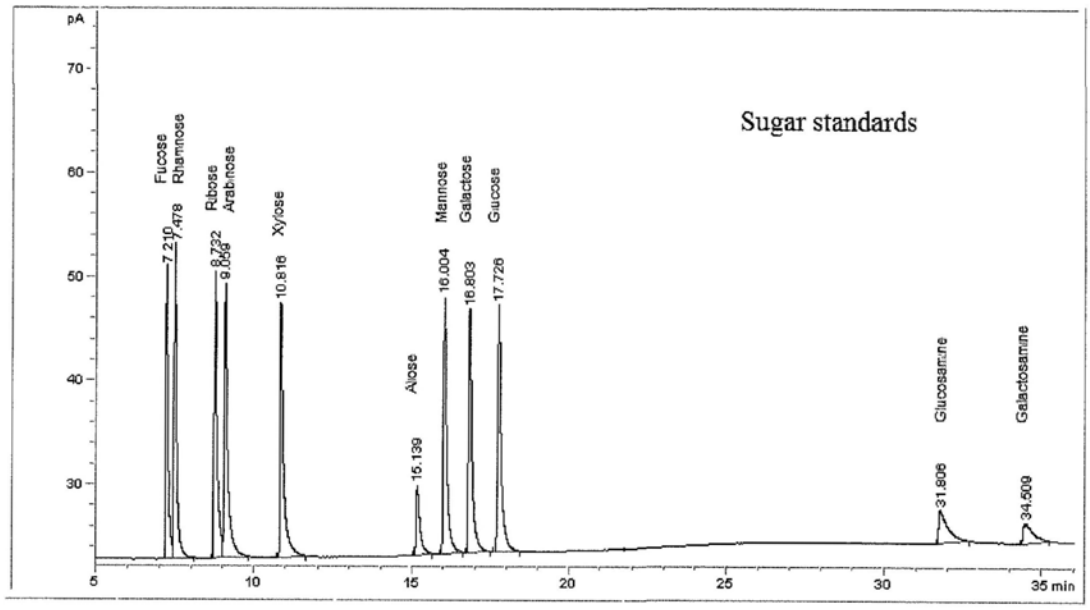


Figure 3.7 The GC chromatograms of sugar standards

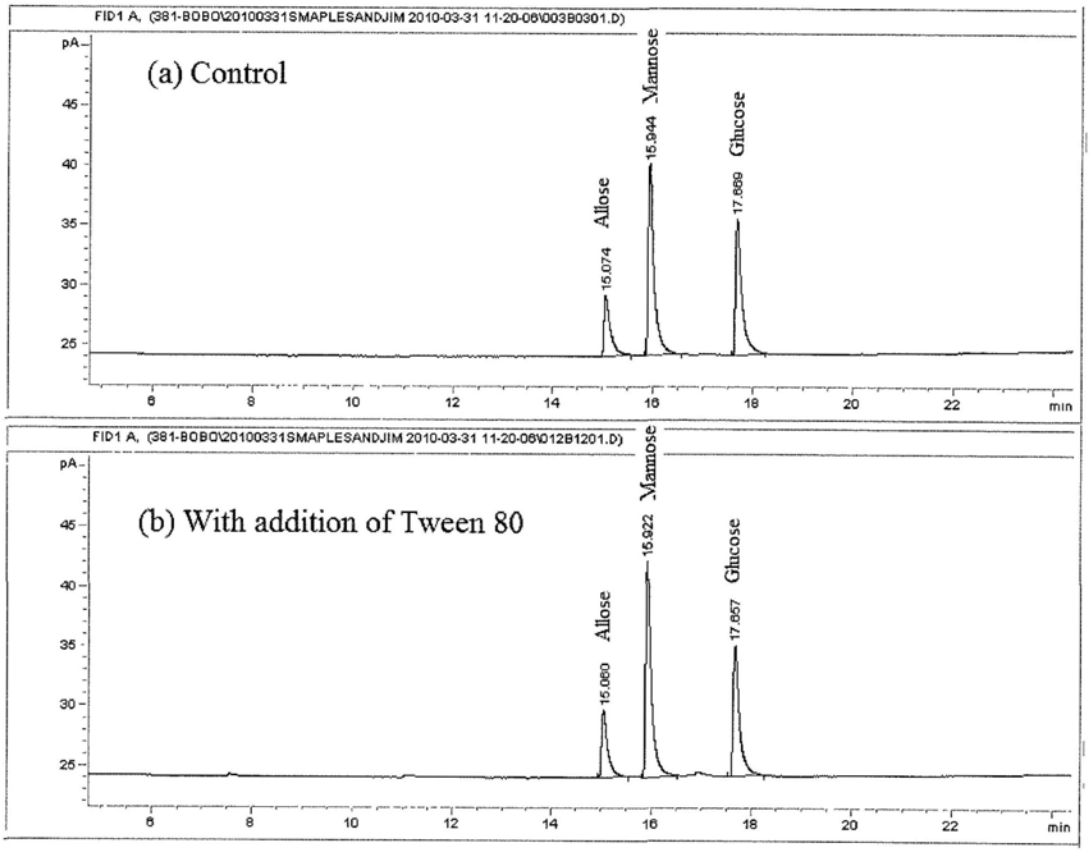


Figure 3.8 The GC chromatograms of EPS (a) without and (b) with the addition of Tween 80

Table 3.5 Monosaccharide composition (%) of EPS with and without the addition of Tween 80 (3.0 g/L added on the 5th day)

Monosaccharide composition (%)	Control	With addition of Tween 80
Fucose	0	0
Rhamnose	0	0
Ribose	0	0
Arabinose	0	0
Xylose	0	0
Mannose	**56.6±1.2** [a]	**57.5±1.7** [a]
Galactose	0	0
Glucose	**43.4±1.2** [b]	**42.5±1.7** [b]
Glucosamine	0	0
Galactosamine	0	0

Data (mean value ± standard deviation, n=3) within different column having the same superscripts have no significant difference (Student's t-test; $p > 0.05$).

3.3.4 Glycosidic linkages

The glycosidic linkages of EPS produced with and without addition of Tween 80 were investigated by the methylation analysis using GC-MS. Both of EPS showed the presence of 1,2,5,6-tetra-*O*-acetyl-3,4- di-*O*-methyl- mannitol; 2,5,6-tri-*O*-acetyl-1,3,4-di-*O*-methyl-mannitol and 1,5-di-*O*-acetyl-2,3,4,6-di-*O*-methyl-glucitol, in a molar ratio of about 2:3:3 and 2:3:3, respectively (Table 3.6). The mannose/glucose ratio of the EPS found in methylation (1.67 : 1) (Table 3.6) was comparable to those found by GC analysis of monosaccharide (1.30-1.35 : 1)(Table 3.5). Results of GC-MS analysis of EPS indicated that the presence of (1→6)-linked mannopyranosyl residues branched at the 2-position, (6→2)-linked mannopyranosyl residues and terminal glucopyranosyl residue in the EPS produced by submerged fermentation of PTR irrespective of addition of Tween 80. It seems that the EPS was mainly consisted of a mannan having a (1→6)-linked main chain of mannopyranosyl residues, almost all of which were branched at *O*-2 with side chain containing two (6→2)-Man*p* residues and a terminal glucopyranosyl residue. A proposed partial structure of the repeating unit for

EPS of PTR is shown in Figure 3.9. Such highly branched polysaccharide should be very soluble in water which may facilitate its biological activities through molecular interaction with cells.

As reported by Rosado et al. (2002), two different EPS were found in *Pleurotus ostreatoroseus*: a mannan having a main chain of (1→6)-linked mannopyranosyl residues, almost all of which were branched at *O*-2 with side chains of different lengths of mannopyranosyl units and a (1→4)-linked galactan. Therefore, it seems that the chemical structure of EPS among different *Pleurotus* species could be very different.

There were other mannans found in fungi in other previous studies. The EPS of *Tremella mesenterica*, a culinary-medicinal yellow brain mushroom, was reported to composed of a (1→3)-α-linked Mannose backbone with branches of various linkages such as (1→4)-linked Glc, (1→2)-linked Xyl, (1→3)-linked Xyl and (1→4)-linked Xyl (Vinogradov et al., 2004). A glucomannan with molecular weight more than 2×10^6 Da isolated by hot water extraction of *Agaricus blazei* was found to be composed of (2→1)-β-linked Mannose backbone and (1→1)-β-linked Glucose side chain (Mizuno et al., 1999). Mannan could also be isolated from the fruiting bodies of *Morchella esculenta* (Duncan et al., 2002) and *Tremella fuciformis* (Gao et al., 1996) with (2→1)-linked and (3→1)-linked backbone, respectively.

Table 3.6 GC-MS data for partially methylated alditol acetates of EPS produced with and without the addition of Tween 80 in submerged fermentation of PTR

Partially methylated sugar	Molar ratio		Linkage type
	With Tween 80	Without Tween 80	
3,4-Me_2-Man*p*	1.0	1.0	→2,6)-Man*p*-(1→
1,3,4-Me_3-Man*p*	1.5	1.5	→6)-Man*p*-(2→
2,3,4,6-Me_4-Glc*p*	1.5	1.5	Glc*p*-(1→

Me: methyl residues; Man*p*: mannopyranosyl residues; Glc*p*: glucopyranosyl residue.

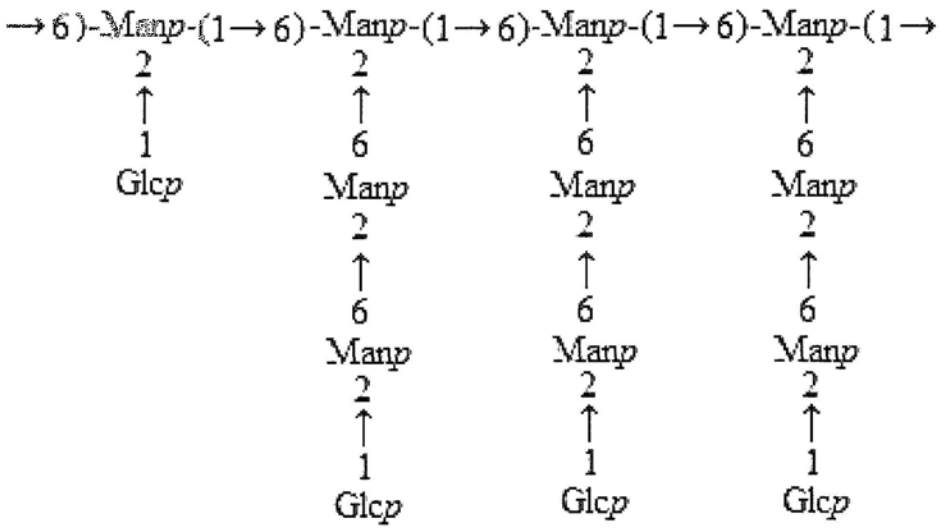


Figure 3.9 A proposed repeating unit of EPS produced by submerged fermentation of PTR

3.3.5 Biological activity

3.3.5.1 Examination of the contamination with endotoxin in EPS

The presence of any endotoxin in the EPS of PTR was examined by the *Limulus* Amebocyte Lysate test kit. Any endotoxin must be removed from samples before the evaluation of their antitumor activities. It was shown that the level of endotoxin in 400 μg/mL of EPS was lower than 0.015 EU/mL. Such level of endotoxin was considered to be extremely low when compared to limit of sensitivity of the kit and their effects on the cell line experiments were insignificant.

3.3.5.2 *In vitro* antitumor activity

Three human caner cell lines were employed to assess the *in vitro* antiproliferative activities of EPS with and without addition of Tween 80. They were originated from different organs and cancer types, including human liver cancer cells (HepG2), human breast cancer cells (MCF-7) and human chronic myelogenous

leukemia cells (K562). All of them are model cell lines used by Drug Research and Development of the National Cancer Institute in screening for potential antitumor agents and studying cell cycle and apoptosis pathway (Hui et al., 2005). Besides, two normal cell lines, one from monkey kidney (Vero) and one from human foreskin (Hs68) were applied to test the cytotoxicity of EPS with and without addition of Tween 80 on the normal cells, if any.

3.3.5.2.1 Effect of EPS on the proliferation of HepG2 cells

The effects of EPS with and without addition of Tween 80 on the proliferation of HepG2 cells are shown in Figure 3.10. In general, the inhibition ratio of the EPS at all tested concentration ranging from 6.35 to 400 μg/mL was found to be lower than 15%. All the EPS could not inhibit the proliferation of HepG2 cells well, irrespective of addition of Tween 80. Although the EPS with addition of Tween 80 showed a little increase in the inhibition ratio on the HepG2 cells compared to that without the addition of Tween 80, the maximal inhibition ratio was only about 12% at 400 μg/mL. Therefore, EPS with and without addition of Tween 80 were not effective in exerting *in vitro* cytotoxicity against HepG2 cells.

3.3.5.2.2 Effect of EPS on the proliferation of MCF-7 cells

The effects of EPS with and without addition of Tween 80 on the proliferation of MCF-7 cells are shown in Figure 3.11. The EPS from PTR inhibited the proliferation of MCF-7 cells in a dose dependent manner. However, the effect of EPS was not substantial because the highest inhibition ratio was only 29.3% at the concentration of 400 μg/mL. The effects of EPS with and without addition of Tween 80 were not significantly different ($p > 0.05$). The present result was not consistent with a previous study in which the polysaccharide isolated from the sclerotium of PTR had strong anti-proliferation effect on the MCF-7 cells (Zhang et al., 2006). This implied that the anti-tumor effects of polysaccharides from PTR were dependent on from which developmental stages the polysaccharides were isolated.

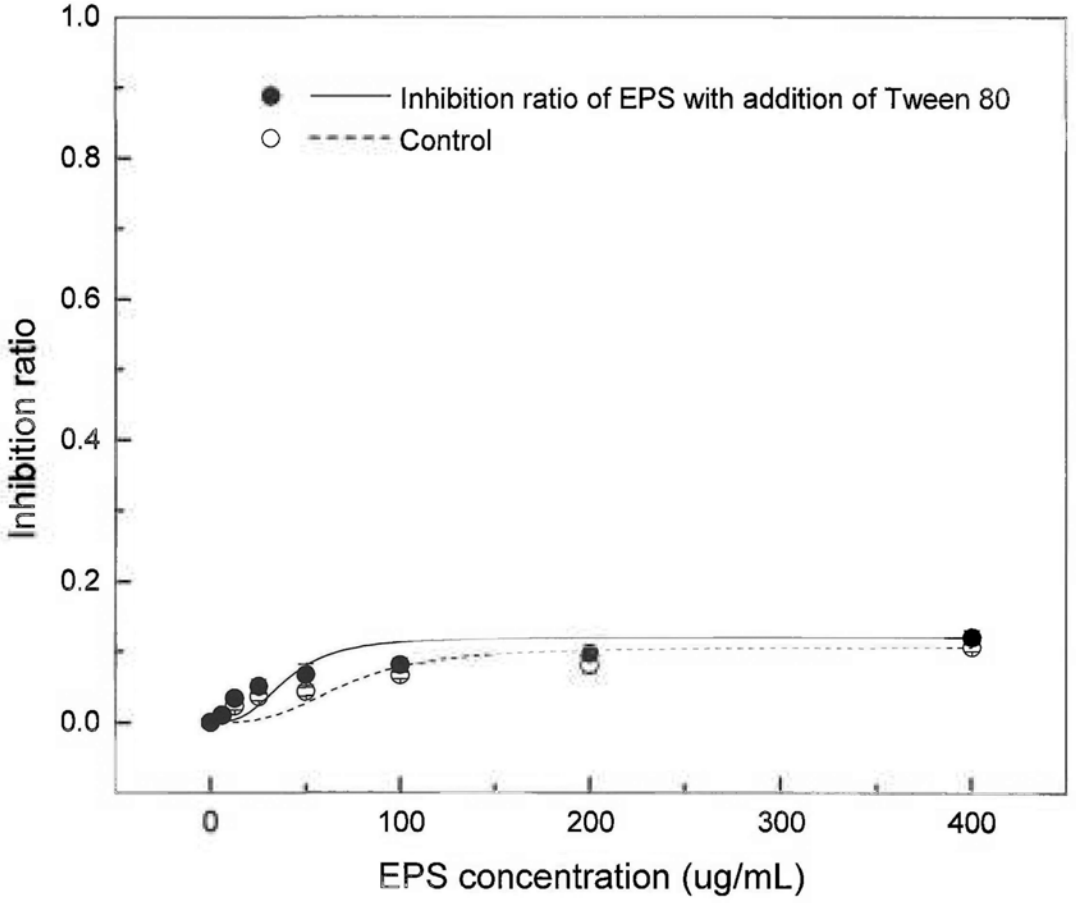


Figure 3.10 Effect of EPS with and without addition of Tween 80 on the proliferation of HepG2 cells. The HepG2 cells were incubated with EPS at concentration of 0, 6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL for 72 h. Results are expressed as mean value ± standard deviation, n=5.

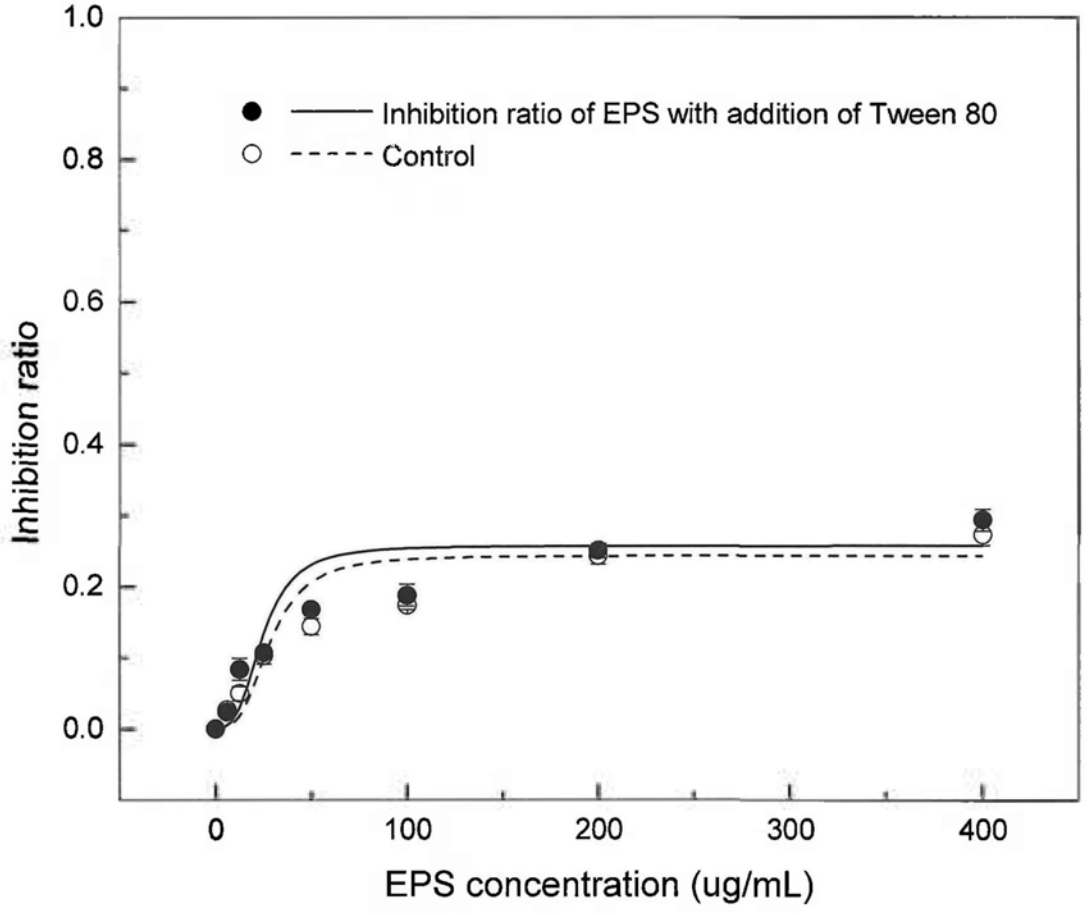


Figure 3.11 Effect of EPS with and without addition of Tween 80 on the proliferation of MCF-7 cells. The MCF-7 cells were incubated with EPS at concentration of 0, 6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL for 72 h. Results are expressed as mean value ± standard deviation, n=5.

