*2005*年 *2*月

碩士學位論文

Effect of Environmental Conditions on Mycelial Growth of *Morchella esculenta*

朝鮮大學校 大學院

化 學 工 學 科

丁 吉 鹿

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곰보버섯 균사체 생육에 미치는 환경요인의 영향

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이 論文을 工學碩士 學位申請論文으로 提出함

2004年　10月　　日

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丁吉鹿의 碩士學位論文을 認准함

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委　員　朝鮮大學校　敎　授　金瑄日　印

委　員　朝鮮大學校　敎　授　車月石　印

2004年　11月　　日

朝鮮大學校 大學院

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초 록

곰보버섯은 Morchelaceae과, Morchela속에 속하는 자낭균류로서 칼로리, 나트륨, 지방 그리고 콜레스테롤이 낮은 반면 단백질, 탄수화물, 섬유, 비타민 및 무기염류 등이 풍부한 영양학적 특성 때문에 많은 나라에서 양송이버섯보다 많이 애용되고 있는 식용버섯이다. 느타리속에 속하는 백영고버섯은 전통적인 중국의 약용버섯인 영지버섯과 같은 형태를 지녔지만 이 버섯은 영양분이 매우 풍부하며, 특히 자실체 내에 면역계를 강화시켜 주는 기능을 갖는 다당류가 19% 함유되어 있어 이 버섯의 유용성은 확대될 전망이다. 곰보버섯과 다른 느타리속 버섯에 대하여 농학 및 유전학적 측면에서 많은 연구가 있었음에도 불구하고 국내에서의 곰보버섯과 백영고버섯에 관한 생물학적 활성 및 배양에 관한 연구는 매우 드문 실정이어서 본 연구에서는 곰보버섯의 균사체 및 세포외 다당류 생산을 위한 액체 배양조건 최적화 및 백영고버섯 자실체 추출물들의 항암, 항산화 효과를 스크리닝하고자 MTT법과 TBA테스트를 이용하여 조사하고자 하였다.

플라스크 배양에서 곰보버섯의 균사생장과 세포외 다당류 생산에 가장 효과적 액체 배양조건을 조사하였다. 곰보버섯의 최적 배양온도와 pH는 각각 25℃와 6.5 이었다. 기본배지를 선발하고자 여러 배지를 선정하여 실험한 결과 그 중에서 BG 배지가 가장 효과적이었다. 50 mL BG 배지용량을 함유한 300 mL 삼각플라스크를 100 rpm으로 진탕배양 하였을 때 최대 균사생장과 세포외 다당류 생산을 보였다. 최적 배양조건하에서 배양하였을 때 최대 균사생장과 세포외 다당류 생산은 각각 배양 9일경에 3.98 g/L와 1.10 g/L로 나

타났다. 영양요구성 실험결과 최적 탄소원은 과당이었고 최적농도는 5% (w/v)이었다. 6종의 질소원을 여러 가지 조합에 따라 과당 5% (w/v)가 함유된 배지에 첨가한 결과 펩톤과 염화암모늄을 1:1의 무게 비로 4% (w/v)로 첨가하였을 때 더욱 효과적이었고, 치적 무기염류는 제이인산칼륨과 황산마그네슘 7수화물이었고, 최적농도는 각각 1.0%와 1.5% (w/v)이었다. 요약하면, 곰보버섯 균사생장과 세포외 다당류 생산을 위한 최적배지는 과당 5%, 1:1 무게비의 펩톤과 염화암모늄 4%, 제이인산칼륨 1.0% 그리고 황산마그네슘 7수화물 1.5%로 나타났다. 5L 발효조에서 곰보버섯 균사생장과 세포외 다당류 생산을 위한 최적 배양조건을 조사하였다. 교반속도 100에서 300 rpm까지 달리하여 조사한 결과 200 rpm에서 최대 균사생장 7.36 g/L와 2.92 g/L를 나타냈으며, 통기량의 경우 1.5 v.v.m.에서 가장 효과적인 균사생장과 세포외 다당류 생산을 보였다. 통기량이 증가할수록 산소 전달속도가 증가하게 되어 곰보버섯의 균사생장과 세포외 다당류 생산에 유리한 환경을 조성하였기 때문인 것으로 사료된다. 최적 배양조건하에서 기본배지를 함유한 5L 발효조에서의 최대 균사생장과 세포외 다당류 생산을 배양 10일경에 각각 8.17 g/L와 3.54 g/L로 나타났고, 영양요구성 실험에서 얻어진 배지조성을 가지고 최적배양한 결과 균사생장과 세포외 다당류 생산은 배양 11일경에 10.2 g/L와 5.24 g/L로 나타났고 그 수율은 기본배지에서 일어난 균사생장과 세포외 다당류 생산보다 각각 20%와 32% 향상된 수율을 보였다.