3.3.5.2.3 Effect of EPS on the proliferation of K562 cells

The effects of EPS with and without addition of Tween 80 on the proliferation of K562 cells are shown in Figure 3.12. The EPS had potent antiproliferative effect to the K562 cells and the inhibition ratio was higher than 15% at all tested concentrations. EPS at the low concentration of 6.5 μg/mL even exerted a strong proliferation inhibitory effect which suggested the bioactive component was very potent. Both EPS, obtained from submerged fermentation with and without addition of Tween 80, could significantly inhibit ($p < 0.05$) the growth of K562 cells in a dose dependent manner, with an estimated IC_{50} value of 43.7 and 47.6 μg/mL, respectively (Figure 3.12). The lower of IC_{50} value in the inhibition of K562 cell proliferation of the EPS produced by addition of Tween 80 suggested a higher potency of cytotoxicity toward these cells. This might be due to the EPS produced with addition of Tween 80 had a signifcantly ($p < 0.05$) lower M_w ($3.18\pm0.09 \times 10^6$) than that of produced without addition of Tween 80 ($4.30\pm0.12 \times 10^6$). Moreover, EPS extracted from the culture medium of PTR had also been reported to have direct cytocidal antitumor activities towards another leukemic cell HL-60 but with a much higher IC_{50} value of 300 μg/mL (Wong et al., 2007).

It was suggested that M_w is very important to the bioactivity of the mushroom polysaccharides. For example, it was found that biological activity of (1→3)-β-glucans was strongly dependent on the molecular weight (Mizuno, 1996). The glucans with high molecular weight ranging from 500 to 2000 kDa seems to be more effective than those with low molecular weight. However, some kinds of mushroom polysaccharides such as (1→3)-α-glucuronoxylomannans from *Tremella fuciformis* Berk, their biological functions are not highly correlated with the molecular weight. Their hydrolysate fractions containing glucuronoxylomannans with molelcular weights ranging from 53 to 1000 Da are as effective as those fractions having higher molecular weights (Gao et al., 1996). It was also reported that differences in molecular weight had no apparent effects on the cytokine stimulating activity of the mushroom polysaccharides (Gao et al., 1996).

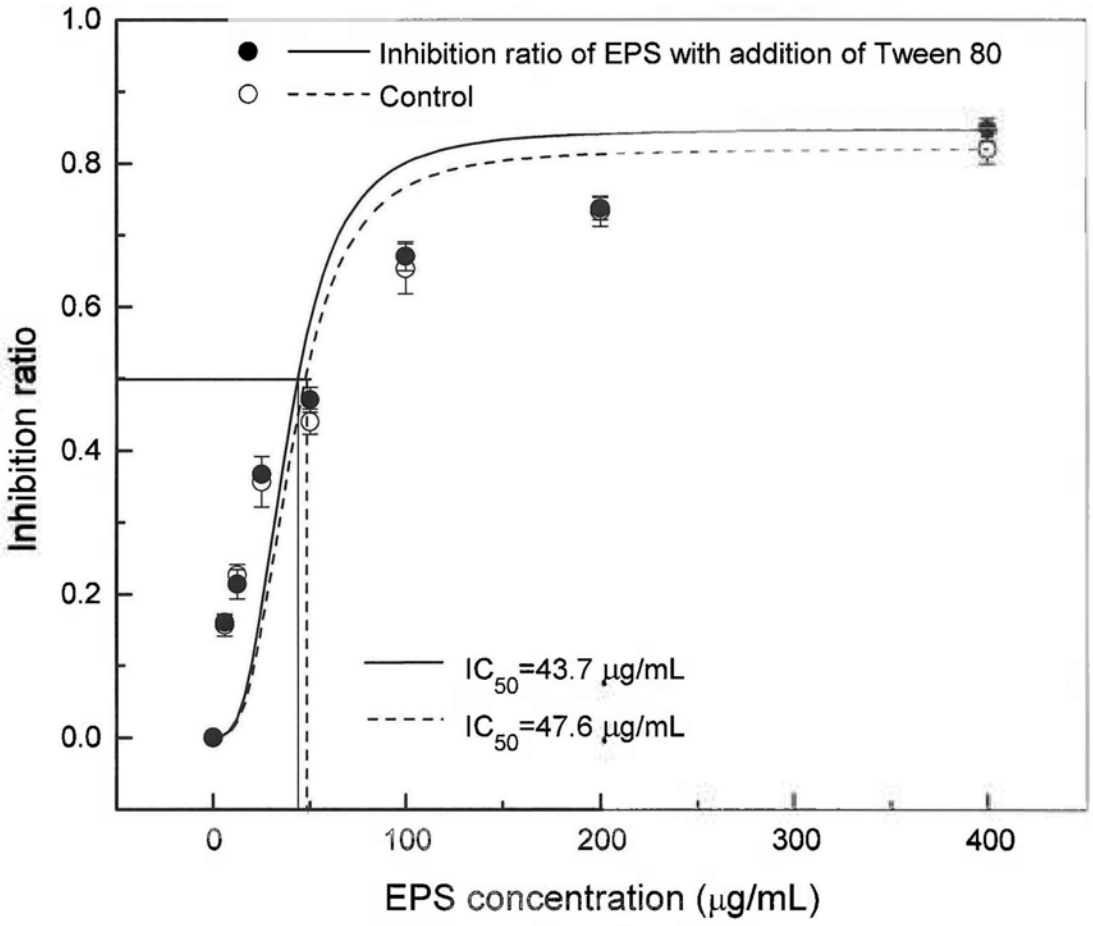


Figure 3.12 Effect of EPS with and without addition of Tween 80 on the proliferation of K562 cells. The K562 cells were incubated with EPS at concentration of 0, 6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL for 72 h. Results are expressed as mean value ± standard deviation, n=5.

3.3.5.2.4 Effect of EPS on the viability of normal cells

A compound is considered to be a potent antitumor agent when it has substantial antiproliferative activities specific to tumor cells and not normal cells. The tumor specificity of the cytotoxic effect of EPS with and without addition of Tween 80 was evaluated by two normal cell lines, human Hs68 and monkey Vero cells. The inhibition ratio found by addition of EPS at the concentration from 6.5 to 400 μg/mL was almost zero, suggesting that their cytotoxic effects were preferential against tumor cells only. This selectivity implicated that the EPS might be used as potential antiproliferative agents.

3.4 Underlying mechanism of effect of Tween 80

In the previous section of 3.2, three kinds of chemical agents including fatty acids, organic solvents and surfactants were studied and their effects varied because of the differences in their chemical nature. Tween 80, a permitted food additive (E433), which is safer than the other three chemical agents and more suitable when used in food application, was selected as the stimulatory agent for further study. The optimum results were achieved when 3.0 g/L Tween 80 was added to the fermentation broth on the 5^{th} day of the fermentation, corresponding to 51.3 and 41.8% significant increase ($p < 0.05$) in PTR mycelial biomass and EPS production, respectively. Subsequently, the chemical structure and biological activity of the EPS produced with and without the addition of Tween 80 were systematic investigated in the section of 3.3.

However, the underlying mechanism of action of Tween 80 are poorly understood and rarely reported. In this section, the study of the detailed underlying mechanisms of the stimulatory effect of Tween 80 on the mycelial biomass and EPS production were conducted by a few new approaches.

3.4.1 Effect of Tween 80 in nutrient uptake

Feeding of nutrition during fermentation is known to be an effective method to enhance the mycelial growth and the production of mushroom EPS. For example, the mycelial biomass and EPS production of *Ganoderma lucidum* increased 26.9% and 68.4%, respectively by feeding of carbon source (lactose) during fermentation (Tang and Zhong, 2002). The question is that whether the addition of Tween 80 as a feeding of carbon source at the late stage of exponential growth phase could lead to the enhancement of mycelial biomass and EPS production in the fermentation of PTR.

Moreover, it is also known that Tween 80 can be utilized as a carbon source by some bacteria. For example, Li et al. (2001, 2004) reported that the use of Tween 80 as a carbon source for *Acinetobacter radioresistens* could significantly increase the production of an extracellular lipase. The question is that whether Tween 80 is

used as a carbon source by the mycelium of PTR.

3.4.1.1 As a feeding of nutrition

To test if Tween 80 could act as a feeding of nutrition, Tween 80 and glucose were added separately into the fermentation broth on the 5th day and the mycelial biomass and EPS production of PTR for the following 3 days were compared.

As shown in Figure 3.13, addition of 0.3% w/v Tween 80 on the 5th day significantly increased the mycelial biomass from 6.86±0.14 to 10.38±0.25 g/L, corresponded to an increase of 51.3%. Meanwhile, the EPS production of PTR significantly increased from 0.73±0.02 to 1.03±0.02 g/L which corresponded to an increase of 41.8%. In contrast, feeding of 0.3% w/v glucose into the culture medium on the 5th day did not have any effect on the mycelial biomass and EPS production, when compared to the control. It suggested that the feeding of nutrition did not influence the process of submerged fermentation of PTR.

The present results were not consistent with those of Shih et al. (2008), in which feeding glucose (when the glucose concentration in the fermentation broth was lower than 0.5% w/v) to the fed-batch fermentation of *Grifola frondosa* could greatly increase the accumulation of mycelial biomass and EPS when compared to that of batch fermentation only. The purpose of nutrition feeding is to keep the mycelial growth without limitation and inhibition by the substrate (Tang and Zhong, 2002). It is therefore considered that feeding of carbon source (lactose) should be performed at its residual concentration between 10 and 5 g/l (during day 8~12) (Tang and Zhong, 2002). A remarkable enhancement of both the productivity and production of polysaccharide was successfully achieved in this way at their experiments.

In the present study, the feeding of glucose into the fermentation broth on the 5th day did not increase the mycelial biomass and EPS production of PTR (Figure 3.13). This might be due to the fact that the initial concentration of glucose (3% w/v) used in the present study was sufficient to provide excess carbon source throughout the whole fermentation period and addition of extra glucose did not have any effect on the growth of the mycelium of PTR. Moreover, it can be concluded that the

addition of Tween 80 did not act as a feeding of carbon source at the late stage of exponential growth phase but could lead to the enhancement of mycelial biomass and EPS production in the fermentation of PTR in the other way.

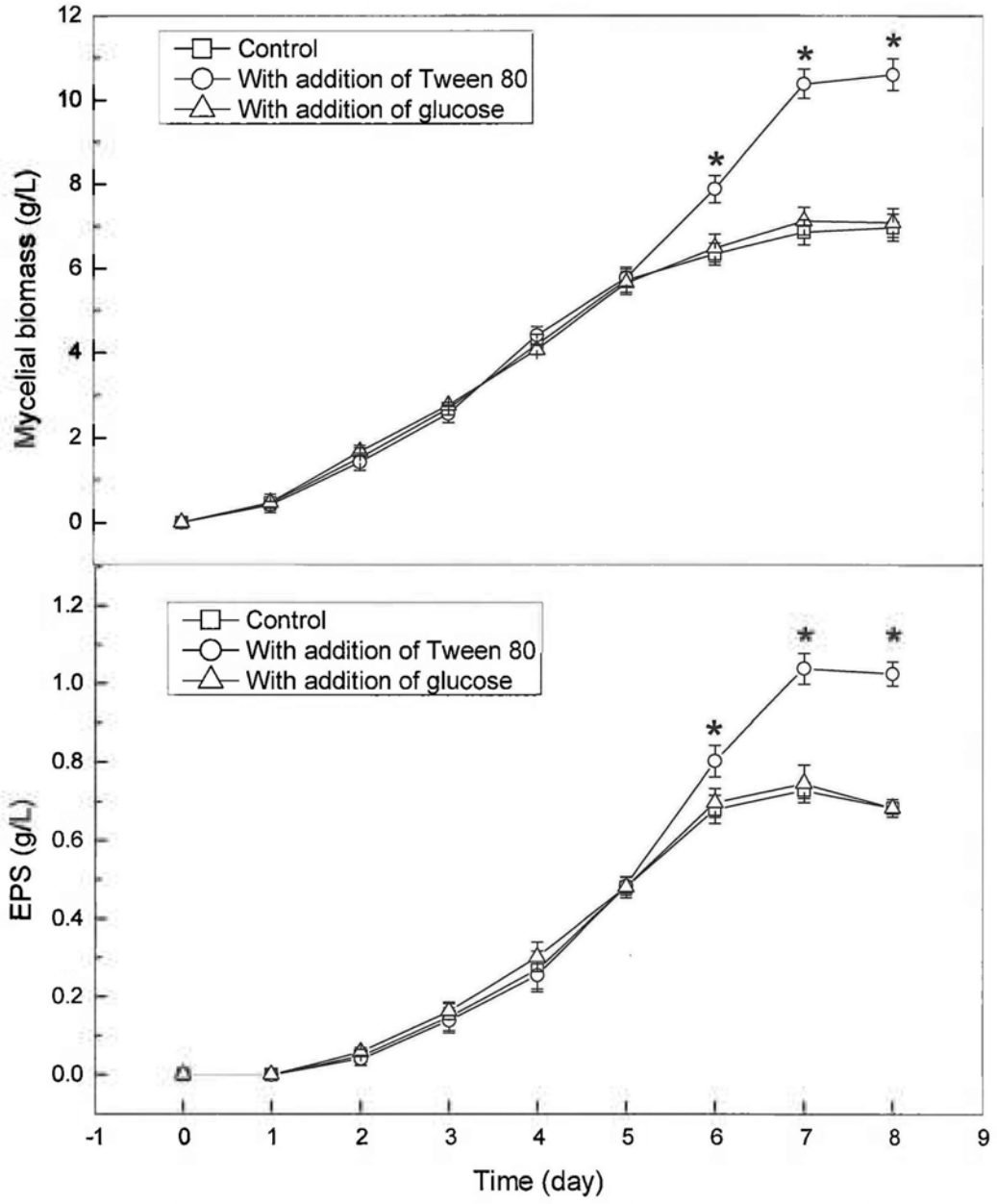


Figure 3.13 Effect of addition of either Tween 80 or glucose on the mycelial biomass and EPS productions in submerged fermentation of PTR

Either Tween 80 or glucose was added (both at a concentration of 0.3% w/v) on the 5th day of fermentation. The culture experiments were carried out in triplicate at 30 ^{o}C. Mycelial biomass and EPS production (mean value ± standard deviation, n=3) denoted with an asterisk have significant difference between control and treatment group (Student's *t*-test; $p < 0.05$).

3.4.1.2 As a carbon source

In this experiment, the original carbon source (glucose) was replaced by Tween 80 to evaluate if PTR could grow in the medium containing Tween 80 as the sole carbon source. Results indicated that there was no growth of mycelium of PTR after 8 days of fermentation when replaced the original carbon source (glucose) by Tween 80 (data not shown).

This result was different from that of Fakhereddine et al. (1998), in which nine moderate thermophilic bacterial strains could grow on a fermentation broth containing Tween 80 as the sole carbon source for producing thermostable lipolytic enzymes. The lipolytic enzyme activity was lost when Tween 80 was removed from the culture medium. It has been shown that Tween 80 could be utilized as a carbon source by *Acinetobacter radioresistens* to increase significantly the production of its extracellular lipase (Li et al., 2001, 2004). It has also been shown that Tween 80 was a suitable carbon source for production of microbial lipases by reducing significantly the fermentation time in *A. radioresistens*. Tween 80 could provide oleic acid, the intrinsic carbon source, for cell growth and lipase production through a mode of controlled release, therefore abates the repression of lipase synthesis (Li et al., 2001, 2004).

All these previous results on Tween 80 were not consistent with the present results. This might be due to the reason that PTR did not possess the enzymes which could utilize Tween 80 as carbon source like the moderate thermophilic bacterial strains and *A. radioresistens* (Li et al., 2001, 2004).

The above results in section 3.4.1.1 and 3.4.1.2 indicated that it was unlikely for Tween 80 to act as a carbon source or a feeding of nutrition to directly enhance the production of mycelial biomass and EPS production found during the submerged fermentation of PTR. However, it has been proposed previously that Tween 80 might be able to increase the uptake efficiency of nutrients from the fermentation broth (Chen et al., 2010). Hence, the role of Tween 80 in nutrient uptake was studied accordingly.

3.4.1.3 Role of Tween 80 in nutrient uptake

To clarify the role of Tween 80 in nutrient uptake, the residual concentration of glucose with and without addition of Tween 80 was determined throughout the submerged fermentation period of PTR (Figure 3.14). The glucose consumption rate (g/day) was estimated by calculating the decrease of glucose concentration in each day and the results are listed in Table 3.7.

In the fermentation broth without the addition of Tween 80, the glucose consumption rate increased in the first 5 days from 0.92±0.03 to 2.15±0.08 g/day, after which an obvious decrease was observed (2.15±0.08 to 0.48±0.03 g/day from the 5^{th} day to the 8^{th} day) (Table 3.7). These results were consistent with that of Figure 3.13, in which the production rate of mycelial biomass and EPS was decreased after 5 days in the fermentation without the addition of Tween 80. In contrast, when Tween 80 was added on the 5^{th} day, the residual glucose concentration decreased sharply from 22.57 g/L to 15.29 g/L (from the 5^{th} day to the 8^{th} day). The glucose consumption rate on the 6^{th}, 7^{th} day and 8^{th} day was 2.27, 3.36 and 1.65 g/day, respectively, which had a 2.6, 3.7 and 3.4 fold increase, respectively when compared to the control.

These results suggested that the glucose consumption rate was significantly increased ($p < 0.05$) after the addition of Tween 80, implying that the uptake efficiency of nutrients from the fermentation broth had been increased and this finding was closely related to the increase of mycelial biomass and EPS production of PTR by the addition of Tween 80.

Table 3.7 Glucose consumption rate (g/day) in submerged fermentation of PTR mycelial cells with and without addition of Tween 80

Time period	Glucose consumption rate (g/day)	
	Control	With addition of Tween 80
1st day	0.92±0.03	0.90±0.02
2nd day	1.21±0.04	1.14±0.03
3rd day	1.55±0.04	1.64±0.06
4th day	1.78±0.06	1.75±0.06
5th day	2.15±0.08	2.17±0.07
6th day	0.86±0.02	2.27±0.09 *
7th day	0.91±0.02	3.36±0.11 *
8th day	0.48±0.03	1.65±0.05 *

Tween 80 was added at a concentration of 0.3% w/v on the 5th day of fermentation. The culture experiments were carried out in triplicate at 30 °C for 8 days. Values are given as mean value ± standard deviation (n=3). Values denoted with an asterisk have significant difference between control and treatment group (Student's *t*-test; $p < 0.05$).

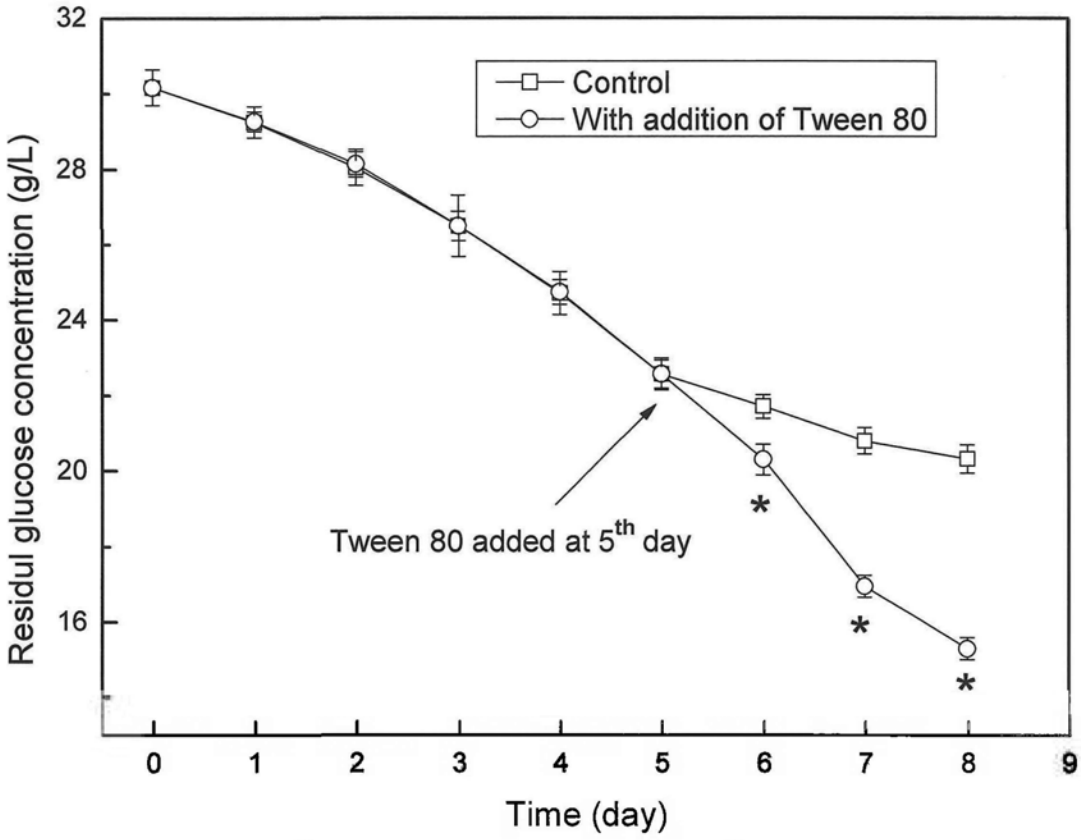


Figure 3.14 Time profile of glucose concentration with and without addition of Tween 80 in submerged fermentation of PTR

Tween 80 was added at a concentration of 0.3% w/v on the 5th day of fermentation. The culture experiments were carried out in triplicate at 30 ^{o}C for 8 days. Values are given as mean value ± standard deviation (n=3). Values denoted with an asterisk have significant difference between control and treatment group (Student's *t*-test; $p < 0.05$).

3.4.2 Effect of Tween 80 in protecting the mycelial pellets from disintegration

The morphology of the mycelium in submerged fermentation is greatly influenced by the culture conditions and exerts significant effects on the production of fungal metabolites (Olsvik and Kristiansen, 1992). In order to obtain maximal mycelial biomass and EPS production, it is important to investigate the relationship between the fungal morphology and the culture conditions (Olsvik and Kristiansen, 1992).

Gehrig et al. (1998) described the general growing process of the mycelial pellet and reported that the production of the antibiotics striatals A, B, and C by basidiomycete *Cyathus striatus* was significantly influenced by the pellet size and morphology. A small pellet consists of an active growing zone which is sufficiently supplied with oxygen and nutrients. With increasing pellet size the oxygen transfer to the center of the pellet is restrained. Long period of limited oxygen supply could lead to the lysis of the mycelium (Gehrig et al., 1998). The pellets with weakening in their mechanical stability could be deformed by the impulse transfer at the stirrer blades or the baffles (Gehrig et al., 1998).

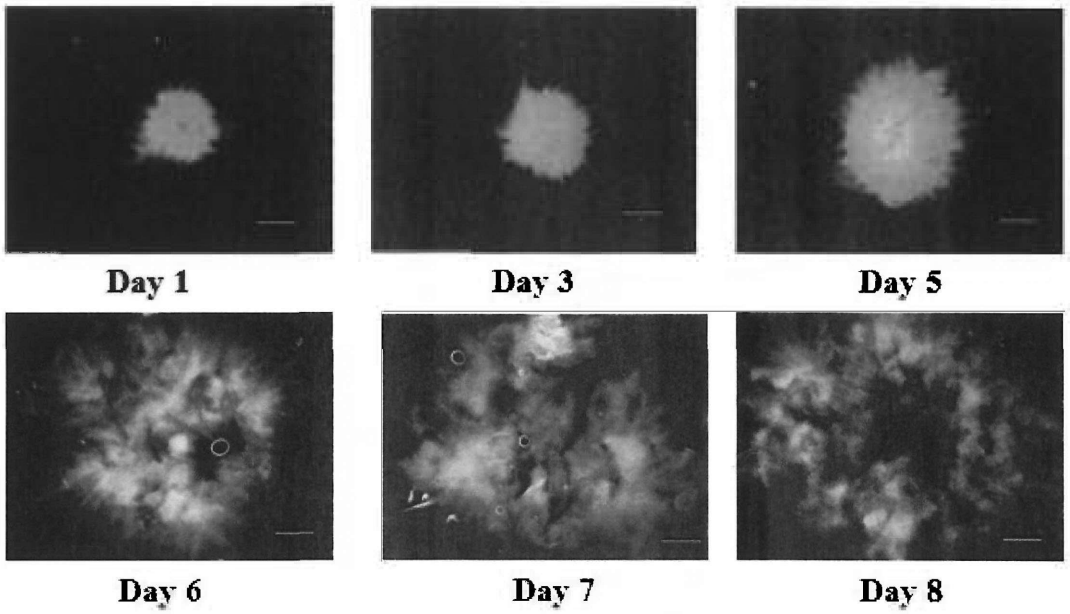
Besides the above information about the relationship between the mycelial morphology and the fungal cell growth, it has been suggested that supplementation of fermentation medium with surface-active substances can alter the physiological properties of *Saccharomyces cerevisiae* including the stimulation of cell growth and an increase in its metabolite production (Benchekroun and Bonaly, 1992). It was observed that surface-active substances (polyoxyalkylene glycols) improved cell growth, viability and alcohol production in *Saccharomyces cerevisiae* the most efficiently when the molecular mass of polyoxyalkylene glycol was between 2000 and 3300 Da (Benchekroun and Bonaly, 1992).

Based on previous studies, mycelial morphology is always considered to be an important index not only for fungal cell growth but also for fungal metabolism. Hence, it is worth investigating whether there were any changes of the mycelial morphology in PTR with and without the addition of Tween 80.

3.4.2.1 Mycelial morphology

The mycelial morphology of PTR with and without the addition of Tween 80 was observed by microscopy and the pictures are shown in Figure 3.15. During the first 5 days of fermentation, the mycelia existed as pellets with a hairy appearance on their surfaces. The diameter of the mycelial pellets increased from about 1.5 mm on the 1st day to 3.0 mm on the 5th day (Figure 3.15). Differences in the morphology between mycelial samples with and without the addition of Tween 80 were observed from the 5th day onward. As shown in Figure 3.15 a, the compact structure of the spherical mycelial pellets in the fermentation broth without the addition of Tween 80 started to get loosen on the 6th day and the mycelia were found dispersed with signs of disintegration on the 8th day. This was consistent with the results of the control shown in Figure 3.13, in which the rate of increase in the mycelial biomass decreased on the 6th day and the growth of mycelial cells almost ceased on the 7th day and thereafter. In contrast, the structure of the spherical mycelial pellets with addition of Tween 80 was still kept in a compact state on the 8th day with the mycelia remained within the core region as shown in Figure 3.15 b. This was in agreement with the result that the mycelial biomass with the addition of Tween 80 still kept on increasing throughout the 8-days fermentation (Figure 3.13).

(a)



(b)

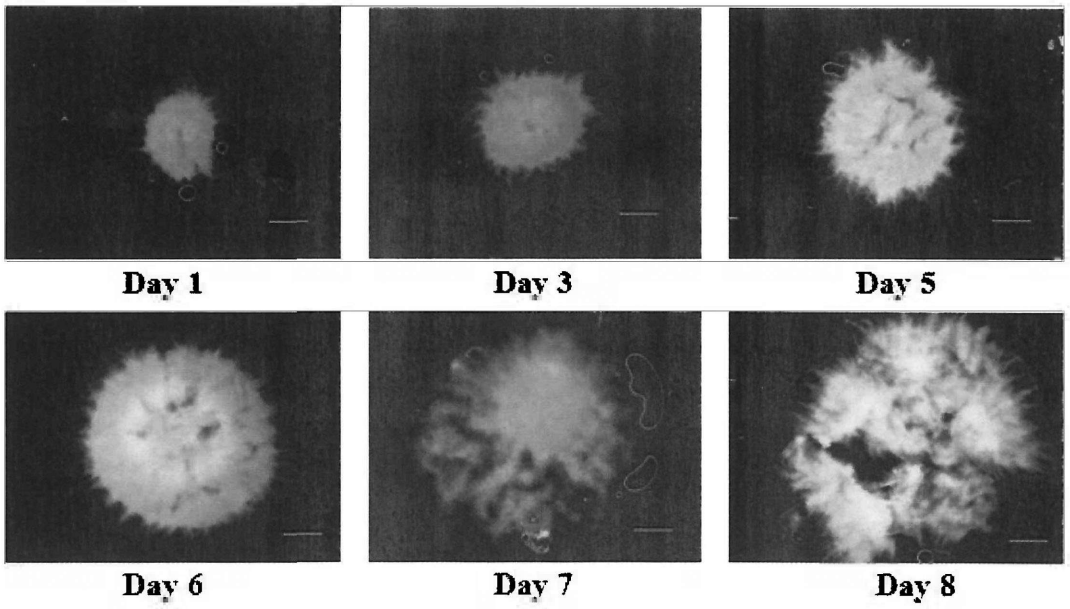


Figure 3.15 Mycelial morphology of PTR by submerged fermentation without (a) and with (b) addition of Tween 80

Tween 80 was added at a concentration of 0.3% w/v on the 5th day of fermentation. The scale bars in pictures represent 1 mm.

Hence, the mycelial morphology observed shown in Figure 3.15 indicated that the addition of Tween 80 could extend the growth of the mycelia for a longer period possibly by maintaining the intact structure of the mycelial pellets and preventing its disintegration due to the shearing forces during the shake-flask experiments. This could be one of the reasons that the rate of increase in mycelial biomass could be maintained to enhance the production of EPS after the 5th day with the addition of Tween 80 (Figure 3.13). This finding was in agreement with the result of Venkatadri and Irvine (1990), in which Tween 80 was able to protect an extracellular ligninase produced by submerged fermentation of the white rot fungus *Phanerochaete chrysosporium* against mechanical inactivation caused by agitation. In another study, Tween 80 could protect the activity of lactate dehydrogenase from denaturation during freeze-thawing (Hillgren et al., 2002).

Moreover, the addition of Tween 80 gave the mycelial pellet a more intact and compact morphology which exerts enhanced the EPS production by submerged fermentation of PTR. There were other similar observations reported previously. In a submerged fermentation of an ascomycete *Paecilomyces japonica*, pellets with high compactness proved to be a more productive morphological form compared with free filamentous mycelia fermentation (Sinha et al. 2001). Another similar study on the roughness and compactness of the mycelial pellets in *Cordyceps militaris* C738 (characterized by use of image analysis between different culture conditions) indicated that larger and more compact pellets were desirable for EPS production by submerged fermentation (Kim et al., 2003).

3.4.2.2 pH value

It has been proposed that the different morphology of fungal mycelia under different pH values was regarded as one of the important factors affecting biomass accumulation and metabolite formation (Hwang et al., 2004). The pH of the culture medium may affect cell membrane functions, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients, and biosynthesis of metabolites (Kim et al., 2003). In general, cells can only grow within a certain pH range, and metabolite formation is also often affected by pH (Kim et al., 2003).

For the mycelial growth and biosynthesis of mushroom EPS in submerged fermentation, the pH value of the fermentation broth was also one of the most critical environmental parameters (Kim et al., 2006, Shu and Lung, 2004, Zou et al., 2009). In order to investigate the protective effect of Tween 80 to the mycelia of PTR, the time-course changes of pH value with and without the addition of Tween 80 were monitored and the results are shown in Figure 3.16. The pH value of the fermentation broth without the addition of Tween 80 was slightly decreased from 5.49 to 5.17 during the first 6 days and then started to increase slightly after that. In contrast, the pH value in the fermentation broth with the addition of Tween 80 on the 5th day exhibited a much larger decrease from 5.28 to 4.32 and this level was maintained until the last day (8th day) of fermentation. It was noted that the pH Tween 80 was 5.80 and hence the decrease of pH value in the fermentation broth was not caused by the inherent pH of Tween 80 itself.

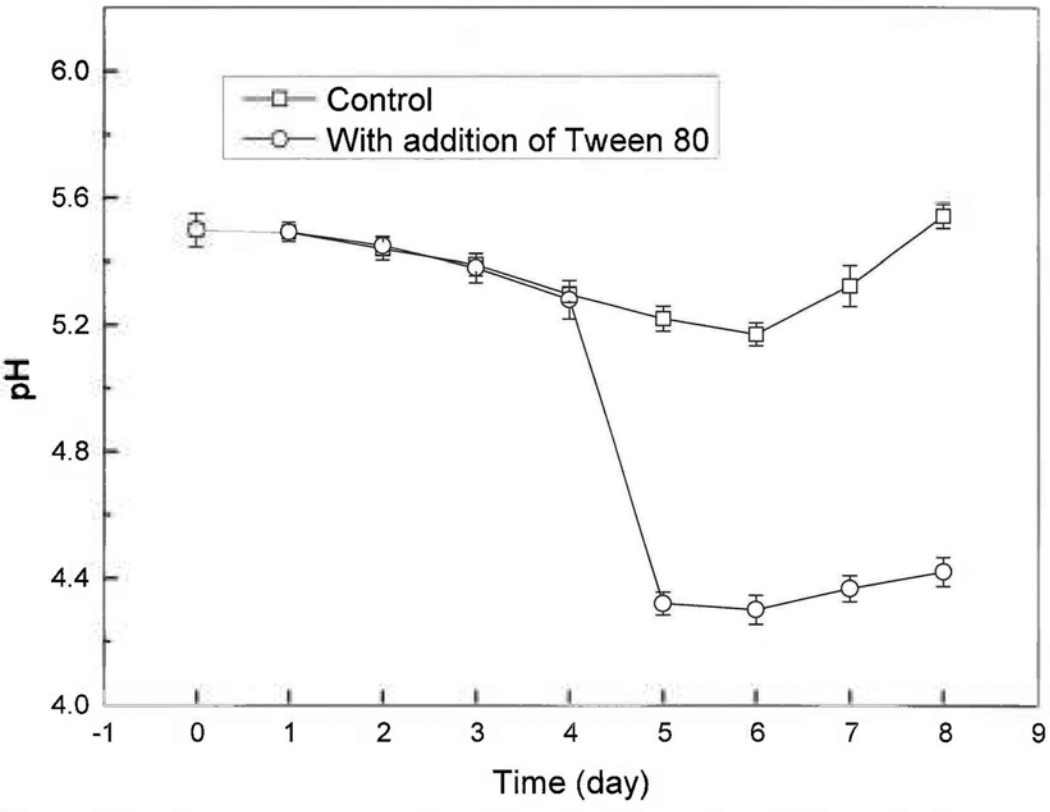


Figure 3.16 Time profiles of pH with and without the addition of Tween 80 in submerged fermentation of PTR

Tween 80 was added at a concentration of 0.3% w/v on the 5th day of fermentation. The culture experiments were carried out in triplicate at 30 °C for 8 days. Values are given as mean value ± standard deviation (n=3).

It is known that when mycelial pellets disintegrate and lysis of mycelium occurs, there is a release of the intracellular components (proteins with buffering effect) that would increase the pH of the fermentation broth. The results in Figure 3.15 and Figure 3.16 suggested that Tween 80 could protect the integrity of the mycelia as indicated by microscopic observation as well as an acidic pH in the fermentation broth and hence enhance the production of mycelial biomass and EPS. These results were in consistent with that of Huang et al. (2009), in which the production of both biomass and EPS were increased significantly in a submerged fermentation of *Ganoderma lucidum* when a low pH (about 2.0) was maintained for an extended period of fermentation (from 7 days to 11 days). In another similar study, lowering the initial pH from 6.5 to 3.5 gradually gave rise to higher production of EPS and a higher production of an intracellular polysaccharide in *Ganoderma lucidum* (Fang and Zhong, 2002).

3.4.3 Effect of Tween 80 on the mushroom cell membrane and its permeability

The fatty acid composition of the cell membrane has a profound effect on its permeability and fluidity. Changes in the membrane composition and properties represent an important factor in the adaptation to different conditions (Nozawa et al., 1974).

Microorganisms own the ability to regulate the permeability and fluidity of their cell membranes by altering fatty acid composition when subjected to different environmental conditions. For example, it was reported that the increase in temperature decreased the ratio of palmitoleic-to-palmitic acid in the fatty acid composition of the cell membrane of *Tetrahymena pyriformis* (Nozawa et al., 1974). Another study reported that salt tolerant yeasts responded to salt stress by decreasing the acyl chain unsaturation of their phospholipids and by increasing the sterol-to-phospholipid ratio, leading to a decrease in membrane fluidity (Hosono, 1992). Furthermore, Reese and Maguire (1969) have reported that Tween 80 stimulated the yield of extracellular enzymes in various fungi (e.g. yield of cellulose by *Trichoderma viride*) by promoting the uptake and exit of substances from the fungal cell through modification of its cell membrane permeability. Hence, the study

of the change in the fatty acid composition in the cell membrane of PTR with and without the addition of Tween 80 was carried out to provide more evidence for this hypothesis.

3.4.3.1 Composition and content of fatty acids of mycelial cells

The fatty acid contents (mg/g dry cell weight) and fatty acid composition (% total fatty acid) of the mycelial cell membrane in PTR with and without the addition of Tween 80 are shown in Table 3.8 and Figure 3.17, respectively. The major components in the mycelial lipids of PTR produced by submerged fermentation (either with or without Tween 80) were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), which were similar with other *Pleurotus* species (Hadar and Cohen-Arazi, 1986). With the addition of Tween 80, the amount of total fatty acids in the mycelial lipids of PTR was significantly enhanced ($p < 0.05$) from 22.1 to 27.8 mg/g whereas the amount of oleic acid (C18:1) was also significantly increased ($p < 0.05$) from 0.6 to 5.1 mg/g dry weight, concomitant with an increase of its percentage from 2.6 to 18.5% in the fatty acid composition (Table 3.8 and Figure 3.17). In the case of linoleic acid (C18:2), no significant difference ($p > 0.05$) was observed in the content of fatty acid although its % composition in total fatty acid was decreased significantly ($p < 0.05$) (Table 3.8 and Figure 3.17). There was no significant change in other fatty acids such as palmitic acid (C16:0), palmitoleic acid (C16:1) and stearic acid (C18:0) in the lipids isolated from the mycelia obtained from the fermentation broth with and without the addition of Tween 80 (Table 3.8 and Figure 3.17). From Table 3.8, it was noted that the ratio of unsaturated-to-saturated fatty acids in the mycelial lipids with the addition of Tween 80 increased from 2.88 to 3.41. It had been reported that an increase in unsaturated fatty acids enhanced the permeability of *Acholeplasma laidlawii* B cells (McElhaney et al., 1973).

Table 3.8 Fatty acid contents (mg/g dry cell weight) of PTR mycelial cells by submerged fermentation with and without the addition of Tween 80

Fatty acids	Control	With Tween 80
C16:0	5.3±0.2	5.8±0.2
C16:1	0.5±0.1	0.6±0.2
C18:0	0.4±0.1	0.5±0.2
C18:1	0.6±0.1	5.1±0.4 *
C18:2	15.3±0.6	15.8±0.5
Total	22.1±0.9	27.8±1.1 *
Unsaturated/saturated #	2.88	3.41

Tween 80 was added at a concentration of 0.3% w/v on the 5th day of fermentation. The culture experiments were carried out in triplicate at 30 °C for 8 days. Values are given as mean value ± standard deviation (n=3). Values denoted with an asterisk have significant difference between control and treatment group (Student's *t*-test; $p < 0.05$).

Ratio of Unsaturated/saturated = (C16:1 + C18:1 + C18:2) / (C16:0 + C18:0)

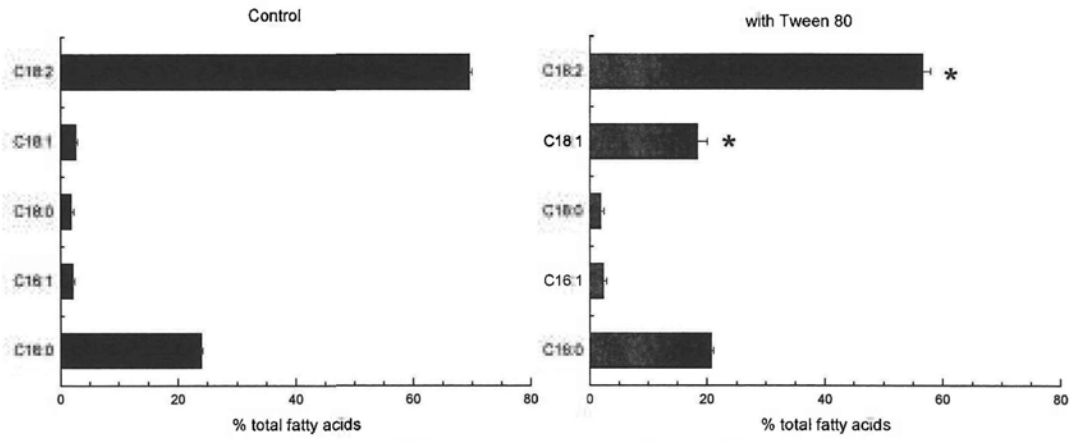


Figure 3.17 Fatty acid composition of PTR mycelial cells by submerged fermentation with and without the addition of Tween 80

Tween 80 was added at a concentration of 0.3% w/v on the 5th day of fermentation. The culture experiments were carried out in triplicate at 30 °C for 8 days. Values are given as mean value ± standard deviation (n=3). Values denoted with an asterisk have significant difference between control and treatment group (Student's *t*-test; $p < 0.05$).