In vitro에서 백영고버섯 추출물의 3종 인간 암세포주의 세포독성에 관하여 조사하였다. 백영고버섯 열수추출물은 자궁암세포와 대장암세포에 대하여 각각 8 mg/mL에서 71%와 73%의 세포독성을 보였고 폐암세포는 6 mg/mL에서 38%의 세포독성을 보였다. 그러나 에탄올 추출물의 경우 폐암세포에 대하여

4 mg/mL에서 열수추출물에서의 세포독성보다 강한 세포독성을 보였다. 따라서 백영고버섯 에탄올 추출물에 함유된 암세포 독성물질을 항암제의 개발을 위하여 많은 연구가 필요하다고 사료된다. 또한 백영고버섯 메탄올과 열수 추출물의 항산화 효과를 조사하기 위하여 TBA 테스트법을 사용하여 검정하였다. 그 결과 메탄올 추출물보다 열수 추출물에서 강한 항산화 효과를 나타냈으며, 40 μL 첨가구에서 37.43%의 항산화 효과를 나타냈다. 따라서, 백영고버섯 추출물은 항암 및 항산화 효과가 있는 것으로 나타나 이에 대한 보다 많은 연구가 수행되어야 할 필요성이 있는 것으로 보인다.

CHAPTER I

Introduction

Mushrooms have long been attracting a great deal of interest in many areas of foods and biopharmaceuticals, and are regarded as popular or effective medicines used to treat various human diseases, such as hepatitis, hypertension, hypercholesterolemia, and gastric cancer [44-45,51-52].

The medicinal property for which mushrooms have been most extensively investigated is their antitumor activity. Most of this research has been conducted in Japan. Whole mushrooms of several species and/or extracts from them have been reported to have an antitumor effect. Among these species are *G. lucidum*, *G. frondosa*, *Lentinus edodes*, *Phlebial radiata*, *Pleurotus citrinopileatus*, and *Schizophyllum commune* [38-39,40,49,64,103]. The antitumor studies conducted with mushrooms thus far are very interesting and do show a potential for providing therapeutic control of tumor. It is, however, difficult to say whether mushrooms could have preventive effects against tumor when consumed as part of the diet. Further epidemiologic and biological research is needed to clarify the role of mushrooms as preventive and curative agents.

Some common edible mushrooms, which are widely consumed in Asian culture, have currently been found to possess antioxidant activity which is well

correlated with their total phenolic content [14]. Another mushroom, *Paxillus panuoides*, was found to contain two *p*-terphenyls which showed potent inhibition effects lipid per-oxidation[101]. These findings suggest that the edible mushrooms might be a potential source of phenolic anti-oxidants. However, investigations of anti-oxidants derived from lesser-known edible mushrooms, which are produced by artificial cultivation methods, are still relatively rare.

Recent studies on exo-polysaccharide from mushrooms have demonstrated many interesting biological activities [45]. There are several antitumor agents produced from mushroom extracts such as Lentinan from *Lentinus edodes* [15], Schizophyllan from *Schizophyllum commune* [85], and Krestin from *Coriolus versicolor* [63], which are presently commercially available.