In the section 3.4.1.3, the glucose consumption rate was significantly increased ($p < 0.05$) after the addition of Tween 80, implying that the uptake efficiency of nutrients from the fermentation broth had been increased that eventually led to an increase of mycelial biomass and EPS production of PTR. The present results also indicated that Tween 80 could significantly increase the unsaturated fatty acid content of oleic acid (C18:1) (Table 3.8 and Figure 3.17). Combined these results, they were similar with previous findings that an increase of unsaturated fatty acids C18:1 in the membranes of the *Aspergillus niger* might be related to the increase in glucose consumption and extracellular enzyme production (Maldonado and Strasser de Saad, 1998).

To the best of our knowledge, there has been no previous study on the relationship between addition of Tween 80 and the fatty acid composition of mycelial cell membrane. The above results suggested that Tween 80 could affect the mycelial cell membrane composition by significantly increasing the fatty acid content with oleic acid (C18:1) in particular, which might increase the permeability of mycelial cells in PTR and enhanced the production of EPS.

3.4.3.2 Concentration of Tween 80

It had been shown that the oleic acid contained in Tween 80 could be incorporated into the cell membranes of some bacteria such as lactic acid bacteria and *Thraustochytrium aureum* (Johnsson et al., 1995; Taoka et al., 2011). It was presumed that Tween 80 could be transported into the bacterial cells and partially utilized as a carbon source (Taoka et al., 2011). However, based on the above results in section 3.4.1.2, the mycelial cells of PTR could not use Tween 80 as a carbon source for its growth. As shown above, Tween 80 could influence the fatty acid composition and significantly increased the content of oleic acid (C18:1) in the mycelial cell membrane of PTR.

A time course experiment on the residual concentration of Tween 80 during submerged fermentation of PTR was performed to investigate whether oleic acid in Tween 80 could be incorporated into the mycelial cell membrane. The initial

concentration of Tween 80 added on the 5th day (0.3 % w/v) was significantly decreased ($p < 0.05$) by 52.9% during the first 3 days (from the 5th day to the 8th day) and was finally kept at a steady value of 0.14% w/v (Figure 3.18). The significant increase in the content of oleic acid (C18:1) in the mycelial cell membrane of PTR (Table 3.8 and Figure 3.17) could be partly explained by the significant decrease ($p < 0.05$) in the concentration of Tween 80 (Figure 3.18). This finding was consistent with the results of a previous study in which by adding Tween 80 into the fermentation broth of *Phanerochaete chrysosporium* the fatty acids (oleic acid) released from Tween 80 through enzymatic hydrolysis were incorporated into its phospholipids, modifying the mycelial membrane permeability (Asther et al., 1988).

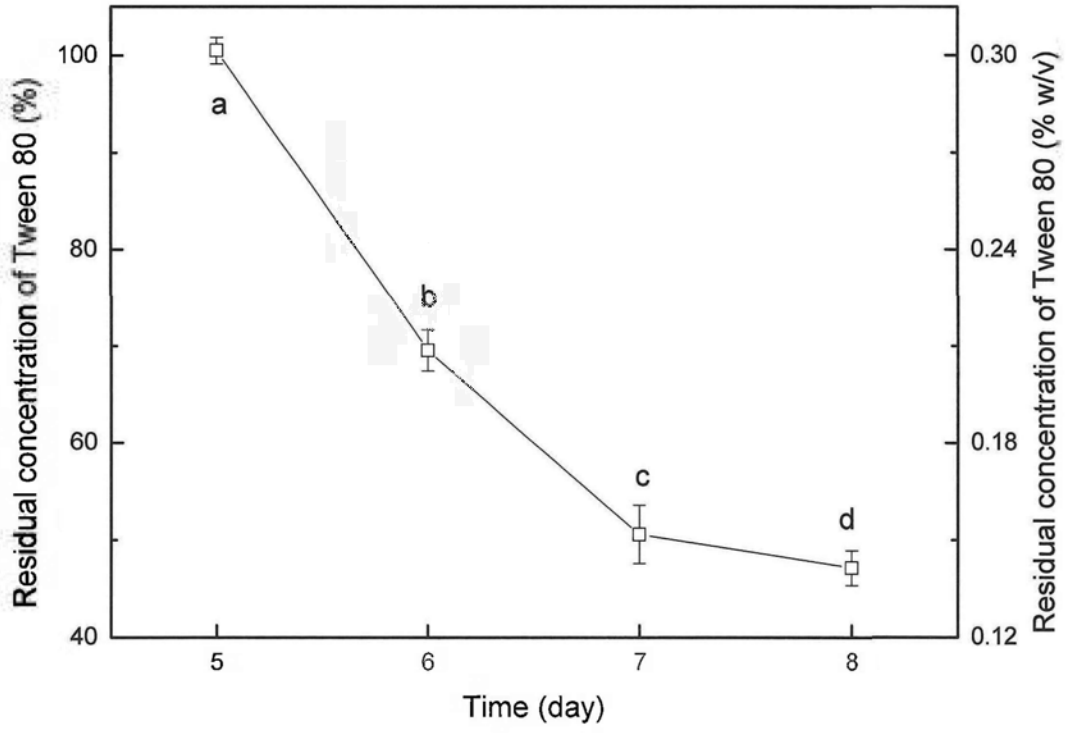


Figure 3.18 Time profile of residual concentration of Tween 80 during submerged fermentation of PTR

Tween 80 was added at a concentration of 0.3% w/v on the 5th day of fermentation. The culture experiments were carried out in triplicate at 30 °C for 8 days. Values (mean value ± standard deviation, n=3) denoted with different superscripts in lowercase letters have significant difference (ANOVA Tukey's test; $p < 0.05$).

Based on these results, it was suggested that the oleic acid contained in Tween 80 could be incorporated into the mycelial cell membrane of PTR, increasing its fatty acid composition (increase in oleic acid) and the cell membrane permeability accordingly.

In order to further confirm the finding that the oleic acid contained in Tween 80 incorporated into the PTR mycelial cell membrane, application of an advanced image analysis technique by confocal laser scanning microscopy (CLSM) was required.

3.4.3.3 Confocal laser scanning microscopy (CLSM) observation

In the previous sections of 3.4.3.1 and 3.4.3.2, it was suggested that the oleic acid contained in Tween 80 could be incorporated into the mycelial cell membrane of PTR, increasing its fatty acid composition and the cell membrane permeability accordingly. In this section, Tween 80 was labeled with a fluorescent dye and the hypothesis of the incorporation of Tween 80 into mycelial cell membrane was verified by an observation of the mycelium under CLSM.

The dye 5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF), which could bind covalently to the hydroxyl group, was used in this study for labeling Tween 80. The labeled Tween 80 was separated from the unlabed dye by heating the mixed solution to the clouding point of Tween 80 at 93 ^{o}C in a water bath. Afterwards, the Tween 80 labeled with 5-DTFA was added at a concentration of 0.3% w/v on the 5th day of fermentation. The culture experiments were carried out at 30 ^{o}C for 8 days. After the submerged fermentation process, mycelial cells of PTR were separated from the fermentation broth and observed under the CLSM. The images of mycelial cells cultured with fluorescent labeled Tween 80 are shown in Figure 3.19.

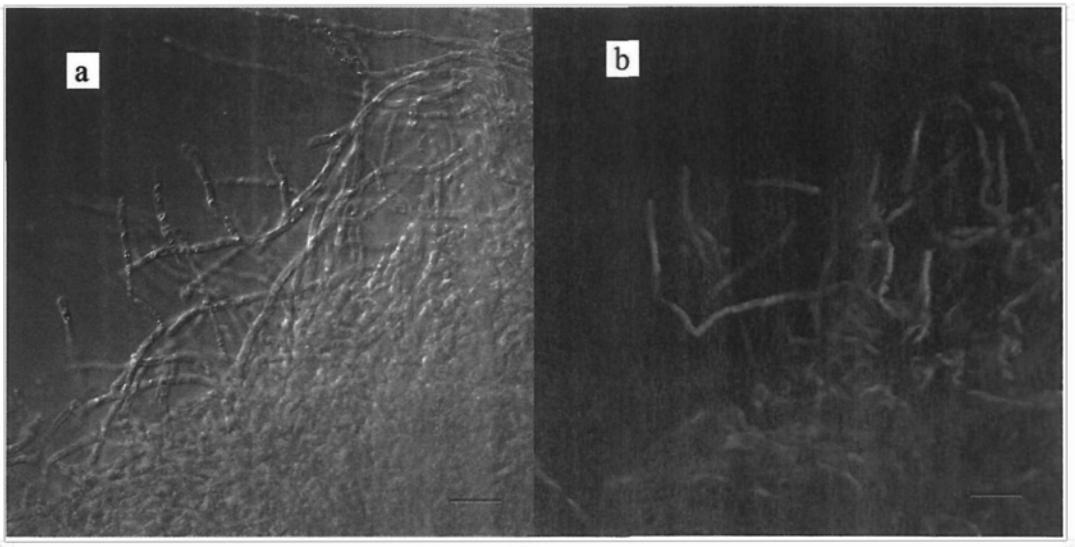


Figure 3.19 CLSM images of mycelial cells of PTR cultured with the addition of Tween 80 (a) ordinary view (b) fluorescent view

A fluorescent dye 5-DTFA which could bind covalently to the hydroxyl group was used to label Tween 80. The scale bar in pictures represents 20 μm.

The mycelial cells were aggregated as a pellet structure with a hairy appearance on their surfaces as shown in the ordinary view of mycelial cells in Figure 3.19 a. The mycelial cells were cultured in the medium containing Tween 80 labeled with a fluorescent dye 5-DTFA. The fluorescent view of mycelial cells shown in Figure 3.19 b indicated the fluorescent stains in some mycelial cells. These fluorescent stains were come from Tween 80 labeled with a fluorescent dye 5-DTFA. These results confirmed that Tween 80 could be incorporated into the mycelial cell of PTR.

Some preliminary results in previous reports had suggested that addition of Tween 80 could be incorporated into cell membrane and affected its composition but lacked direct evidence of proof (Nemec and Jernejc, 2002; Taoka et al., 2011). For the first time, the use of CLSM, together with fluorescent labeled Tween 80 by 5-DTFA, had proved that Tween 80 could be incorporated into the mycelial cell membrane of PTR. This finding could give a strong support to the hypothesis that Tween 80 could be incorporated into the mycelial cell membrane and then further influenced fatty acid composition of mycelial cells and the cell membrane permeability accordingly.

3.5 Proteomics of PTR mycelial cells with and without the addition of Tween 80

3.5.1 Proteomics by One-dimensional gel analysis

3.5.1.1 One-dimensional gel profile

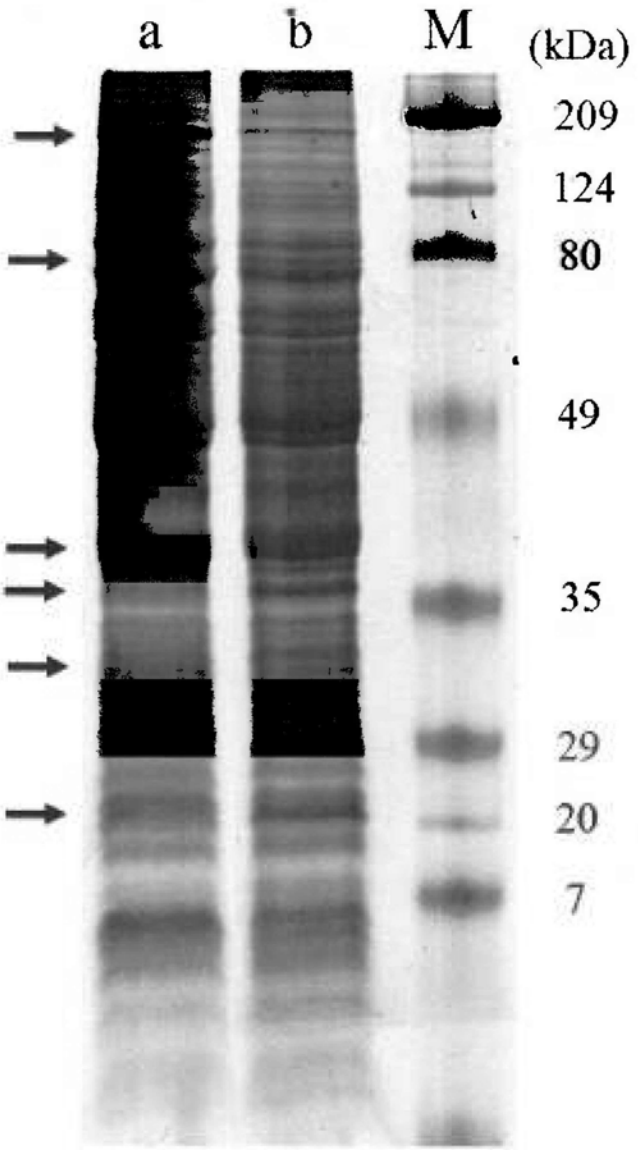


Figure 3.20 SDS-PAGE of mycelial proteins of PTR by submerged fermentation without (a) and with (b) the addition of Tween 80. M: Makers of Bio-Rad standards Some protein bands with differential expression level by the addition of Tween 80 were denoted with the arrows.

The SDS-PAGE of mycelial proteins of PTR by submerged fermentation without and with the addition of Tween 80 is shown in Figure 3.20. Forty microgram of proteins were loaded and separated by the SDS-PAGE. In general, the major bands of proteins appeared in the gels with or without Tween 80 added were similar. There were some differences in the expression level of several protein bands with the addition of Tween 80 (Figure 3.20).

The gels with and without the addition of Tween 80 were digestion with trypsin, the tryptic peptides of the gel bands were analyzed by nESI-LC-MS/MS. Acquired MS/MS profiles were searched against NCBI nonredundant protein database (fungi) using the MASCOT search engine. The functional categories of matched proteins from PTR mycelial cells produced with and without the addition of Tween 80 are listed in Table 3.9.

One-dimensional gel analysis has successfully identified 51 differentially expressed proteins from SDS-PAGE gels with and without the addition of Tween 80 (Table 3.9). These 51 differentially expressed proteins belonged to ten functional categories according to their biological process as shown in Figure 3.21. These ten functional categories included translation, ribosomal structure and biogenesis (37.3%), post-translational modification, protein turnover, chaperones (11.8%), energy production and conversion (9.8%), amino acid transport and metabolism (9.8%), carbohydrate transport and metabolism (7.8%), chromatin structure and dynamics (5.9%), cytoskeleton (3.9%), signal transduction (3.9%), DNA repair (2.0%) and unclassified (7.8%). Among them, 28 proteins were only identified on the SDS-PAGE gel from PTR mycelial cell produced without the addition of Tween 80 while 23 proteins were only appeared on the gel with the addition of Tween 80. The followings are the details of the possible functional roles of some of these proteins in the PTR mycelial cells.

3.5.1.2 Functional analysis of proteins with different expression level

3.5.1.2.1 Translation elongation factor proteins

There were eleven translation elongation factor proteins differentially expressed in the two comparative gels with and without the addition of Tween 80 (Table 3.9). Translation elongation factor proteins associate with ribosomes cyclically during the elongation phase of protein synthesis, and catalyze formation of the acyl bond between the incoming amino-acid residue and the peptide chain (Parker, 2003). They play two important roles during the elongation cycle of protein biosynthesis on the ribosome. First, translation elongation factors are involved in bringing aminoacyl-tRNA (aa-tRNA) to the ribosome during protein synthesis. Second, an elongation factor is involved in translation, the step in elongation at which the peptidyl-tRNA is moved from one ribosomal site to another as the mRNA moves through the ribosome. Both steps result in the hydrolysis of guanosine triphosphate (GTP), and the conformation of the elongation factors changes depending on whether they are bound to GTP or to guanosine diphosphate (GDP) (Parker, 2003). The differential expression level in these proteins may be attributed to translational channeling and compartmentalization of protein synthesis in higher eukaryotic cells and they may contribute to the coordinate regulation of multiple cellular processes including growth, division, and transformation (Negrutskii and El'skaya, 1998).

3.5.1.2.2 Ribosomal proteins

Eight ribosomal proteins such as 60S ribosomal protein L23a, 40S ribosomal protein S14, 40S ribosomal protein S11 with differential expression level were found in this category by 1D gel analysis with the addition of Tween 80 (Table 3.9). In general, ribosomal proteins are involved in ribosome biogenesis and/or for different stages of the translation process (Ferreira-Cerca et al., 2005). Accordingly, ribosomal proteins might be very important in transport of the ribosomal precursors, RNA folding, protein assembly, rRNA processing, stabilization of the subunit structure, and/or interaction with other factors required for either ribosome

biogenesis or translation. Furthermore, they can also play a key role in cotranslational processes like cotranslational translocation or the interaction with protein folding factors at the exit tunnel of the ribosome (Ferreira-Cerca et al., 2005). Ribosomal proteins can also contribute to important enzymatic activities for ribosome function like the mRNA helicase activity of bacterial ribosomes (Takyar et al., 2005). Hence, the differential expression level in these translation-related proteins suggested the protein synthesis was affected by the addition of Tween 80.

3.5.1.2.3 Heat shock proteins

Six of the proteins identified in the category of posttranslational modification, protein turnover and chaperones belong to the heat shock protein family. One of the six proteins was only expressed in the control gel while the other five proteins were only appeared in the gel with the addition of Tween 80 (Table 3.9). This suggested that an increasing trend in the expression level of heat shock proteins from PTR mycelial cells produced with the addition of Tween 80. Heat shock proteins were either constitutively expressed or activated when exposed to elevated temperatures or other environmental stress conditions, such as contacted with toxins, infection, starvation or other nutrition deficiency (Narberhaus, 2002). The mechanism by which environmental stress condition induces the heat shock factor has not been clear understood. Apart from the stress response ability, HSP70 played essential roles under normal condition including assisting folding of newly synthesized proteins, guiding translocation of proteins across organelle membrane, disassembling oligomeric protein structures and facilitating proteolytic degradation of unstable proteins (Bakau and Horwich, 1998). HSP60 possessed similar functions and was also found to be responsible for assembly of peptides that were imported into mitochondria, as well as export of certain secretory proteins (Plesofsky-Vig and Brambl, 1995).

At the later growth phase, the environmental stress of submerged fermentation on the PTR mycelial cells was increased and might result in the increase number of damaged proteins or other cellular components. Consequently, the demand for heat shock proteins had to be increased to provide additional repair of misfolded proteins, protecting cellular proteins, and maintaining cellular viability

under conditions of intensive stress. In section 3.4.2, it was indicated that the addition of Tween 80 could extend the growth of the mycelia for a longer period possibly by maintaining the intact structure of the mycelial pellets and preventing its disintegration caused by the shearing forces during the shake-flask experiments. Combining all these results, it could be suggested that the addition of Tween 80 might protect PTR mycelial cells by up-regulating the expression level of heat shock proteins.

3.5.1.2.4 Tricarboxylic acid cycle (TCA cycle) related proteins

As mentioned earlier, the addition of Tween 80 could significantly increase the EPS production in submerged fermentation of PTR (section 3.1), it is necessary to investigate the proteins involved in the function of carbohydrate transport and metabolism. A total of four proteins had different expression levels in this function category (Table 3.9). Among them, YALI0E34793p (only identified in the control gel) and ATP citrate lyase isoform 2 (only appeared in the gel with the addition of Tween 80) were involved in the TCA cycle which are involved in aerobic respiration in all living cells.

YALI0E34793p possesses the ATP citrate synthase activity which catalyzes the conversion of oxaloacetate and acetyl-CoA into citrate and CoA, the first step of the TCA cycle. In contrast, ATP citrate lyase isoform 2 owns the citrate lyase activity which catalyzes the reaction of citrate and CoA to form oxaloacetate and acetyl-CoA, along with the hydrolysis of ATP. These two proteins are responsible for the same reaction but in opposite direction. YALI0E34793p (only identified in the control gel) promotes the TCA cycle while ATP citrate lyase isoform 2 (only appeared in the gel with the addition of Tween 80) suppresses the TCA cycle. It was reported that the activity of TCA cycle enzymes is influenced by the nutritional condition of the cell and a variety of stress-inducing stimuli (Vuong et al., 2005; Sadykov et al., 2008). It was also found that the PIA (an N-acetylglucosamine exopolysaccharide) synthesis by *Staphylococcus epidermidis* was increased when the TCA cycle activity was suppressed by changing the environmental and nutritional conditions (Vuong et al., 2005). It had been proposed that the mechanism by which *S. epidermidis* can perceive external environmental change is through alterations in its TCA cycle

activity leading to changes in the intracellular levels of biosynthetic intermediates, ATP, or the redox status of the cell (Vuong et al., 2005; Sadykov et al., 2008).

In the present study, the activity of TCA cycle was suppressed due to the decreasing expression of YALI0E34793p and the increasing expression of ATP citrate lyase isoform 2 with the addition of Tween 80, which might further exert a stimulatory effect on the production of EPS. This finding was consistent with previous studies (Vuong et al., 2005; Sadykov et al., 2008) and was very important for understanding the mechanism of the stimulatory effect of Tween 80. More in-depth investigations on the intracellular levels of this bioprocess are required.

3.5.1.2.5 Signal transduction related protein

Mitogen-activated protein kinase (MAPK) plays a central role in signal transduction pathways that control intracellular events including acute responses to hormones and major developmental changes in organisms (Pearson et al., 2001). MAPK can be activated by a wide range of extracellular signals (mitogens, osmotic stress and heat shock) and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis (Pearson et al., 2001). The MAPK signaling cascades convey information to effectors, coordinate incoming information from other signaling pathways, amplify signals, and allow for a variety of response patterns (Pearson et al., 2001). They respond to different stimuli by phosphorylating cytoplasmic components and nuclear transcription factors depending on the cellular context. Down-regulation of MAPK pathways may occur through dephosphorylation by serine/threonine phosphatases, tyrosine phosphatases, or dual-specificity phosphatases and through feedback inhibitory mechanisms that involve the phosphorylation of upstream kinases (Weston et al., 2002). In the present study, the increase in expression level of this protein might reflect the response of mycelial cells to the environmental stimuli by the addition of Tween 80 and more studies on this hypothesis are required.

Table 3.9 The functional categories of proteins from PTR mycelial cells produced with and without the addition of Tween 80 by 1D gel analysis

Protein number	Accession number	Protein name	Organism	Theoretical Mw	Score	Matching peptide	Control	Addition of Tween 80
Translation, ribosomal structure and biogenesis								
1	gi|156558207	Elongation factor	*Hebeloma cylindrosporum*	50418	243	5	+	–
2	gi|154819731	Translation elongation factor 1-α	*Hypocrea microcitrina*	36280	112	2	+	–
3	gi|32563370	Translation elongation factor 1-α	*Hanseniaspora guilliermondii*	41491	142	3	+	–
4	gi|148788034	Elongation factor 1-α	*Calocybe ionides*	24982	95	3	+	–
5	gi|53831018	Translation elongation factor 1-α	*Beauveria bassiana*	46819	94	3	+	–
6	gi|156099458	Translation elongation factor 1-α	*Brettanomyces naardenensis*	35687	54	2	+	–
7	gi|242206798	60S ribosomal protein L23a	*Postia placenta* Mad-698-R	17820	147	1	+	–
8	gi|39940004	40S ribosomal protein S14	*Magnaporthe oryzae* 70-15	16175	74	1	+	–

9	gi|170086746	Predicted protein	*Laccaria bicolor* S238N-H82	29630	101	2	+	–
10	gi|169864162	Ras2	*Coprinopsis cinerea* okayama7#130	40999	77	2	+	–
11	gi|50421395	DEHA2D17512p	*Debaryomyces hansenii* CBS767	17896	59	1	+	–
12	gi|320590768	Translation elongation factor 1-α	*Grosmannia clavigera* kw1407	50173	227	5	–	+
13	gi|58758721	Translation elongation factor 1-α	*Peniophorella praetermissa*	33838	132	3	–	+
14	gi|209402353	Translation elongation factor 1-α	*Absidia psychrophilia*	39947	306	6	–	+
15	gi|73990901	Translation elongation factor 1-α	*Hanseniaspora clermontiae*	33610	298	6	–	+
16	gi|134284926	Elongation factor 1-α	*Dactylellina parvicollis*	27758	199	4	–	+
17	gi|242212409	40S ribosomal protein S11	*Postia placenta* Mad-698-R	17791	100	4	–	+
18	gi|169862655	60s ribosomal protein l27	*Coprinopsis cinerea* okayama7#130	15893	97	2	–	+

19	gi|242206289	60S ribosomal protein L18A	*Postia placenta* Mad-698-R	20198	50	3	–	+
Post-translational modification, protein turnover, chaperones								
20	gi|172713	70kDa heat shock protein	*Saccharomyces cerevisiae*	37593	54	1	+	–
21	gi|20260807	Hsp70 protein 1	*Rhizopus stolonifer*	71127	84	3	–	+
22	gi|6323288	Hsp60p	*Saccharomyces cerevisiae* S288c	60999	69	1	–	+
23	gi|299754101	Heat shock protein	*Coprinopsis cinerea* okayama7#130	67280	66	2	–	+
24	gi|255724128	Heat shock 70 kDa protein C precursor	*Candida tropicalis* MYA-3404	74647	78	2	–	+
25	gi|6320950	Ssa4p	*Saccharomyces cerevisiae* S288c	69723	123	3	–	+
Energy production and conversion								
35	gi|50554781	YALI0E35112p	*Yarrowia lipolytica*	81172	54	1	+	–
27	gi|50424093	DEHA2F06226p	*Debaryomyces hansenii* CBS767	53847	77	2	–	+
37	gi|118596522	Acetaldehyde dehydrogenase	*Aciculosporium take*	32548	70	1	–	+

29	gi|212532319	Electron transport oxidoreductase	*Penicillium marneffei* ATCC 18224	74142	48	1	–	+
30	gi|169861381	ATP synthase d subunit	*Coprinopsis cinerea* okayama7#130	19369	60	1	–	+
			Amino acid transport and metabolism					
31	gi|299740078	Adenosylhomocysteinase	*Coprinopsis cinerea* okayama7#130	47514	100	2	+	–
32	gi|164662415	Hypothetical protein MGL_0104	*Malassezia globosa* CBS 7966	48007	99	2	+	–
33	gi|169845471	Adenosine kinase	*Coprinopsis cinerea* okayama7#130	36958	48	1	+	–
34	gi|322697160	Tyrosine-protein phosphatase non-receptor type 6	*Metarhizium acridum* CQMa 102	111872	54	1	+	–
35	gi|169862561	Glycine hydroxymethyltransferase	*Coprinopsis cinerea* okayama7#130	53167	123	3	–	+
			Carbohydrate transport and metabolism					
36	gi|50554757	YALI0E34793p	*Yarrowia lipolytica*	71441	64	1	+	–
37	gi|170096058	Transaldolase	*Laccaria bicolor* S238N-H82	35700	78	2	+	–

38	gi|299739746	ATP citrate lyase isoform 2	*Coprinopsis cinerea* okayama7#130	126895	288	7	–	+
39	gi|67523969	Hypothetical protein AN2440.2	*Aspergillus nidulans* FGSC A4	28792	48	1	–	+
			Chromatin structure and dynamics					
40	gi|331246889	Structural maintenance of chromosomes protein 3	*Puccinia graminis f. sp. tritici* CRL 75-36-700-3	141104	53	1	+	–
41	gi|169849973	Histone H4	*Coprinopsis cinerea* okayama7#130	11359	115	4	+	–
42	gi|50400217	Histone H4	*Mortierella alpina*	11402	98	2	–	+
			Cytoskeleton					
43	gi|170106465	Actin-1	*Laccaria bicolor* *4*S238N-H82	41947	95	3	+	–
44	gi|113292	Actin	*Candida albicans*	41954	70	2	+	–
			Signal transduction					
45	gi|170091548	14-3-3 protein	*Laccaria bicolor* S238N-H82	29025	206	5	+	–
46	gi|6224710	Mitogen-activated protein kinase	*Cochliobolus heterostrophus*	41057	56	1	–	+

			DNA repair					
47	gi|115442758	DNA damage checkpoint protein rad24	*Aspergillus terreus* NIH2624	29253	95	3	+	–
			Unclassified					
48	gi|156066428	1-Cys peroxiredoxin isozyme	*Taiwanofungus camphoratus*	18314	54	1	+	–
49	gi|50980804	Peptidyl-prolyl cis-trans isomerase	*Paxillus involutus*	17649	56	1	+	–
50	gi|3549613	ADP/ATP carrier protein	*Candida parapsilosis*	32914	102	2	+	–
51	gi|169851712	Manganese superoxide dismutase	*Coprinopsis cinerea* okayama7#130	22418	71	2	–	+

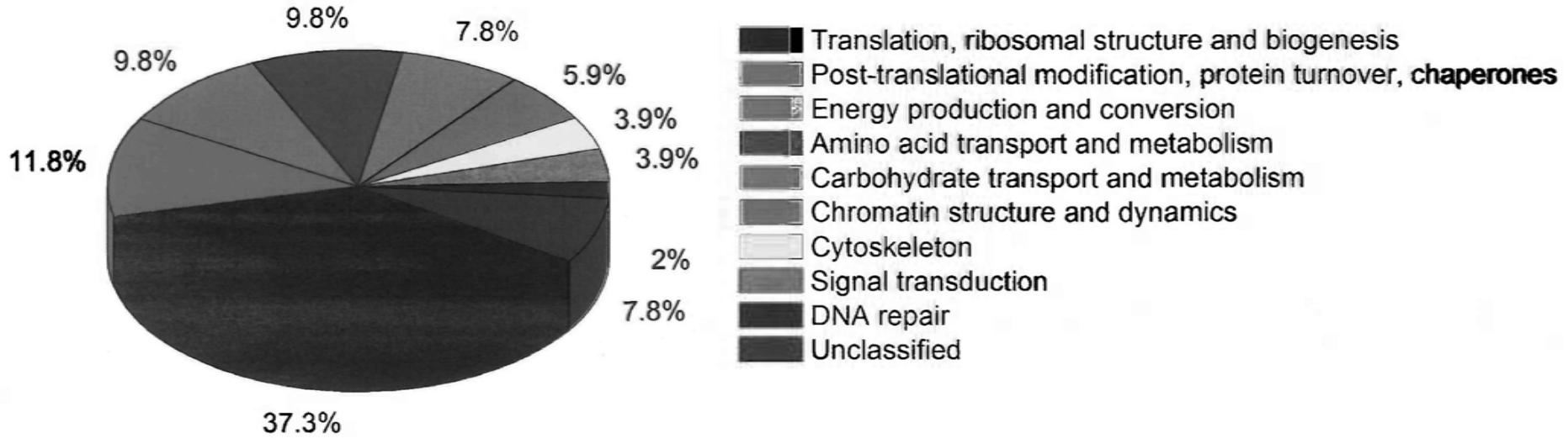


Figure 3.21 The ten functional categories of the differentially expressed proteins from PTR mycelial cells produced with and without the addition of Tween 80

3.5.2 Proteomics by Two-dimensional gel analysis

3.5.2.1 Two-dimensional gel profile

In order to achieve a higher level of resolution and higher loading capacity than that can be achieved in a 1D separation, 2D gels are the most common multidimensional separation technique used to separate proteins in complex mixtures. Therefore, the total proteins isolated from the mycelial cells with and without the addition of Tween 80 were separated in the 2D gels and further identified by the MALDI-TOF/TOF MS analysis.

The representative gel images of the proteins isolated from the mycelial cells without and with the addition of Tween 80 are shown in Figure 3.22 and 3.23, respectively. The gel with the addition of Tween 80 was compared with the one without addition of Tween 80 as reference. Approximately 260 protein spots on the two gels were revealed by the ImageMaster analysis. Among these 260 protein spots, those with a change of at least 2.5-fold increase and at least 0.4-fold decrease in the normalized volume ratio were considered as differentially expressed. With the addition of Tween 80, a total of 40 spots were differentially expressed (Figure 3.23), in which 24 spots having at least 2.5-fold increase and 16 spots having at least 0.4-fold decrease. The identification data for these 40 differentially expressed proteins is shown in Table 3.10. The possible functional roles of some of these proteins are discussed in the followings.

3.5.2.2 Functional analysis of proteins with different expression level

3.5.2.2.1 Membrane related protein

The hypothetical protein TRV_04216 (spot 10) and fatty acid synthase alpha subunit FasA (spot 19) were two membrane related proteins with increasing expression level by the addition of Tween 80. The hypothetical protein TRV_04216 protein was believed to have membrane related function but its exact activity was unknown while the fatty acid synthase alpha subunit FasA is mainly for fatty acid biosynthesis. Fatty acid synthases possess the ability to produce long-chain fatty acids which play a central role in the cell to incorporate lipids into membranes or storage bodies (Tsitsigiannis et al., 2004). It has been found that fatty acid synthase alpha subunit (fasA) encodes the central enzyme in *de novo* lipogenesis, catalyzing the synthesis of acetyl coenzyme A (acetyl-CoA) and malonyl-CoA into long-chain fatty acids (Brown et al., 1996; Tsitsigiannis et al., 2004).

In section 3.4.3, it was shown that the amount of total fatty acids in the mycelial lipids of PTR was significantly enhanced ($p < 0.05$) from 22.1 to 27.8 mg/g with the addition of Tween 80. When combining with the results of increasing expression level of fatty acid synthase found in the 2D gel analysis, it could be suggested that the addition of Tween 80 could exert an effect on the fatty acid synthase of PTR mycelial cells to influence the fatty acid content.

3.5.2.2.2 Cell communication protein

Hypothetical protein PGUG_02954 (spot 24) and Phospholipase D1 (spot 38) are both phosphatidylinositol binding proteins and function in cell communication. In general, cell communication proteins are involved in the process that mediates interactions between a cell and its surroundings, such as signaling or attachment between one cell and another cell, between a cell and an extracellular matrix, or between a cell and any other aspect of its environment (Rasmussen, 1970). These two cell communication proteins are bind with phosphatidylinositol, which play important roles in membrane trafficking, lipid signaling and cell signaling. With the

addition of Tween 80, the expression level of hypothetical protein PGUG_02954 was increased while that of Phospholipase D1 was decreased. However, it was not clear about the origin of the change of expression level in these two cell communication proteins. It could only be proposed that the addition of Tween 80 might act as an extracellular stimulus to the PTR mycelial cells through the cell communication proteins.

3.5.2.2.3 ATP-binding cassette transporter protein

ATP-dependent bile acid permease (spot 32) is one kind of ATP-binding cassette (ABC) transporter protein. ABC transporters are transmembrane proteins that use the energy from ATP hydrolysis to conduct a number of biological processes such as transport different kinds of substrates across extra- and intracellular membranes and also non-transport-related processes such as translation of RNA and DNA repair (Davidson et al., 2008). ABC transporters can mediate the uptake of various substrates including ions, amino acids, peptides, sugars, and other molecules which are mostly hydrophilic into the cell. They can also function as pumps that extrude toxins and some metabolic products such as lipids, polysaccharides and teichoic acid out of the cell (Davidson and Chen, 2004). The most noteworthy function of ABC transporter is that they also play important roles in extracellular polysaccharide biosynthesis pathway (Zhou et al., 1998). Although the exact function of ATP-dependent bile acid permease was not clear understood, it seems interesting to connect the differential expression of this ABC transporter protein with the addition of Tween 80, which led to a significant increase in the production of EPS. However, the hypothetic interaction of Tween 80 and ABC transporter protein needs more in-depth studies.

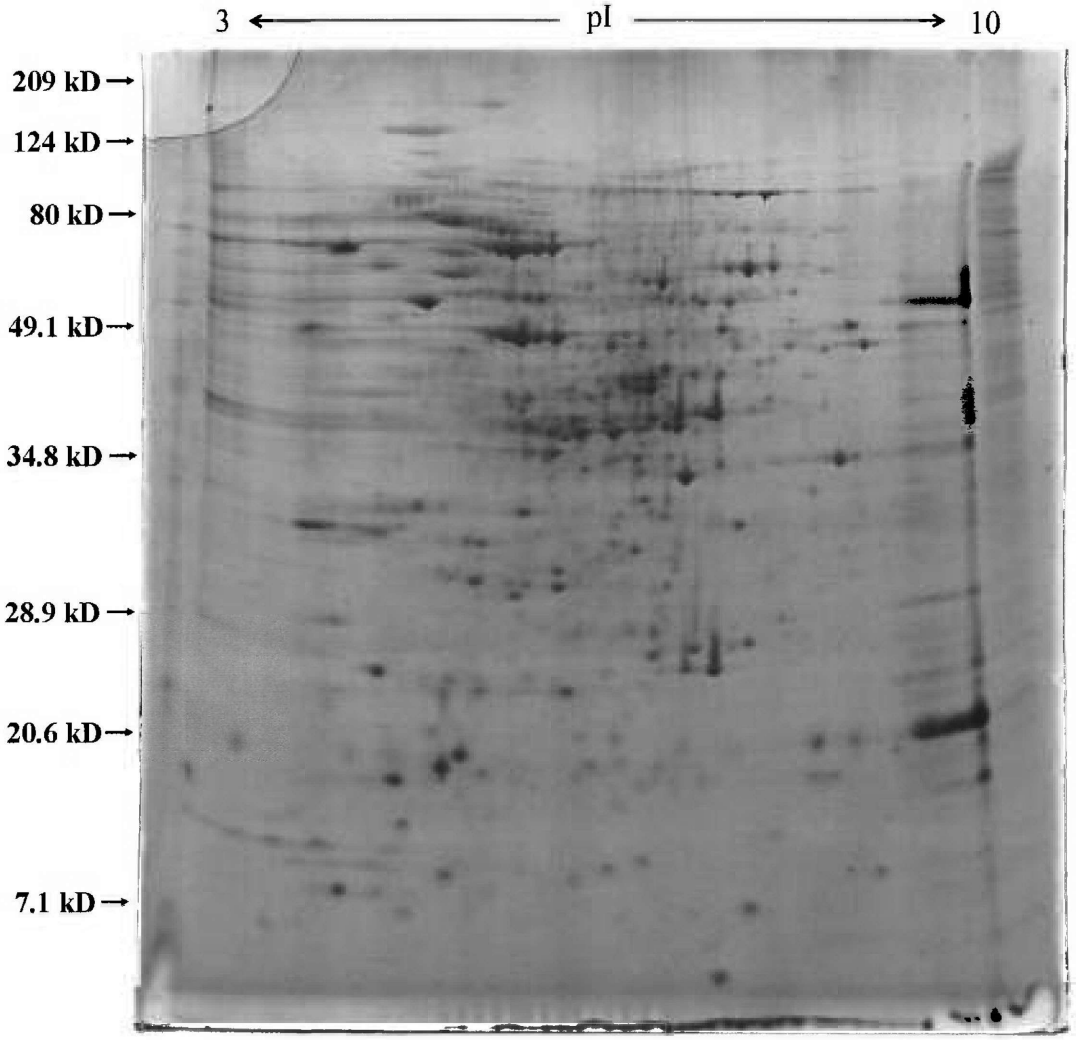


Figure 3.22 Representative 2D gel of PTR mycelial proteins produced without the addition of Tween 80 (reference gel)

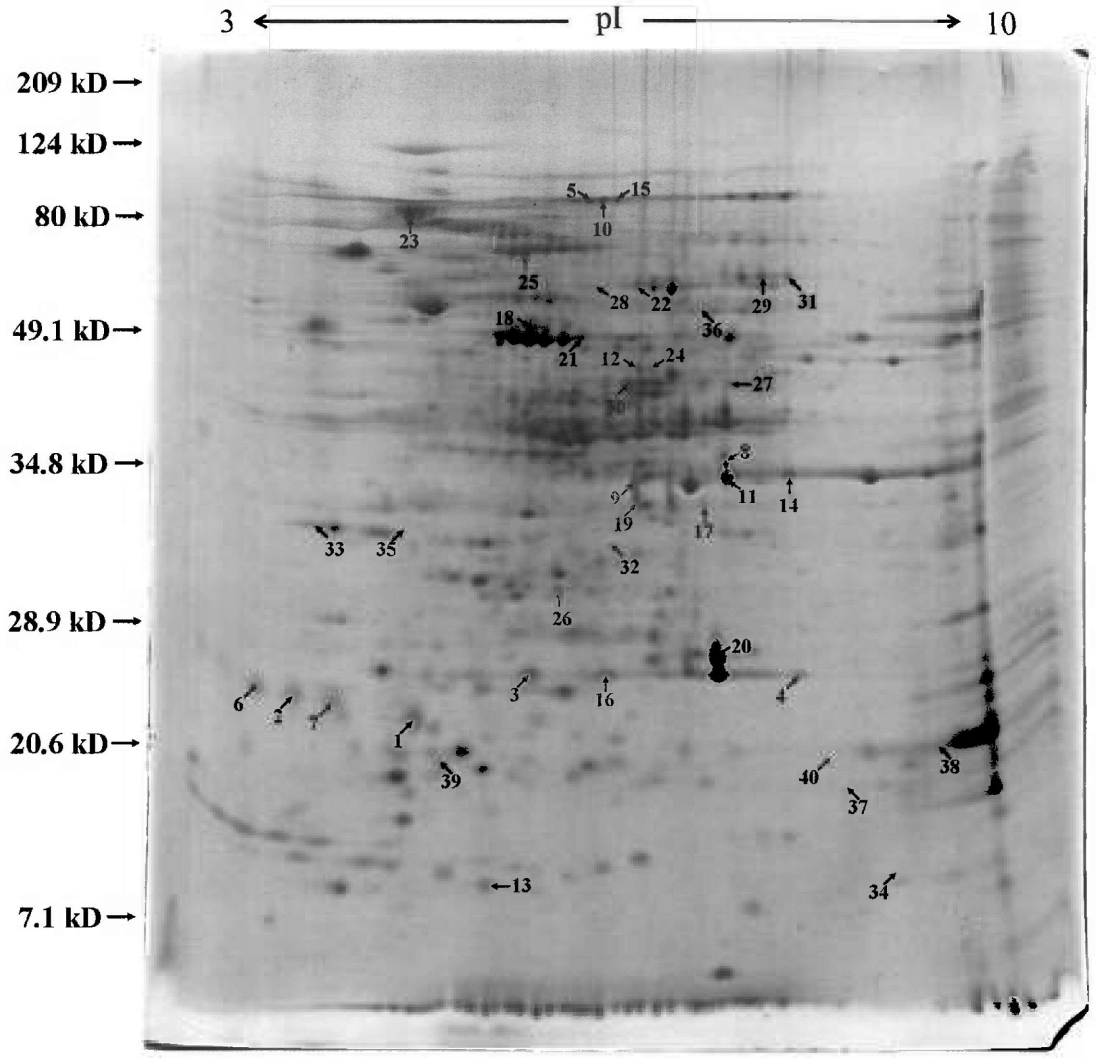


Figure 3.23 Representative 2D gel of PTR mycelial proteins produced with the addition of Tween 80

Protein spots with differentially expression are denoted on the gel image. Spots 1-24 are the proteins with an up-regulation of expression while spots 25-40 are the proteins with a down-regulation of expression when compared to the reference gel.