Many investigators have exerted their efforts to cultivate this mushrooms on solid artificial media rather than submerged culture [71,73]. Because it normally takes 6 months to complete a fruiting body culture in solid state fermentation, many attempts are being made to obtain useful cellular materials or to produce effective substances from a submerged mycelial culture [81,88]. Submerged cultures give rise to potential advantages of higher mycelial production in a compact space and shorter time with lesser chances of contamination [23,97]. Although several investigators have attempted to obtain optimal submerged culture conditions for mycelial growth and exo-biopolymer production from several mushrooms, the nutritional requirements and environmental conditions for submerged culture have not been demonstrated extensively [18,50,52-53,68,83].

Morchella esculenta, a wild edible mushroom, is a Ascomycotina fungus

belonging to the genus Morchellaceae, and the family Morchella (Fig.1) [6,72-73,80]. *Morchella esculenta* is low in calories, sodium, fat and cholesterol, while rich in protein, carbohydrate, fiber, vitamins and minerals. These nutritional properties make more a attractive food stuff than *Agaricus bisporous* in the many countries [66]. There are no reports on cultivation of this mushroom in Korea.

Pleurotus nebrodensis Inzenga originated from the Gobi Desert in the Xinjiang Autonomous Region of China which makes it more suitable for arid climates. This mushroom is by Chinese called Western Paradise White *Garnoderma* (Fig. 2). In recent years, Jinxin Mushroom Corporation Limited has successfully introduced this white *Ganoderma* to the Beijing market. To find it wild in nature is extremely rare and when something is scarce, it is precious. So this is the reason for the name, Western Paradise White *Ganoderma*. As shown in Fig. 1-8, it is shaped like the traditional Chinese medicine, *Ganoderma*, but its color white, and more importantly it is rich in nutrients. A unique feature of the white *Ganoderma* mushroom is its fat and thick layer. According to the examination report of the Chinese Food Examination Center, white *Ganoderma* contains 14.7% protein and the content of vitamin C, D and E is several times higher than in other types of mushroom. The content of polysaccharide in white *Ganoderma* reaches 19% and has the ability to strengthen the immune system [35]. Although there have been many reports on different *Pleurotus* genus, there are no reports on cultivation and biological activities of *Pleurotus nebrodensis* Inzenga in Korea.

Therefore, the objectives of this work are to determine the optimal culture

conditions in liquid-state fermentation for production of *Morchella esculenta*, and to investigate the effects of *Pleurotus ferulae* various extracts on viability of human cancer cell lines and antioxidative activity for screening the substances contained *Pleurotus nebrodensis* Inzenga.

Fig. 1. Fruiting body of *Morchella esculenta*

Fig. 2. Fruiting body of *Pleurotus nebrodensis* Inzenga

CHAPTER II

Literature Survey

II-1. World production of mushroom

Mushroom science is the discipline that is concerned with the principles and practices of mushroom cultivation. As is true in any branch of science, it is essential to establish the facts upon which principles can be derived for future developments of the discipline. Consistent production of successful mushroom crops will be built upon scientific knowledge and practical experience [7].

There are at least 12,000 species of fungi that can be considered as mushrooms with at least 2,000 species showing various degrees of edibility [10]. furthermore, over 200 species of mushroom hae been collected from the wild and utilized for various traditional medical purposes mostly in the Far East. To date, about 35 mushroom species have been cultivated commercially and of these, about 20 are cultivated on an industrial scale (Table 1). The majority of these cultivate species are both edible and possess medicinal properties. However, two of the major medicinal mushrooms, viz. *Ganoderma lucidum* and *Trametes* (Coriolus).65 spp. are distinctly inedible.