Table 3.10 Identification of differentially expressed proteins from PTR mycelial cells produced with and without the addition of Tween 80 by 2D gel analysis

Spot no.	Accession no.	Protein name	Organism	Theoretical Mw	Theoretical pI	Expect	Coverage	Functional category
Proteins with up-regulation of expression by addition of Tween 80								
1	gi|322710763	Filament-forming protein	*Metarhizium anisopliae* ARSEF 23	228390	5.26	0.0031	16%	Protein transport
2	gi|154305665	Hypothetical protein BC1G_07647	*Botryotinia fuckeliana* B05.10	106740	4.64	0.039	13%	Unknown
3	gi|294658522	DEHA2F11440p	*Debaryomyces hansenii* CBS767	166127	6.22	0.02	11%	DNA replication
4	gi|225681776	Conserved hypothetical protein	*Paracoccidioides brasiliensis* Pb03	49025	10.35	0.0042	29%	Unknown
5	gi|242210320	Predicted protein	*Postia placenta* Mad-698-R	60611	6.56	0.00069	22%	Unknown
6	gi|212528240	α-glucosidase, putative	*Penicillium marneffei* ATCC 18224	93623	6.11	0.0013	17%	Carbohydrate metabolism
7	gi|2494190	Probable DNA polymerase	*Podospora anserina*	139248	9.31	0.023	19%	DNA replication
8	gi|312219760	Hypothetical protein	*Leptosphaeria maculans*	49894	7.68	0.033	30%	Oxidoreductase activity
9	gi|224223947	Transcription elongation factor 1-α	*Tremellogaster surinamensis*	33683	8.21	0.027	32%	Protein biosynthesis
10	gi|302660041	Hypothetical protein	*Trichophyton verrucosum*	108678	9.53	0.0031	18%	Membrane

		TRV_04216	HKI 0517					
11	gi|116191971	Hypothetical protein CHGG_05703	*Chaetomium globosum* CBS 148.51	57171	8.33	0.008	27%	Acid phosphatase activity
12	gi|330936475	Hypothetical protein PTT_18236	*Pyrenophora teres f. teres* 0-1	134292	6.21	0.013	20%	Unknown
13	gi|170089925	Predicted protein	*Laccaria bicolor* S238N-H82	49897	5.83	0.025	26%	Unknown
14	gi|151944277	Formin, involved in spindle orientation	*Saccharomyces cerevisiae* YJM789	219969	6.21	0.034	14%	Actin cytoskeleton organization
15	gi|71005980	Hypothetical protein UM01509.1	*Ustilago maydis* 521	156873	8.97	0.046	14%	Protein phosphorylation
16	gi|261203089	DNA repair protein Pso2/Snm1	*Ajellomyces dermatitidis* SLH14081	93765	7.71	0.0036	16%	DNA repair
17	gi|258564420	Conserved hypothetical protein	*Uncinocarpus reesii* 1704	54520	8.09	0.02	25%	Catalytic activity
18	gi|255724388	Hypothetical protein CTRG_01429	*Candida tropicalis* MYA-3404	303062	6.20	0.022	13%	Cellular protein metabolism
19	gi|239609121	Fatty acid synthase alpha subunit FasA	*Ajellomyces dermatitidis* ER-3	208952	6.41	0.0026	17%	Fatty acid biosynthesis
20	gi|258574259	U3 small nucleolar ribonucleoprotein protein	*Uncinocarpus reesii* 1704	22823	9.71	0.026	40%	Ribonucleoprotein
21	gi|134119046	Hypothetical protein	*Cryptococcus neoformans*	169293	8.01	0.045	11%	DNA replication

		CNBN2030	*var. neoformans* B-3501A					
22	**gi|169861758**	Hypothetical protein CC1G_01425	*Coprinopsis cinerea* okayama7#130	**107587**	**5.77**	**0.038**	**20%**	**Unknown**
23	**gi|302674856**	Hypothetical protein SCHCODRAFT_83451	*Schizophyllum commune* H4-8	**42084**	**9.19**	**0.016**	**19%**	DNA repair
24	gi|146418519	Hypothetical protein PGUG_02954	*Meyerozyma guilliermondii* ATCC 6260	60703	6.51	0.021	24%	Cell communication, phosphatidylinositol binding
Proteins with down-regulation of expression by addition of Tween 80								
25	gi|242761847	Protein phosphatase 2C, putative	*Talaromyces stipitatus* ATCC 10500	59941	5.56	0.043	24%	Protein dephosphorylation
26	gi|45185126	ABL104Cp	*Ashbya gossypii ATCC* 10895	61183	6.61	0.038	19%	Heme biosynthesis, 5-aminolevulinate synthase activity
27	gi|189196182	Diphosphomevalonate decarboxylase	*Pyrenophora tritici-repentis* Pt-1C-BFP	42671	6.18	0.015	28%	Isoprenoid biosyntheis
28	gi|149241192	Hypothetical protein LELG_02840	*Lodderomyces elongisporus* NRRL YB-4239	123792	6.12	0.025	12%	DNA replication
29	gi|302654630	Hypothetical protein TRV_06856	*Trichophyton verrucosum* HKI 0517	196699	8.64	0.0046	21%	Signal transduction
30	gi|71004018	Hypothetical protein UM00528.1	*Ustilago maydis* 521	157568	6.46	0.0057	16%	Transcription
31	gi|269859601	LSU ribosomal protein L3P	*Enterocytozoon bieneusi*	37072	9.90	0.04	46%	Translation, ribosomal structure

			H348					
32	gi|299739205	ATP-dependent bile acid permease	*Coprinopsis cinerea* okayama7#130	155080	6.30	0.0023	17%	ATP-binding cassette transporter
33	gi|255710927	KLTH0A06666p	*Lachancea thermotolerans*	35627	7.78	0.03	19%	Protein folding
34	gi|116207878	Hypothetical protein CHGG_03232	*Chaetomium globosum* CBS 148.51	78673	8.17	0.03	25%	Ubiquitin-dependent protein catabolic process
35	gi|156841725	Hypothetical protein Kpol_1051p25	*Vanderwaltozyma polyspora* DSM 70294	228541	5.43	0.0043	21%	Motor activity
36	gi|302655226	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	*Trichophyton verrucosum* HKI 0517	37071	6.52	0.038	29%	Lyase activity
37	gi|116182398	Hypothetical protein CHGG_01827	*Chaetomium globosum* CBS 148.51	102752	7.7	0.035	18%	Transport activity
38	gi|239613261	Phospholipase D1	*Ajellomyces dermatitidis* ER-3	206738	7.08	0.034	12%	Cell communication, phosphatidylinositol binding
39	gi|190405561	α-1,3-mannosyltransferase	*Saccharomyces cerevisiae* RM11-1a	88511	6.81	0.039	24%	Protein glycosylation
40	gi|329351119	Polyprotein	*Verticillium dahliae* VdLs.17	130217	9.05	0.024	14%	Unknown

"Unknown" represents the protein with similarity to hypothetical proteins or known proteins of which functions have not yet been determined.

4 Summary and Conclusions

4.1 Summary of Results

In general, the addition of 3 kinds of stimulatory agents including fatty acids, organic solvents and surfactants could significantly affect the production of mycelial biomass and EPS in submerged fermentation of PTR in different extent due to their chemical nature. Use of palmitic acid, methanol, hexane and Tween 80 as additives could stimulate the EPS production in PTR. Among these, palmitic acid is the most expensive agent and may not be economical to be used in for large scale fermentation. Although organic solvents are less expensive than other stimulatory agents and could be eliminated by simple methods like evaporation, they may have safety concerns when use in food or pharmaceutical area. Among the various surfactants used, Tween 80, a permitted food additive (E433), which is safer than the other three chemical agents and more suitable when used in food application, was selected as the targeted stimulatory agent for more in-depth investigations.

In the study of the effect of different concentrations and addition time of Tween 80 on mycelial biomass and EPS production, a concentration of 3.0 g/L Tween 80 when added to the fermentation broth on the 5^{th} day gave a significant increase of 51.3 and 41.8% ($p < 0.05$) in the yield of mycelial biomass and EPS, respectively.

The chemical characteristics including the carbohydrate and protein content, monosaccharide composition and glycosidic linkages of the EPS (a highly branched glucomannan) produced from the fermentation of PTR mycelium with addition of Tween 80 was not significantly different ($p > 0.05$) from those of the control. However, the M_w of EPS produced by the addition of Tween 80 in fermentation broth had a significantly ($p < 0.05$) lower M_w ($3.18 \pm 0.09 \times 10^6$) than that of the control ($4.30 \pm 0.12 \times 10^6$).

Both EPS, with and without addition of Tween 80, could significantly inhibit ($p < 0.05$) the growth of K562 cells in a dose dependent manner, with an estimated IC_{50} value of 43.7 and 47.6 μg/mL, respectively. The lower of IC_{50} value in the inhibition of K562 cell proliferation of the EPS produced by addition of Tween 80 suggested a higher potency of cytotoxicity toward these cells, probably due to its lower M_w.

The mechanism by which Tween 80 could increase the mycelial growth and EPS production in PTR were investigated by 3 different approaches. Firstly, the addition of Tween 80 did not act as a feeding of carbon source or a carbon source that could directly lead to the enhancement of mycelial biomass and EPS production in the fermentation of PTR. However, the glucose consumption rate was significantly increased ($p < 0.05$) after the addition of Tween 80, implying that the efficiency of nutrient uptake from the fermentation broth had been increased that could eventually lead to an increase of mycelial biomass and EPS production in PTR. Secondly, the comparative mycelial morphology indicated that the addition of Tween 80 could extend the growth period of the mycelia possibly by maintaining the intact structure of the mycelial pellets and preventing its disintegration caused by the shear forces during the shake-flask experiments. Moreover, the addition of Tween 80 could maintain the pH of the fermentation broth at a more acidic level which was favorable for the growth of the mycelia and EPS production in PTR. Thirdly, Tween 80 could affect the mycelial cell membrane composition by significantly increasing the fatty acid content of oleic acid (C18:1). A time course experiment on the residual concentration of Tween 80 during submerged fermentation of PTR had shown that the initial concentration of Tween 80 added on the 5^{th} day (0.3 % w/v) was significantly decreased ($p < 0.05$) by 52.9% from the 5^{th} day to the 8^{th} day. The significant increase in the content of oleic acid (C18:1) in the mycelial cell membrane of PTR could be partly explained by the significant decrease ($p < 0.05$) in the concentration of Tween 80. Furthermore, by the use of CLSM together with fluorescent labeled Tween 80 by 5-DTFA, it was demonstrated for the first time that oleic acid contained in Tween 80 could be incorporated into the mycelial cell membrane of PTR, altering its fatty acid composition and increase cell membrane permeability to facilitate the extracellular transport of EPS to the fermentation broth.

The effect of addition of Tween 80 on the PTR mycelial proteins was studied by the use of proteomics including both 1D and 2D gel analysis. A total of 51 differentially expressed proteins belonged to 10 functional categories such as translation elongation factor proteins, ribosomal proteins, heat shock proteins, tricarboxylic acid cycle (TCA cycle) related proteins and signal transduction related protein were identified by 1D gel analysis. In the 2D gel analysis, a total of 40 differentially expressed proteins in the mycelium were identified and among them the membrane related proteins, cell communication proteins and ATP-binding cassette transporter proteins were found closely related to the effect of the addition of Tween 80.

Based on the above findings, the underlying mechanism by which the addition of Tween 80 could stimulate the PTR mycelial cell growth and EPS production is proposed as shown in Figure 4.1. Firstly, the addition of Tween 80 could significantly increase the glucose consumption rate which was essential to increase the mycelial growth. Secondly, the addition of Tween 80 could up-regulate the expression of FasA, which controls the synthesis of long chain fatty acid to increase the fatty acid composition (oleic acid, C18:1) and the membrane permeability of the mycelial cells. Moreover, the addition of Tween 80 down-regulated the expression of YALI0E34793p and up-regulated the expression of ATP citrate lyase, which suppressed the TCA cycle activity, resulted in an increase of EPS production. The up-regulation of heat shock proteins could help to reduce the environmental stresses on the mycelial cells by maintaining its intact structure and preventing its disintegration. In addition, MAPK proteins might act as the coordinator for all these signal transduction processes.

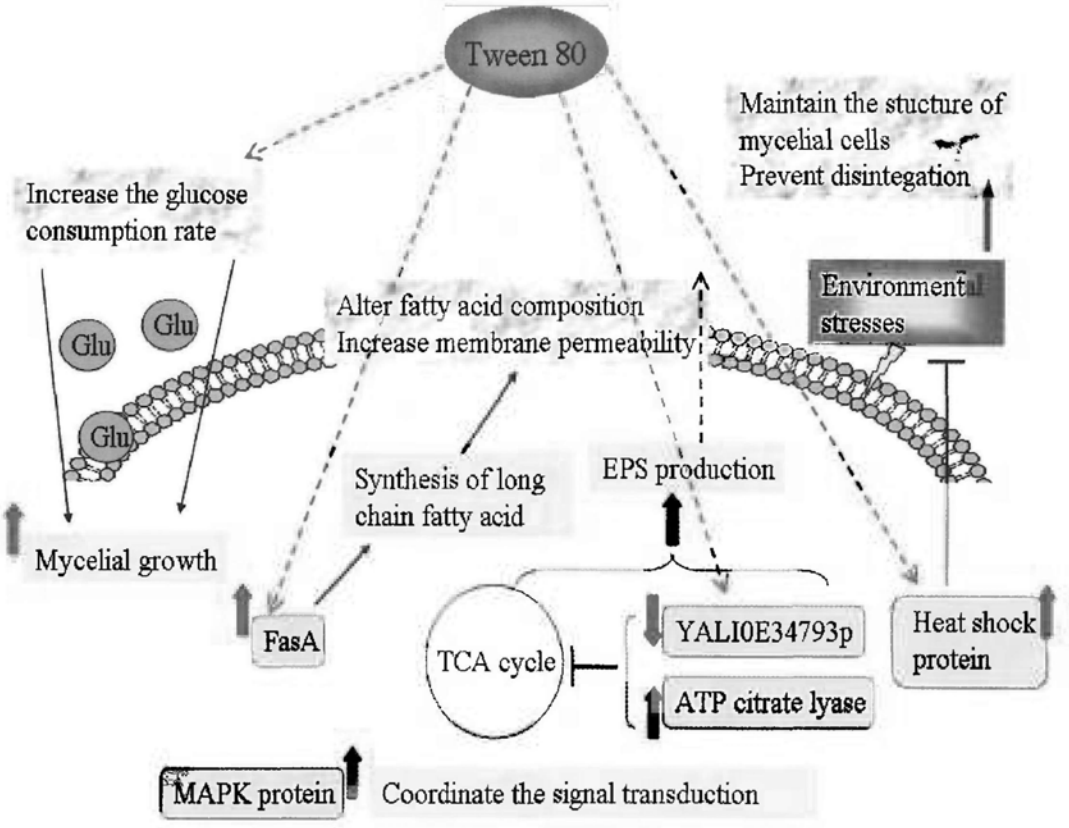


Figure 4.1 A partial model showing the proposed mechanisms by which Tween 80 influences the growth of PTR mycelia and EPS production

4.2 Conclusions and future works

Commercial cultivation of the fruiting body and sclerotium of the edible and medicinal mushroom PTR for bioactive polysaccharides is rare and time-consuming. In this study, the use of stimulatory agents to enhance the production of EPS from submerged fermentation of PTR mycelial cells has been clearly demonstrated.

Among the various stimulatory agents tested, Tween 80, a surfactant which is a permitted food additive, could significantly increase the PTR mycelial production of a bioactive EPS which is a glucomannan with similar chemical structure to that to the control.

The possible underlying mechanism by which Tween 80 can enhance the mycelial growth and EPS production is proposed in a partial model shown in Figure 4.1 based on the physical, chemical and proteomic analyses.

Although some related proteins have been revealed in the regulation of PTR mycelial growth and EPS production with the addition of Tween 80, many proteins isolated from the 1D and 2D gels are still with hypothetical or unknown functions, which is due to the lack of information of the mushroom genome and proteome. With the functional genomic study on PTR is underway, it is anticipated that the cellular and molecular mechanisms of the effect of addition of stimulatory agent on the mycelial cell growth and EPS production could be elucidated in more details in the future by more in-depth proteomic analysis combined with other molecular techniques.

In general, the findings in this project advance our knowledge in biotechnology and open up new strategies to increase the yield of useful metabolites by fermentation. In particular, the present results provide new insights into the understanding of the mechanism by which stimulatory agents can enhance the production of useful bioactive metabolites from submerged fermentation of mushroom/fungal mycelium.

References

Aebersold, R. and Goodlett, D. R. (2001). Mass spectrometry in proteomics. *Chemical Reviews*, 101, 269-296.

Anumula, K. R. and Taylor, P. B. (1992). A comprehensive procedure for preparation of partially methylated alditol acetates from glycoprotein carbohydrates. *Analytical Biochemistry*, 203, 101-108.

Arora, D. (1986). Indentification and termininology, In: Mushrooms Demystified: A Comprehensive Guide to The Fleshy Fungi. Berkeley: Ten Speed Press. p. 4-9.

Asther, M., Corrieu, G., Drapron, R. and Odier, E. (1987). Effect of Tween 80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA-12. *Enzyme and Microbial Technology*, 9, 245-249.

Asther, M., Lesage, L., Drapron, R., Corrieu, G. and Odier, E. (1988). Phospholipid and fatty acid enrichment of *Phanerochaete chrysosporium* INA-12 in relation to ligninase production. *Applied Microbiology and Biotechnology*, 27, 393-398.

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

Bakau, B. and Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell*, 92, 351-366.

Benchekroun, K. and Bonaly, R. (1992). Physiological properties and plasma membrane composition of *Saccharomyces cerevisiae* grown in sequential batch culture and in the presence of surfactants. *Applied Microbiology and Biotechnology*, 36, 673-678.

Blakeney, A. B., Harris, P. J., Henry, R. J. and Stone, B. A. (1983). A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydrate Research*, 113, 291-299.

Bohn, J. A. and BeMiller, J. N. (1995). β-D-Glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydrate Polymers*, 28, 3-14.

Brekhman, I. I. and Fulder, S. (1980). Man and biologically active substances: the effect of drugs, diet, and pollution on health. New York: Pergamon Press. p. 67-89.

Brown, D. W., Adams, T. H. and Keller, N. P. (1996). *Aspergillus* has distinct fatty acid synthases for primary and secondary metabolism. *Proceedings of the National Academy of Sciences*, 93, 14873-14877.

Brummer, Y. and Cui, S. W. (2005). Understanding carbohydrate analysis, In: Food carbohydrates: chemistry, physical properties, and applications, edited by S. W. Cui. Boca Raton: Taylor & Francis Press. p. 79-91.

Carlisle, M. J., Watkinson, S. C. and Gooday, G. W. (1994). The fungi.London: Academic Press. p. 124-131.