Table 1. World production of cultivated edible and medicinal mushrooms in different years (Chang, 1999b)

	1981		1986		1990		1994		1997	
	Fresh Wt × 10^3		Fresh Wt × 10^3		Fresh Wt × 10^3		Fresh Wt × 10^3		Fresh Wt × 10^3	
Species	Metric tons	%	Metric tons	%	Metric tons	%	Metric tons	%	Metric tons	%
Agaricus bisporus/ bitorquis	900.0	71.6	1,227.0	56.2	1,420.0	37.8	1,846.0	37.6	1,955.9	31.8
Lentinus edodes	180.0	14.3	314.0	14.4	393.0	10.4	826.2	16.8	1,564.4	25.4
Pleurotus spp.	35.0	2.8	169.0	7.7	900.0	23.9	797.4	16.3	875.6	14.2
Auricularia spp.	10.0	0.8	119.0	5.5	400.0	10.6	420.1	8.5	485.3	7.9
Volvariella volvacea	54.0	4.3	178.0	8.2	207.0	5.5	298.8	6.1	180.8	3.0
Flammulina velutipes	60.0	4.8	100.0	4.6	143.0	3.8	156.2	4.7	284.7	4.6
Tremella spp.	-	-	40.0	1.8	105.0	2.8	54.8	3.2	130.5	2.1
Hypisizygus spp.	-	-	-	-	22.6	0.6	27.0	1.1	74.2	1.2
Pholiota spp.	17.0	1.3	25.0	1.1	22.0	0.6	14.2	0.6	55.5	0.9
Grifola frondosa	-	-	-	-	7.0	0.2	238.8	0.3	33.1	0.5
Others	1.2	0.1	10.0	0.5	139.4	3.7	4,909.3	4.8	518.4	8.4
Total	1,357.2	100.0	2,182.0	100.0	3,763.0	100.0	30.5	100.0		100.0
Increasing %			73.6		72.5				25.4	

Overall, the world production of cultivated edible and/or medicinal mushrooms was recorded as $4{,}909.3\times10^3$ tons in 1994 increasing to $6{,}158.4\times10^3$ in 1997 with and estimated value in excess of 14 billion US dollars [11].

Mushroom cultivation is a worldwide practice (Table 2). In percentage terms, output yield of the leading 10 species cultivated made up c 92% of total world production of these six species, viz: *Agaricus bisporus* (31.8%); *Lentinus edodes* (25.4%), *Pleurotus* spp. (14.2%), *Auricularia auricula* (7.9%), *Flammulina velutipes* (4.6%), and *Volvariella volvaceae* (7.9%), made up 87% of the total production. It can be further observed that by late 1994, of these six species only *Agaricus* and *Pleurotus* were cultivated worldwide to be joined in 1997 by *Lentinus*. The other three of the major six species are grown almost exclusively in Asia [11].

World production of mushrooms over the last two decades has shown a phenomenal pattern of growth (Table 1), with a 5 times increase in tonnage. While the white butoon mushroom (*Agaricus bisporus*) still retains the highest overall world production, its relative contribution is decreasing due to the dramatic increase in the other species, viz; *Lentinus* and *Pleurotus* in particular. In 1981, *Agaricus* production represented 72% of world production but by 1997 this had dropped to 32%. Overall, world production of mushrooms is increasingly being dominated by species that are both edible and have medicinal properties.

It is pertinent to note that world production of mushrooms is now dominated by China with over 64% of total production. China has become a major produce and consumer of both edible and medicinal mushrooms.

Table 2. World population of cultivated edible and medicinal mushrooms in 1997 (Metric tons) (Chang, 1999b)

	China	Japan	Rest of Asia	North America	Latin America	EU	Rest of Europe	Africa	China	Total	%
									5440		
Agaricus bisporus	330000	-	68400	425300	51600	875000	115200	36000	0	1955000	31.8
Lentinus edodes	1397000	115300	47400	3600	300	500	300	-	-	1564400	25.4
Pleurotus spp.	760000	13300	88400	1500	200	6200	5800	200	-	875600	14.2
Auricularia spp.	48000	-	5300	-	-	-	-	-	-	485300	7.9
Volvariella volvacea	12000	-	60800	-	-	-	-	-	-	180800	3.0
Flammulina spp.	15000	10900	25700	-	-	-	-	-	-	284700	4.6
Tremella spp.	13000	-	500	-	-	-	-	-	-	130500	2.1
Hypsizygus marmoreus	2100	7200	100	-	-	-	-	-	-	74200	1.2
Pholioto nameko	3100	24500	-	-	-	-	-	-	-	55500	0.9
Grifola frondosa	2000	3100	-	-	-	-	-	-	-	33100	0.5
Hericium erinaceus	800	-	-	-	-	-	-	-	-		
Coprinus comatus	500	-	-	-	-	-	-	-	-	520800	8.4
Others	514900	2900	800	400	-	200	100	-	200		
Total	3918300	368000	297400	430800	52100	881900	121400	36200	5460		
%	63.6	6.0	4.8	7.0	0.8	14.3	2.0	0.6	0	6160800	100
									0.9		

Furthermore, China is also the major producer of the non-edible medicinal mushrooms, e.g. *Wolfiporia (Poria) cocos* (10,000 tons) and *Ganoderma lucidum* (4,000 tons). At least 10 new species of edible or medicinal mushrooms have been brought into cultivation in China in recent years and although as yet on a small scale, the potential, especially for mushrooms of medicinal value, is quite significant. Because of their historical background in the use of wild mushrooms, both as food and in Chinese traditional medicines, it is to be expected that China will continue to develop methods for cultivation of an increasing number of, as yet, uncultivatable mushrooms for medicinal exploitation. The traditional acceptance of mushrooms in herbal medicine and in expanding pharmaceutical industries, will ensure that China will continue to be a major exploiter of medicinal mushroom technology [96]. China is also investing heavily in fermenter technology for growing mushroom mycelium of medicinal species.

Historically, mushrooms were gathered from the wild for consumption and for medicinal use. China has been the source of many early cultivations of mushrooms, e.g. *Auricularia auricula* (600 AD), *Flammulina velutipes* (800 AD), *Lentinus edodes* (1000 AD) and *Tremella fuciformis* (1800). *Agaricus bisporus* was first cultivated in France in c 1600 while *Pleurotus ostreatus* was first grown in US in 1900. While mushroom cultivation now spans many centuries, it is only over the last 2-3 decades that there have been manor expansions in basic research and practical knowledge leading to the creation of a major worldwide industry [7].

The cultivation of *Agaricus bisporus* is an outstanding example of a

biotechnological enterprise that challenges the combined skills of industrial and biological technologies. *A bisporus* cultivation in Western countries has achieved its current pre-eminence in the mushroom industries because of a solid foundation in basic scientific research in all aspects of *Agaricus biology* (genetics, physiology, biochemistry), bio-process technology and, above all, the use of modern management principles [8]. This foundation made possible a highly technical approach involving the creation and utilization of specialized equipment and advanced engineering technology. While *Agaricus bisporus* is a highly tasty and nutritious mushroom, it does not appear to have been used for any specific medical conditions. However, much fundamental knowledge has been acquired in recent years which will be of considerable value for other cultivations.

II-2. Mushrooms as functional foods or dietary supplements

Functional foods come in a plethora of name forms, e.g. dietary supplements, natra- or nutri-ceuticals, medical foods, vita foods, pharma foods, phytochemicals, mycochemicals, biochemopreventatives, designer foods and foods for specific health uses [31-32]. Such complex designations could well be an impediment to their rightful maturation ad consumer acceptance [102]. There continues to be much confusion over these names especially in the commercial world. However, the term dietary supplement (DS) is now being more widely accepted and recognised. The term DS was formally defined by the US administration in 1994 as a product intended to supplement the diet to enhance health. A DS includes one or more of the following dietary ingredients: a mineral, amino acid, vitamin, herb or other botanical; or it is a dietary substance used to supplement the diet by increasing the total dietary intake and is intended for ingestion in the form of a capsule, powder, soft-gel or gel cap and not represented as a conventional food or as a sole item of a meal or the diet [20].