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

Chang, S. T. and Miles, P. G. (1992). Mushroom biology-a new discipline. *The Mycologist*, 6, 64-73.

Chang, S. T. and Miles, P. G. (1989). Edible mushrooms and their cultivation.Florida: CRC press. p. 45-58.

Chang, S. and Quimio, T. H. (1992). Introduction to mushroom science, In: Tropical mushrooms: biological nature and cultivation methods. Hong Kong: Chinese

University Press. p. 3-10.

Chen, H. B., Huang, H. C., Chen, C. I., I, Y. P. and Liu, Y. C. (2010). The use of additives as the stimulator on mycelial biomass and exopolysaccharide productions in submerged culture of *Grifola umbellata*. *Bioprocess and Biosystems Engineering*, 33, 401-406.

Chen, W., Zhao, Z., Chen, S. F. and Li, Y. Q. (2008). Optimization for the production of exopolysaccharide from *Fomes fomentarius* in submerged culture and its antitumor effect *in vitro*. *Bioresource Technology*, 99, 3187-3194.

Chihara, G., Hamuro, J., Maeda, Y. Y., Arai, Y. and Fukuoka, F. (1970). Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Research*, 30, 2776-2781.

Cho, E. J., Oh, J. Y., Chang, H. Y. and Yun, J. W. (2006). Production of exopolysaccharides by submerged mycelial culture of a mushroom *Tremella fuciformis*. *Journal of Biotechnology*, 127, 129-140.

Christie, W. W. (2003). Lipid analysis. Isolation, separation, identification and structural analysis of lipids. Bridgwater: Oily Press. p. 51-70.

Couto, S. R. and Sanroman, M. (2006). Application of solid-state fermentation to food industry-A review. *Journal of Food Engineering*, 76, 291-302.

Crisan, E. V. and Sands, A. (1978). Nutritional value, In: The biology and cultivation of edible mushrooms, edited by S. T. Chang and W. A. Hayes. New York: Academic Press. p. 137-168.

Cui, J. and Chisti, Y. (2003). Polysaccharopeptides of *Coriolus versicolor*: physiological activity, uses, and production. *Biotechnology Advances*, 21, 109-122.

Davidson, A. L. and Chen, J. (2004). ATP-binding cassette transporters in bacteria.

Annual Review of Biochemistry, 73, 241-268.

Davidson, A. L., Dassa, E., Orelle, C. and Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiology and Molecular Biology Reviews*, 72, 317-364.

De Leon, A. (2003). Periplasmic penicillin G acylase activity in recombinant *Escherichia coli* cells permeabilized with organic solvents. *Process Biochemistry*, 39, 301-305.

DeHoog, C. L. and Mann, M. (2004). Proteomics. *Annual Review of Genomics and Human Genetics*, 5, 267.

Delahunty, C. and Yates Iii, J. R. (2005). Protein identification using 2D-LC-MS/MS. *Methods*, 35, 248-255.

Deshaies, R. J., Seol, J. H., McDonald, W. H., Cope, G., Lyapina, S., Shevchenko, A., Shevchenko, A., Verma, R. and Yates, J. R. (2002). Charting the protein complexome in yeast by mass spectrometry. *Molecular and Cellular Proteomics*, 1, 3-10.

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

Dudman, W. F. (1977). The role of surface polysaccharides in natural environments, In: Surface carbohydrates of the prokaryotic cell, edited by I. W. Sutherland. New York: Academic Press. p. 357-414.

Duncan, C. J. G., Pugh, N., Pasco, D. S. and Ross, S. A. (2002). Isolation of a galactomannan that enhances macrophage activation from the edible fungus *Morchella esculenta*. *Journal of Agricultural and Food Chemistry*, 50, 5683-5685.

Eyal, J. (1991). Mushroom mycelium grown in submerged culture–potential food

applications, In: Biotechnology and Food Ingredients, edited by I. Goldberg and R. Williams. New York: Van Nostrand Reinhold. p. 31-64.

Fakhereddine, L., Kademi, A., A t-Abdelkader, N. and Baratti, J. C. (1998). Microbial growth and lipolytic activities of moderate thermophilic bacterial strains. *Biotechnology Letters*, 20, 879-883.

Fan, L., Soccol, A. T., Pandey, A. and Ricardo Soccol, C. (2007). Effect of nutritional and environmental conditions on the production of exo-polysaccharide of *Agaricus brasiliensis* by submerged fermentation and its antitumor activity. *LWT-Food Science and Technology*, 40, 30-35.

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

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

Fasidi, I. O. and Ekuere, U. U. (1993). Studies on *Pleurotus tuber-regium* (Fries) Singer: cultivation, proximate composition and mineral contents of sclerotia. *Food Chemistry*, 48, 255-258.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. and Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science*, 246, 64-71.

Feofilova, E. P. (1998). Current trends in studies of biologically active substances of basidial fungi. *Applied Biochemistry and Microbiology*, 34, 541-550.

Ferreira-Cerca, S., Poll, G., Gleizes, P. E., Tschochner, H. and Milkereit, P. (2005). Roles of eukaryotic ribosomal proteins in maturation and transport of pre-18S rRNA and ribosome function. *Molecular Cell*, 20, 263-275.

Francesca, G., Francesca, M., Tania, G., Marina, B., Maurizio, S. and Alessandra, M. (2010). Effect of different glucose concentrations on proteome of *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta (BBA)*, 1804, 1516-1525.

Franz, G. (1989). Polysaccharides in pharmacy: current applications and future concepts. *Planta Medica*, 55, 493-497.

Galindo, E. and Salcedo, G. (1996). Detergents improve xanthan yield and polymer quality in cultures of *Xanthomonas campestris*. *Enzyme and Microbial Technology*, 19, 145-149.

Gao, Q., Seljelid, R., Chen, H. and Jiang, R. (1996). Characterisation of acidic heteroglycans from *Tremella fuciformis* Berk with cytokine stimulating activity. *Carbohydrate Research*, 288, 135-142.

Gehrig, I., Bart, H. J., Anke, T. and Germerdonk, R. (1998). Influence of morphology and rheology on the production characteristics of the basidiomycete *Cyathus striatus*. *Biotechnology and Bioengineering*, 59, 525-533.

Gibbs, P. A., Seviour, R. J. and Schmid, F. (2000). Growth of filamentous fungi in submerged culture: problems and possible solutions. *Critical Reviews in Biotechnology*, 20, 17-48.

Gomes, J. and Menawat, A. S. (2000). Precise control of dissolved oxygen in bioreactors-a model-based geometric algorithm. *Chemical Engineering Science*, 55, 67-78.

Graves, P. R. and Haystead, T. A. J. (2002). Molecular biologist's guide to proteomics. *Microbiology and Molecular Biology Reviews*, 66, 39-63.

Hadar, Y. and Cohen-Arazi, E. (1986). Chemical composition of the edible mushroom *Pleurotus ostreatus* produced by fermentation. *Applied and Environmental Microbiology*, 51, 1254-1352.

Hao, L., Xing, X., Li, Z., Zhang, J., Sun, J., Jia, S., Qiao, C. and Wu, T. (2010). Optimization of effect factors for mycelial growth and exopolysaccharide production by *Schizophyllum commune*. *Applied Biochemistry and Biotechnology*, 160, 621-631.

Haq, I. U., Ali, S., Qadeer, M. A. and Iqbal, J. (2003). Stimulatory effect of alcohols (methanol and ethanol) on citric acid productivity by a 2-deoxy-glucose resistant culture of *Aspergillus niger* GCB-47. *Bioresource Technology*, 86, 227-233.

Hara, C., Kumazawa, Y., Inagaki, K., Kaneko, M., Kiho, T. and Ukai, S. (1991). Mitogenic and colony-stimulating factor-inducing activities of polysaccharide fractions from the fruit bodies of *Dictyophora indusiata* Fisch. *Chemical and Pharmaceutical Bulletin*, 39, 1615-1616.

Hartke, A., Bouché, S., Giard, J. C., Benachour, A., Boutibonnes, P. and Auffray, Y. (1996). The lactic acid stress response of *Lactococcus lactis* subsp. lactis. *Current Microbiology*, 33, 194-199.

Hetland, G., Johnson, E., Lyberg, T., Bernardshaw, S., Tryggestad, A. and Grinde, B. (2008). Effects of the medicinal mushroom *Agaricus blazei Murill* on immunity, infection and cancer. *Scandinavian Journal of Immunology*, 68, 363-370.

Hillgren, A., Lindgren, J. and Alden, M. (2002). Protection mechanism of Tween 80 during freeze-thawing of a model protein, LDH. *International Journal of Pharmaceutics*, 237, 57-69.

Hobbs, C. R. (2000). Medicinal value of *Lentinus edodes* (Berk.) Sing.(Agaricomycetideae). A literature review. *International Journal of Medicinal Mushrooms*, 2, 287-302.

Horie, K., Rakwal, R., Hirano, M., Shibato, J., Nam, H. W., Kim, Y. S., Kouzuma, Y., Agrawal, G. K., Masuo, Y. and Yonekura, M. (2008). Proteomics of two cultivated mushrooms *Sparassis crispa* and *Hericium erinaceum* provides insight into their numerous functional protein components and diversity. *The Journal of*

Proteome Research, 7, 1819-1835.

Hosono, K. (1992). Effect of salt stress on lipid composition and membrane fluidity of the salttolerant yeast *Zygosaccharomyces rouxii*. *Journal of General Microbiology*, 138, 91-96.

Hsieh, C., Liu, C. J., Tseng, M. H., Lo, C. T. and Yang, Y. C. (2006). Effect of olive oil on the production of mycelial biomass and polysaccharides of *Grifola frondosa* under high oxygen concentration aeration. *Enzyme and Microbial Technology*, 39, 434-439.

Hsieh, C., Wang, H. L., Chen, C. C., Hsu, T. H. and Tseng, M. H. (2008). Effect of plant oil and surfactant on the production of mycelial biomass and polysaccharides in submerged culture of *Grifola frondosa*. *Biochemical Engineering Journal*, 38, 198-205.

Huang, H. C., Chen, C. I., Hung, C. N. and Liu, Y. C. (2009). Experimental analysis of the oil addition effect on mycelia and polysaccharide productions in *Ganoderma lucidum* submerged culture. *Bioprocess and Biosystems Engineering*, 32, 217-224.

Huang, N. L. (1982). Cultivation of *Tremella fuciformis* in Fujian, China. *Mushroom Newsletter for the Tropics*, 2, 2-4.

Huang, N., Guo, M. and Huang, L. (1996). Tiger milk mushroom (*Pleurotus tuber-regium* (Fr.) Sing.) and its cultivation. *Edible Fungi China*, 16, 13-14.

Hui, K. P. Y., Sit, W. H. and Wan, J. M. F. (2005). Induction of S phase cell arrest and caspase activation by polysaccharide peptide isolated from *Coriolus versicolor* enhanced the cell cycle dependent activity and apoptotic cell death of doxorubicin and etoposide, but not cytarabine in HL-60 cells. *Oncology Reports*, 14, 145-155.

Humfeld, H. (1948). The production of mushroom mycelium (*Agaricus campestris*) in Submerged Culture. *Science*, 107, 373.

Hurkman, W. J. and Tanaka, C. K. (1986). Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiology*, 81, 802.

Hwang, H. J., Kim, S. W., Xu, C. P., Choi, J. W. and Yun, J. W. (2004). Morphological and rheological properties of the three different species of basidiomycetes *Phellinus* in submerged cultures. *Journal of Applied Microbiology*, 96, 1296-1305.

Ilori, M. O., Fasida, I. O. and Isikhuemhen, O. S. (1997). XVIII. Mushroom research and commercial cultivation in Nigeria. *Food Reviews International*, 13, 489-496.

Isikhuemhen, O. S. and Nerud, F. (1999). Preliminary studies on the ligninolytic enzymes produced by the tropical fungus *Pleurotus tuber-regium* (Fr.) Sing. *Antonie van Leeuwenhoek*, 75, 257-260.

Johnsson, T., Nikkila, P., Toivonen, L., Rosenqvist, H. and Laakso, S. (1995). Cellular fatty acid profiles of *Lactobacillus* and *Lactococcus* strains in relation to the oleic acid content of the cultivation medium. *Applied and Environmental Microbiology*, 61, 4497-4499.

Jonathan, S. G. and Fasidi, I. O. (2003). Studies on *Psathyerella atroumbonatai* (Pegler), a Nigerian edible fungus. *Food Chemistry*, 81, 481-484.

Jong, S. C., Birmingham, J. M. and Pai, S. H. (1991). Immunomodulatory substances of fungal origin. *Journal of Immunology and Immunopharmacology*, 11, 115-122.

Karas, M. and Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Analytical Chemistry*, 60, 2299-2301.

Kiho, T., Yoshida, I., Nagai, K., Ukai, S. and Hara, C. (1989). α-D-Glucan from an alkaline extract of *Agrocybe cylindracea*, and antitumor activity of its O-(carboxymethyl) ated derivatives. *Carbohydrate Research*, 189, 273-279.

Kim, H. M., Park, M. K. and Yun, J. W. (2006). Culture pH affects

exopolysaccharide production in submerged mycelial culture of *Ganoderma lucidum*. *Applied Biochemistry and Biotechnology*, 134, 249-262.

Kim, H. O., Lim, J. M., Joo, J. H., Kim, S. W., Hwang, H. J., Choi, J. W. and Yun, J. W. (2005). Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. *Bioresource Technology*, 96, 1175-1182.

Kim, S. W., Hwang, H. J., Xu, C. P., Choi, J. W. and Yun, J. W. (2003). Effect of aeration and agitation on the production of mycelial biomass and exopolysaccharides in an enthomopathogenic fungus *Paecilomyces sinclairii*. *Letters in Applied Microbiology*, 36, 321-326.

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

Kim, Y. O., Han, S. B., Lee, H. W., Ahn, H. J., Yoon, Y. D., Jung, J. K., Kim, H. M. and Shin, C. S. (2005). Immuno-stimulating effect of the endo-polysaccharide produced by submerged culture of *Inonotus obliquus*. *Life Sciences*, 77, 2438-2456.

Kues, U. and Liu, Y. (2000). Fruiting body production in *basidiomycetes*. *Applied Microbiology and Biotechnology*, 54, 141-152.

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

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J. and Yun, J. W. (2003). Biological activities of the polysaccharides produced from submerged culture of the edible *Basidiomycete Grifola frondosa*. *Enzyme and Microbial Technology*, 32, 574-581.

Lee, K. M., Lee, S. Y. and Lee, H. Y. (1999). Bistage control of pH for improving exopolysaccharide production from mycelia of *Ganoderma lucidum* in an air-lift fermentor. *Journal of Bioscience and Bioengineering*, 88, 646-650.

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

Lestan, D., Cernilec, M., Strancar, A. and Perdih, A. (1993). Influence of some surfactants and related compounds on ligninolytic activity of *Phanerochaete chrysosporium*. *FEMS Microbiology Letters*, 106, 17-22.

Li, C. Y., Cheng, C. Y. and Chen, T. L. (2001). Production of *Acinetobacter radioresistens* lipase using Tween 80 as the carbon source. *Enzyme and Microbial Technology*, 29, 258-263.

Li, C. Y., Cheng, C. Y. and Chen, T. L. (2004). Fed-batch production of lipase by *Acinetobacter radioresistens* using Tween 80 as the carbon source. *Biochemical Engineering Journal*, 19, 25-31.

Lim, J. M. and Yun, J. W. (2006). Enhanced production of exopolysaccharides by supplementation of toluene in submerged culture of an edible mushroom *Collybia maculata* TG-1. *Process Biochemistry*, 41, 1620-1626.

Lin, E. S., Wang, C. C. and Sung, S. C. (2006). Cultivating conditions influence lipase production by the edible *Basidiomycete Antrodia cinnamomea* in submerged culture. *Enzyme and Microbial Technology*, 39, 98-102.

Liu, F., Ooi, V., Liu, W. K. and Chang, S. T. (1996). Immunomodulation and antitumor activity of polysaccharide-protein complex from the culture filtrates of a local edible mushroom, *Tricholoma lobayense*. *General Pharmacology*, 27, 621-624.

Lo, H. C., Tsai, F. A., Wasser, S. P., Yang, J. G. and Huang, B. M. (2006). Effects of

ingested fruiting bodies, submerged culture biomass, and acidic polysaccharide glucuronoxylomannan of *Tremella mesenterica* Retz.: Fr. on glycemic responses in normal and diabetic rats. *Life Sciences*, 78, 1957-1966.

Maldonado, M. C. and Strasser de Saad, A. M. (1998). Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. *Journal of Industrial Microbiology & Biotechnology*, 20, 34-38.

Mann, M., Hendrickson, R. C. and Pandey, A. (2001). Analysis of proteins and proteomes by mass spectrometry. *Annual Review of Biochemistry*, 70, 437-473.

McElhaney, R. N., De Gier, J. and Van der Neut-Kok, E. (1973). The effect of alterations in fatty acid composition and cholesterol content on the nonelectrolyte permeability of *Acholeplasma laidlawii* B cells and derived liposomes. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 298, 500-512.

Miles, P. G. and Chang, S. (1997). Mushroom Biology: Concise Basics and Current Developments.Singapore: World Scientific Pub Co Inc. p. 161–175

Mitchell, D. A. and Lonsane, B. K. (1992). Definition, characteristics and potential, In: Solid Substrate Cultivation, edited by H. W. Doelle, D. A. Mitchell and C. E. Rolz. Elsevier Applied Biotechnology Series. p. 1-13.

Miyazaki, T. and Nishijima, M. (1981). Studies on fungal polysaccharides. XXVII. Structural examination of a water-soluble, antitumor polysaccharide of *Ganoderma lucidum*. *Chemical and Pharmaceutical Bulletin*, 29, 3611-3616.

Mizuno, M., Shiomi, Y., Minato, K., Kawakami, S., Ashida, H. and Tsuchida, H. (2000). Fucogalactan isolated from *Sarcodon aspratus* elicits release of tumor necrosis factor-α and nitric oxide from murine macrophages. *Immunopharmacology*, 46, 113-121.

Mizuno, T. (1997). Anti-tumor mushrooms. *Ganoderma lucidum Grifora frondosa, Lentinus edodes, and Agaricus blazei, Gendaishorin, Tokyo*, 188-193.

Mizuno, T. (1998). Bioactive substances in *Yamabushitake*, the *Hericium erinaceum* fungus, and its medicinal utilization. *Food and Food Ingredient Japanese Journal*, 167, 105-114.

Mizuno, T. (1996). Development of antitumor polysaccharides from mushroom fungi. *Food and Food Ingredient Japanese Journal*, 167, 69-85.

Mizuno, T., Zhuang, C., Abe, K., Okamoto, H., Kiho, T., Ukai, S., Leclerc, S. and Meijer, L. (1999). Antitumor and hypoglycemic activities of polysaccharides from the sclerotia and mycelia of *Inonotus obliquus* (Pers.: Fr.) Pil.(Aphyllophoromycetideae). *International Journal of Medicinal Mushrooms*, 1, 301-316.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, 55-63.

Mukherjee, R. and Nandi, B. (2004). Improvement of *in vitro* digestibility through biological treatment of water hyacinth biomass by two *Pleurotus* species. *International Biodeterioration & Biodegradation*, 53, 7-12.

Narberhaus, F. (2002). α-Crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network. *Microbiology and Molecular Biology Reviews*, 66, 64-91.

Nascimento, A. E., Nascimento, A. E. and Campos Takaki, G. M. (1997). Effects of tween 80 on protease production by *candida lipolytica*. *Boletim do Instituto de Microbiologia*, 12, 75-78.

Negrutskii, B. S. and El'Skaya, A. V. (1998). Eukaryotic translation elongation factor 1α: Structure, expression, functions, and possible role in aminoacyl-tRNA channeling. *Progress in Nucleic Acid Research and Molecular Biology*, 60, 47-78.

Nemec, T. and Jernejc, K. (2002). Influence of Tween 80 on lipid metabolism of an *Aspergillus niger* strain. *Applied Biochemistry and Biotechnology*, 101, 229-238.

Ngai, P. H. K. and Ng, T. B. (2003). Lentin, a novel and potent antifungal protein from shitake mushroom with inhibitory effects on activity of human immunodeficiency virus-1 reverse transcriptase and proliferation of leukemia cells. *Life Sciences*, 73, 3363-3374.

Nozawa, Y., Iida, H., Fukushima, H., Ohki, K. and Ohnishi, S. (1974). Studies on Tetrahymena membranes: Temperature-induced alterations in fatty acid composition of various membrane fractions in *Tetrahymena pyriformis* and its effect on membrane fluidity as inferred by spin-label study. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 367, 134-147.