Functional food science is now considered as a part of nutritional science in which the primary objectives are to maintain good health, improve homeostasis and to create the conditions for disease risk reduction. In this way it should be seen to be quite distinct from the medical and pharmaceutical sciences where the objectives medicine seeks to eliminate disease rather than to fortify the patient. In essence, functional food science aims:

1. to identify beneficial interactions between the presence or absence of a food

component (macro-nutrient, micronutrient or so-called non-nutrient) and a specific function or functions in the body;

2. to understand their mechanisms so as to support hypotheses to be treated in protocols relevant for human studies. This will require multidisciplinary research programs containing the expertise of scientific partners including biochemists, nutritionists, the medical profession and process technologists.

Functional foods are set to play an increasingly important role in national efforts in developed nations to curtail medical expenditure and also to improve the dietary habits of the populace. Consumers are becoming increasingly more health conscious and discerning in the types of foodstuffs that are purchased. It is now not possible to overlook the critical role that diet, including functional foods, can play in general health and wellbeing. Many types of cancer can now be linked to inappropriate diets. In contrast, regular consumption of fruits and vegetables (now viewed as classical examples of functional foods) are now considered as essential ingredients in cancer prevention programmes [82].

Mushrooms have long been valued as highly tasty and nutritional foods by many societies throughout the world [7]. Early civilizations, by trial and error built up a practical knowledge of those suitable to eat and those to be avoided, e.g. poisonous or even psychotropic. In many parts of the world, especially Europe, wild mushrooms are regularly collected and used directly as a main source of food or added to soups, stews and teas. Mushrooms are considered to be a good source of digestible proteins with protein content above most vegetables and some what less than most meats and milk.

Protein content can vary from 10-40% no a dry weight basis. Mushrooms contain all the essential amino acids, but can be limiting in the sulphur-containing amino acids, cystine and methionine [5,9]. Fresh mushrooms contain 3-21% carbohydrates and 3-35% fiber on a dry weight basis. Thus, a considerable proportion of the carbohydrate of mushrooms consists of dietary fiber which cannot easily be digested by humans and which function essentially as dietary fiber; in this was the calorific value of most mushrooms is low.

Mushrooms probably contain every mineral present in their growth substrate including substantial quantities of phosphorous and potassium, lesser amount냴 calcium and iron. Mushrooms appear to be an excellent source of vitamins especially thiamine (B_1), riboflavin (B_2), niacin, biotin and ascorbic acid (VitC). Vitamins A and D are relatively uncommon although several species contain detectable amounts of β-carotine and ergosterol which converts to active vitamin D when exposed to ultraviolet irradiation. While crude fat in mushrooms contains all the main classes of lipid compounds including free fatty acids, mono-, di- and tri-glycerides, sterols, sterol esters ad phospholipids, levels are generally low, around 2-8% of dry weight [5]. Without doubt, edible mushrooms in fresh, cooked or processed forms are a nutritionally sound, tasteful food source for most people and can be a significant dietary component for vegetarians [5]. In China, the term Yakuzen is generally used for medicinal food dishes of mushrooms.

However, in the Orient several thousand years ago, there was the recognition that many edible and certain non-edible mushrooms could have valuable health

benefits [1,33]. The edible mushrooms which demonstrate medicinal or functional properties include species of *Lentinus* (Lentinula), *Auricularia*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus* and *Tremella* while others are known only for their medicinal properties, e.g. *Ganoderma* and *Trametes* (*Coriolus*) - these are definitely non-edible due to their coarse and hard texture or bitter taste. The historical evolution of usage of these essentially scarce, forest-obtained medicinal mushrooms would most certainly not have been as whole mushrooms but as hot water extracts, concentrates, liquors or powders and used in health tonics, tinctures, teas, soups and herbal formulae.

These mushroom dietary supplements are used extensively in traditional Chinese medicine in various combinations, often with other herbal products, to treat many medical conditions. A limited number of highly purified polysaccharide compounds derived from certain medicinal mushrooms are now being used, particularly in Japan, as pharmaceutical grade products.

II-3 Extraction, development and chemistry of anti-cancer compounds from medicinal mushrooms

II-3-1 Extraction, fractionation, purification and chemical modification

There is a broad similarity in the various methods that have been developed to extract the anti-cancer polysaccharides from mushroom fruit-bodies, mycelium and liquid media [62].

In the initial step dried mushroom powder or mycelium is repeatedly heated in 80% ethanol to extract and eliminate low molecular weight substances. Crude fraction 1, 11 and 111 are obtained from the remaining ethanol extract residue by extraction with water (100℃, 3h), 1% ammonium oxalate (100℃, 6h) and 5% sodium hydroxide (80℃, 6h) in that order (Fig. 3). Further purification of the polysaccharide are achieved by a combination of techniques including ethanol concentration, fractional precipitation, acidic precipitation with acetic acid, ion-exchange chromatography, gel filtration and affinity chromatography (Fig. 4).

There is a growing interest in increasing the activity of medicinal mushroom polysaccharides by various chemical modifications and perhaps creating a range of semisynthetic compounds not unlike the penicillin story. Chemical modification can be achieved by oxido-reductohydrolysis (Smith degradation) and also by formolysis. Some positive improvements in activity have been recorded but it is still at a very early stage [62].