Nwe, N., Chandrkrachang, S., Stevens, W. F., Maw, T., Tan, T. K., Khor, E. and Wong, S. M. (2002). Production of fungal chitosan by solid state and submerged fermentation. *Carbohydrate Polymers*, 49, 235-237.

Oda, K., Kakizono, D., Yamada, O., Iefuji, H., Akita, O. and Iwashita, K. (2006). Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. *Applied and Environmental Microbiology*, 72, 3448-3457.

Okhuoya, J. A. and Okogbo, F. O. (1990). Induction of edible sclerotia of *Pleurotus tuber-regium* (FR) Sing, in the laboratory. *Annals of Applied Biology*, 117, 295-298.

Olsvik, E. S. and Kristiansen, B. (1992). Influence of oxygen tension, biomass concentration, and specific growth rate on the rheological properties of a filamentous fermentation broth. *Biotechnology and Bioengineering*, 40, 1293-1299.

Ooi, V. E. C. and Liu, F. (1999). A review of pharmacological activities of mushroom polysaccharides. *International Journal of Medicinal Mushrooms*, 1, 195-206.

Oso, B. A. (1977). *Pleurotus tuber-regium* from Nigeria. *Mycologia*, 69, 271-279.

Palleschi, A., Bocchinfuso, G., Coviello, T. and Alhaique, F. (2005). Molecular dynamics investigations of the polysaccharide scleroglucan: first study on the triple helix structure. *Carbohydrate Research*, 340, 2154-2162.

Pandey, A. (2003). Solid-state fermentation. *Biochemical Engineering* , 13, 81-84.

Pandey, A., Selvakumar, P., Soccol, C. R. and Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes. *Current Science*, 77, 149-162.

Papagianni, M. (2004). Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances*, 22, 189-259.

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

Parker, J. (2003). Elongation Factors; Translation. *Encyclopedia of Genetics*, 610-611.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K. and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine Reviews*, 22, 153-183.

Peng, Y., Zhang, L., Zeng, F. and Kennedy, J. F. (2005). Structure and antitumor activities of the water-soluble polysaccharides from *Ganoderma tsugae* mycelium. *Carbohydrate Polymers*, 59, 385-392.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analytical Biochemistry*, 83, 346-356.

Pitarch, A., Sánchez, M., Nombela, C. and Gil, C. (2003). Analysis of the *Candida albicans* proteome: I. Strategies and applications. *Journal of Chromatography B*, 787,

101-128.

Plesofsky-Vig, N. and Brambl, R. (1995). Disruption of the gene for hsp30, an α-crystallin-related heat shock protein of *Neurospora crassa*, causes defects in thermotolerance. *Proceedings of the National Academy of Sciences*, 92, 5032.

Pokhrel, C. P. and Ohga, S. (2007). Submerged culture conditions for mycelial yield and polysaccharides production by *Lyophyllum decastes*. *Food Chemistry*, 105, 641-646.

Ramachandran, S., Patel, A. K., Nampoothiri, K. M., Francis, F., Nagy, V., Szakacs, G. and Pandey, A. (2004). Coconut oil cake-a potential raw material for the production of α-amylase. *Bioresource Technology*, 93, 169-174.

Rasmussen, H. (1970). Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science*, 170, 404-412.

Rau, U. (2004). Glucans secreted by fungi. *Turkish Electronic Journal of Biotechnology*, 2, 30-36.

Reddy, R. M., Reddy, P. G. and Seenayya, G. (1999). Enhanced production of thermostable [beta]-amylase and pullulanase in the presence of surfactants by *Clostridium thermosulfurogenes* SV2. *Process Biochemistry*, 34, 87-92.

Reese, E. T. and Maguire, A. (1969). Surfactants as stimulants of enzyme production by microorganisms. *Applied and Environmental Microbiology*, 17, 242-245.

Riley, G. L., Tucker, K. G., Paul, G. C. and Thomas, C. R. (2000). Effect of biomass concentration and mycelial morphology on fermentation broth rheology. *Biotechnology and Bioengineering*, 68, 160-172.

Roberts, I. S. (1996). The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Reviews in Microbiology*, 50, 285-315.

Rosado, F. R., Carbonero, E. R., Kemmelmeier, C., Tischer, C. A., Gorin, P. A. J. and Iacomini, M. (2002). A partially 3-O-methylated (1→ 4)-linked α-D-galactan and α-D-mannan from *Pleurotus ostreatoroseus* Sing. *FEMS Microbiology Letters*, 212, 261-265.

Rosado, F. R., Germano, S., Carbonero, E. R., da Costa, S. M. G., Iacomini, M. and Kemmelmeier, C. (2003). Biomass and exopolysaccharide production in submerged cultures of *Pleurotus ostreatoroseus* Sing and *Pleurotus ostreatus* "florida"(Jack.: Fr.) Kummer. *Journal of Basic Microbiology*, 43, 230-237.

Sadykov, M. R., Olson, M. E., Halouska, S., Zhu, Y., Fey, P. D., Powers, R. and Somerville, G. A. (2008). Tricarboxylic acid cycle-dependent regulation of *Staphylococcus epidermidis* polysaccharide intercellular adhesin synthesis. *Journal of Bacteriology*, 190, 7621.

Sasaki, T. and Takasuka, N. (1976). Further study of the structure of lentinan, an anti-tumor polysaccharide from *Lentinus edodes*. *Carbohydrate Research*, 47, 99-104.

Sharon, N. and Lis, H. (1993). Carbohydrates in cell recognition. *Scientific American*, 268, 82.

Shih, I. L., Chou, B. W., Chen, C. C., Wu, J. Y. and Hsieh, C. (2008). Study of mycelial growth and bioactive polysaccharide production in batch and fed-batch culture of *Grifola frondosa*. *Bioresource Technology*, 99, 785-793.

Shih, I. L., Pan, K. and Hsieh, C. (2006). Influence of nutritional components and oxygen supply on the mycelial growth and bioactive metabolites production in submerged culture of *Antrodia cinnamomea*. *Process Biochemistry*, 41, 1129-1135.

Shimada, Y., Yamakawa, A., Morita, T. and Sugiyama, K. (2003). Effects of dietary eritadenine on the liver microsomal Δ6-desaturase activity and its mRNA in rats. *Bioscience, Biotechnology, and Biochemistry*, 67, 1258-1266.

Shu, C. H. and Lung, M. Y. (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. *Process Biochemistry*, 39, 931-937.

Silva, C. C., Dekker, R. F. H., Silva, R. S. S. F., Silva, M. L. C. and Barbosa, A. M. (2007). Effect of soybean oil and Tween 80 on the production of botryosphaeran by *Botryosphaeria rhodina* MAMB-05. *Process Biochemistry*, 42, 1254-1258.

Sinha, J., Bae, J. T., Park, J. P., Kim, K. H., Song, C. H. and Yun, J. W. (2001). Changes in morphology of *Paecilomyces japonica* and their effect on broth rheology during production of exo-biopolymers. *Applied Microbiology and Biotechnology*, 56, 88-92.

Sinha, J., Tae Bae, J., Pil Park, J., Hyun Song, C. and Won Yun, J. (2001). Effect of substrate concentration on broth rheology and fungal morphology during exo-biopolymer production by *Paecilomyces japonica* in a batch bioreactor. *Enzyme and Microbial Technology*, 29, 392-399.

Stanbury, P. F., Whitaker, A. and Hall, S. J. (1995). Principles of Fermentation Technology.Oxford: Butterworth-Heinemann. p. 15-37.

Stasinopoulos, S. J. and Seviour, R. J. (1990). Stimulation of exopolysaccharide production in the fungus *Acremonium persicinum* with fatty acids. *Biotechnology and Bioengineering*, 36, 778-782.

Stutzenberger, F. J. (1987). Component-specific stimulation of cellulase secretion in *Thermomonospora curvata* by the surfactant Tween 80. *Journal of Applied Microbiology*, 63, 239-244.

Sugiyama, K., Kawagishi, H., Tanaka, A., Saeki, S., Yoshida, S., Sakamoto, H. and Ishiguro, Y. (1992). Isolation of plasma cholesterol-lowering components from ningyotake (*Polyporus confluens*) mushroom. *Journal of Nutritional Science and Vitaminology*, 38, 335.

Supelco (2000). Chromatography products catalog. p. 160-161.

Suresh, M. (2009). Microbial exopolysaccharides: variety and potential applications, In: Microbial Production of Biopolymers and Polymer Precursors, edited by B. H. A. Rehm. New Zealand: Caister Academic Press. p. 229.

Sutherland, I. W. (2002). Polysaccharides from microorganisms, plants and animals, In: Biopolymers, edited by E. J. Vandamme. Wiley Online Library. p. 1-19.

Svensater, G., Sjogreen, B. and Hamilton, I. R. (2000). Multiple stress responses in *Streptococcus* mutans and the induction of general and stress-specific proteins. *Microbiology*, 146, 107-117.

Takyar, S., Hickerson, R. P. and Noller, H. F. (2005). mRNA helicase activity of the ribosome. *Cell*, 120, 49-58.

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

Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S. and Hayashi, M. (2011). Effect of Tween 80 on the growth, lipid accumulation and fatty acid composition of *Thraustochytrium aureum* ATCC 34304. *Journal of Bioscience and Bioengineering*, 111, 420-424.

Todd, J. F. J. (1995). Recommendations for nomenclature and symbolism for mass spectroscopy. *International Journal of Mass Spectrometry and Ion Processes*, 142, 209-240.

Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R. and Keller, N. P. (2004). Endogenous lipogenic regulators of spore balance in *Aspergillus nidulans*. *Eukaryotic Cell*, 3, 1398-1411.

Ukai, S., Kiho, T., Hara, C., Morita, M., Goto, A. and Imaizumi, N. (1983).

Polysaccharides in fungi. XIII. Antitumor activity of various polysaccharides isolated from *Dictyophora indusiata*, *Ganoderma japonicum*, *Cordyceps cicadae*, *Auricularia auricula-judae*, and *Auricularia* species. *Chemical and Pharmaceutical Bulletin*, 31, 741-744.

Ukawa, Y., Ito, H. and Hisamatsu, M. (2000). Antitumor effects of (1→ 3)-β-glucan and (1→6)-β-glucan purified from newly cultivated mushroom, *Hatakeshimeji* (*Lyophyllum decastes* Sing.). *Journal of Bioscience and Bioengineering*, 90, 98-104.

Vaidyanathan, S., Macaloney, G., Vaughan, J., McNeil, B. and Harvey, L. M. (1999). Monitoring of submerged bioprocesses. *Critical Reviews in Biotechnology*, 19, 277-316.

Vardar-Sukan, F. (1998). Foaming: consequences, prevention and destruction. *Biotechnology Advances*, 16, 913-948.

Venkatadri, R. and Irvine, R. L. (1990). Effect of agitation on ligninase activity and ligninase production by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*, 56, 2684-2691.

Viniegra-Gonzalez, G., Favela-Torres, E., Aguilar, C. N., Rómero-Gomez, S. J., Diaz-Godinez, G. and Augur, C. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochemical Engineering Journal*, 13, 157-167.

Vinogradov, E., Petersen, B. O., Duus, J. O. and Wasser, S. (2004). The structure of the glucuronoxylomannan produced by culinary-medicinal yellow brain mushroom (*Tremella mesenterica* Ritz.: Fr., *Heterobasidiomycetes*) grown as one cell biomass in submerged culture. *Carbohydrate Research*, 339, 1483-1489.

Vuong, C., Kidder, J. B., Jacobson, E. R., Otto, M., Proctor, R. A. and Somerville, G. A. (2005). *Staphylococcus epidermidis* polysaccharide intercellular adhesin production significantly increases during tricarboxylic acid cycle stress. *Journal of Bacteriology*, 187, 2967.

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

Wang, G., Zhang, J., Mizuno, T., Zhuang, C., Ito, H., Mayuzumi, H., Okamoto, H. and Li, J. (1993). Antitumor active polysaccharides from the Chinese mushroom Songshan lingzhi, the fruiting body of *Ganoderma tsugae*. *Bioscience, Biotechnology, and Biochemistry*, 57, 894-900.

Wang, H., Ng, T. B. and Ooi, V. E. C. (1998). Lectins from mushrooms. *Mycological Research*, 102, 897-906.

Wang, J. C., Hu, S. H., Liang, Z. C. and Yeh, C. J. (2005). Optimization for the production of water-soluble polysaccharide from *Pleurotus citrinopileatus* in submerged culture and its antitumor effect. *Applied Microbiology and Biotechnology*, 67, 759-766.

Wasser, S. P. (1997). The fungus among us. *Eretz Magazine*, 1, 52-69.

Wasser, S. P. and Weis, A. L. (1999a). Medicinal properties of substances occurring in higher *Basidiomycetes* mushrooms: current perspectives (Review). *International Journal of Medicinal Mushrooms*, 1, 31-62.

Wasser, S. P. and Weis, A. L. (1999b). Therapeutic effects of substances occurring in higher *Basidiomycetes* mushrooms: a modern perspective. *Critical Reviews in Immunology*, 19, 65-96.

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

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

Weiner, R., Langille, S. and Quintero, E. (1995). Structure, function and immunochemistry of bacterial exopolysaccharides. *Journal of Industrial Microbiology and Biotechnology*, 15, 339-346.

Weng, Y., Xiang, L., Matsuura, A., Zhang, Y., Huang, Q. and Qi, J. (2010). Ganodermasides A and B, two novel anti-aging ergosterols from spores of a medicinal mushroom *Ganoderma lucidum* on yeast via UTH1 gene. *Bioorganic and Medicinal Chemistry*, 18, 999-1002.

Weston, C. R., Lambright, D. G. and Davis, R. J. (2002). Signal transduction. MAP kinase signaling specificity. *Science*, 296, 2345.

Whitfield, C. (1988). Bacterial extracellular polysaccharides. *Canadian Journal of Microbiology*, 34, 415-420.

Wilkins, J. C., Homer, K. A. and Beighton, D. (2001). Altered protein expression of *Streptococcus oralis* cultured at low pH revealed by two-dimensional gel electrophoresis. *Applied and Environmental Microbiology*, 67, 3396-3405.

Wong, K. H., Cheung, P. C. K. and Wu, J. Z. (2003). Biochemical and microstructural characteristics of insoluble and soluble dietary fiber prepared from mushroom sclerotia of *Pleurotus tuber-regium*, *Polyporus rhinocerus*, and *Wolfiporia cocos*. *Journal of Agricultural and Food Chemistry*, 51, 7197-7202.

Wong, S. M., Wong, K. K., Chiu, L. C. M. and Cheung, P. C. K. (2007). Non-starch polysaccharides from different developmental stages of *Pleurotus tuber-regium* inhibited the growth of human acute promyelocytic leukemia HL-60 cells by cell-cycle arrest and/or apoptotic induction. *Carbohydrate Polymers*, 68, 206-217.

Wu, J. Z., Cheung, P. C. K., Wong, K. H. and Huang, N. L. (2003). Studies on

submerged fermentation of *Pleurotus tuber-regium* (Fr.) Singer--Part 1: physical and chemical factors affecting the rate of mycelial growth and bioconversion efficiency. *Food Chemistry*, 81, 389-393.

Wu, J., Cheung, P. C. K., Wong, K. and Huang, N. (2004). Studies on submerged fermentation of *Pleurotus tuber-regium* (Fr.) Singer. Part 2: effect of carbon-to-nitrogen ratio of the culture medium on the content and composition of the mycelial dietary fibre. *Food Chemistry*, 85, 101-105.

Wymelenberg, A. V., Minges, P., Sabat, G., Martinez, D., Aerts, A., Salamov, A., Grigoriev, I., Shapiro, H., Putnam, N. and Belinky, P. (2006). Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. *Fungal Genetics and Biology*, 43, 343-356.

Xiao, J., Chen, D., Wan, W., Hu, X., Qi, Y. and Liang, Z. (2006). Enhanced simultaneous production of mycelia and intracellular polysaccharide in submerged cultivation of *Cordyceps jiangxiensis* using desirability functions. *Process Biochemistry*, 41, 1887-1893.

Xu, C. P. and Yun, J. W. (2003). Optimization of submerged - culture conditions for mycelial growth and exo-biopolymer production by *Auricularia polytricha* (wood ears fungus) using the methods of uniform design and regression analysis. *Biotechnology and Applied Biochemistry*, 38, 193-199.

Yamamoto, T., Yamashita, T. and Tsubura, E. (1981). Inhibition of pulmonary metastasis of Lewis lung carcinoma by a glucan, Schizophyllan. *Invasion and Metastasis*, 1, 71-84.

Yang, B. K., Ha, J. Y., Jeong, S. C., Jeon, Y. J., Ra, K. S., Das, S., Yun, J. W. and Song, C. H. (2002). Hypolipidemic effect of an exo-biopolymer produced from submerged mycelial culture of *Auricularia polytricha* in rats. *Biotechnology Letters*, 24, 1319-1325.

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

Yang, H., Wu, T. and Zhang, K. (2004). Enhancement of mycelial growth and polysaccharide production in *Ganoderma lucidum* (the Chinese medicinal fungus,Lingzhi') by the addition of ethanol. *Biotechnology Letters*, 26, 841-844.

Zhang, B. B. and Cheung, P. C. K. (2011). Use of stimulatory agents to enhance the production of bioactive exopolysaccharide from *Pleurotus tuber-regium* by submerged fermentation. *Journal of Agricultural and Food Chemistry*, 59, 1210-1216.

Zhang, M., Cheung, P. C. K. and Zhang, L. (2001). Evaluation of mushroom dietary fiber (nonstarch polysaccharides) from sclerotia of *Pleurotus tuber-regium* (Fries) Singer as a potential antitumor agent. *Journal of Agricultural and Food Chemistry*, 49, 5059-5062.

Zhang, M., Zhang, L., Cheung, P. C. K. and Ooi, V. E. C. (2004). Molecular weight and anti-tumor activity of the water-soluble polysaccharides isolated by hot water and ultrasonic treatment from the sclerotia and mycelia of *Pleurotus tuber-regium*. *Carbohydrate Polymers*, 56, 123-128.

Zhang, M., Cheung, P. C. K., Chiu, L. C. M., Wong, E. Y. L. and Ooi, V. E. C. (2006). Cell-cycle arrest and apoptosis induction in human breast carcinoma MCF-7 cells by carboxymethylated β-glucan from the mushroom sclerotia of *Pleurotus tuber-regium*. *Carbohydrate Polymers*, 66, 455-462.

Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C. and Raetz, C. R. H. (1998). Function of Escherichia coli MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *Journal of Biological Chemistry*, 273, 12466-12475.

Zhuang, C., Mizuno, T., Ito, H. and Shimura, K. (1994). Chemical modification and antitumor activity of polysaccharides from the mycelium of liquid-cultured *Grifola*

frondosa. *Nippon Shokuhin Kogyo Gakkai-Shi*, 41, 733-740.

Zhuang, C., Mizuno, T., Ito, H., Shimura, K., Sumiya, T. and Kawade, M. (1994). Antitumor activity and immunological property of polysaccharides from the mycelium of liquid-cultured *Grifola frondosa*. *Nippon Shokuhin Kogyo Gakkai-Shi*, 41, 724-732.

Zhuang, C., Mizuno, T., Shimada, A., Ito, H., Suzuki, C., Mayuzumi, Y., Okamoto, H., Ma, Y. and Li, J. (1993). Antitumor protein-containing polysaccharides from a Chinese mushroom Fengweigu or Houbitake, *Pleurotus sajor-caju* (Fr.) Sings. *Bioscience, Biotechnology, and Biochemistry*, 57, 901-906.

Zoberi, M. H. (1973). Some edible mushroom from Nigeria. *Nigerian Field*, 38, 81-90.

Zou, X., Guo, X. and Sun, M. (2009). pH control strategy in a shaken minibioreactor for polysaccharide production by medicinal mushroom *Phellinus linteus* and its anti-hyperlipemia activity. *Bioprocess and Biosystems Engineering*, 32, 277-281.

Related publications

Zhang, B. B. and Cheung, P. C. K. (2011). Use of stimulatory agents to enhance the production of bioactive exopolysaccharide from *Pleurotus tuber-regium* by submerged fermentation. *Journal of Agricultural and Food Chemistry*, 59, 1210-1216.

Zhang, B. B. and Cheung, P. C. K. (2011). A mechanistic study of the enhancing effect of Tween 80 on the mycelial growth and exopolysaccharide production by *Pleurotus tuber-regium*. *Bioresourse Technology*, 102, 8323-8326.

Zhang, B. B. and Cheung, P. C. K. (2010) Effects of stimulating agents on the production of mushroom mycelial biomass and exopolysaccharide by submerged fermentation. *Abstract of the Institute of Food Technologists Annual Meeting*, Chicago, USA.