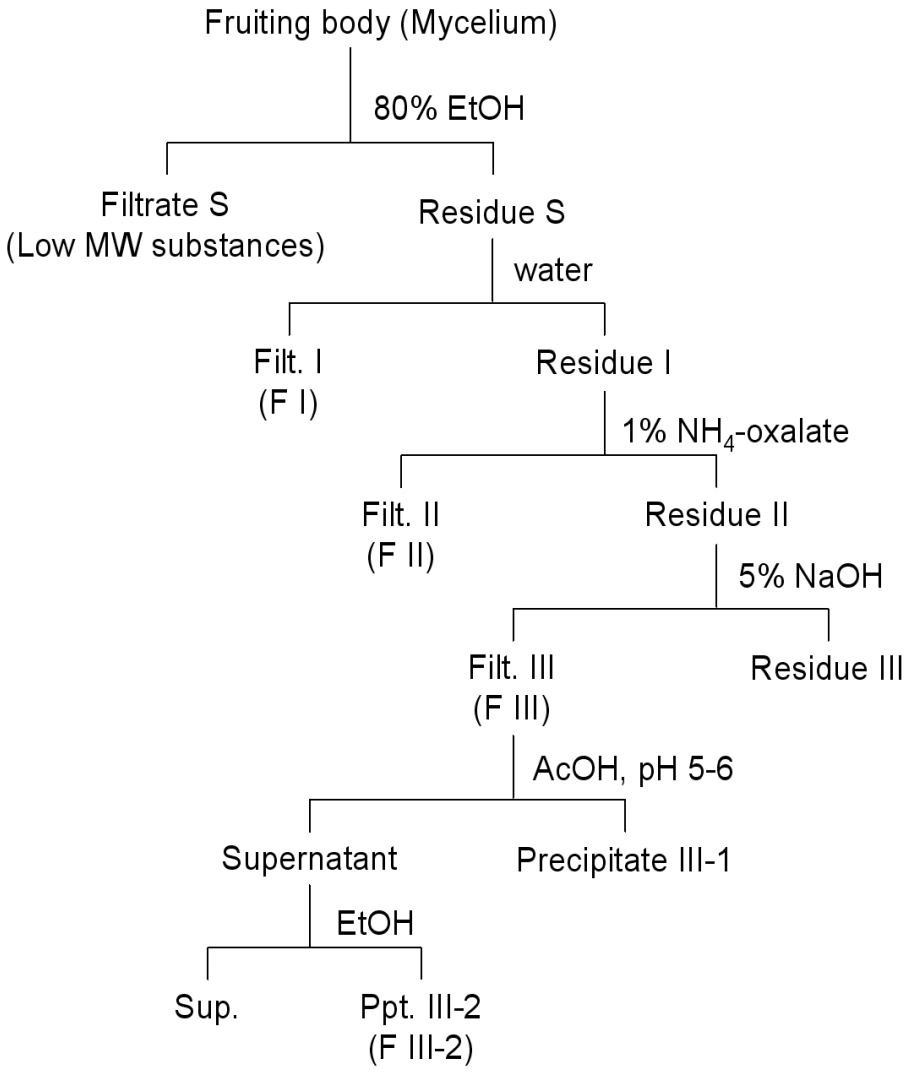

Fig. 3. Fractional preparation of polysaccharides from mushrooms

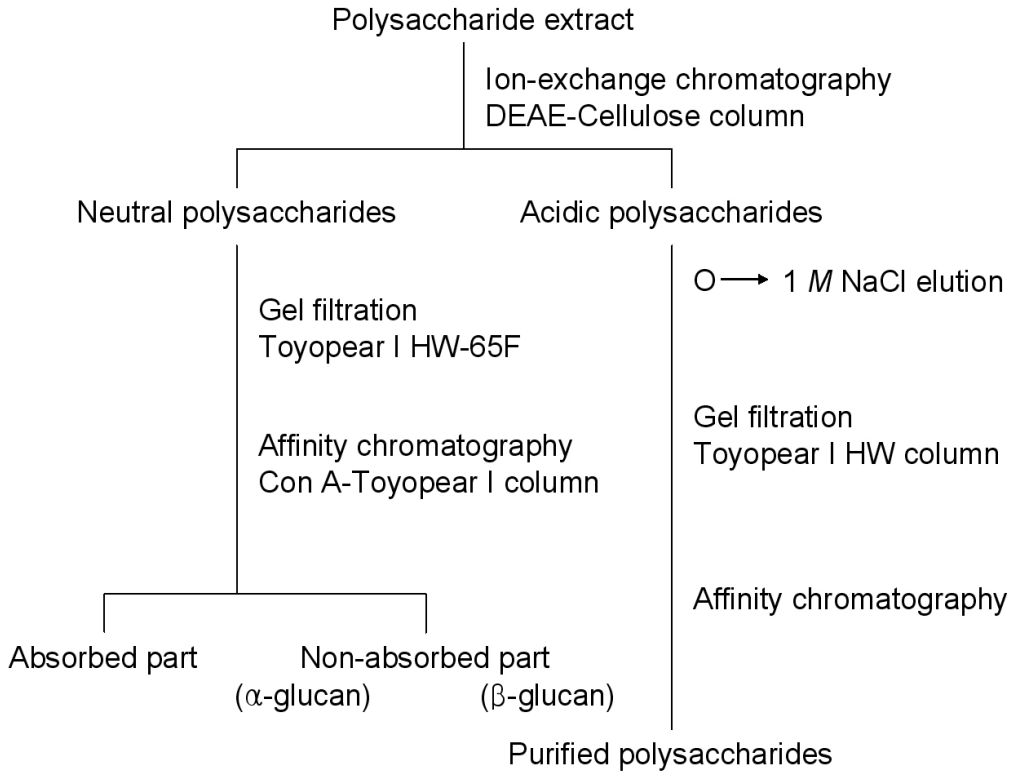

Fig. 4. Fraction purification of polysaccharides by chromatography

A recent study by Yap and Ng (2001) [99] has established a more efficient procedure for the extraction of β-D-glucans from *Lentinus edodes* (Fig. 5). The β -D-glucan was isolated through ethanol precipitation and freeze-drying in liquid nitrogen. Purity testing, using a carbohydrate analysis column, gave 87.5% purity. From a commercial aspect this method is less time-consuming, more efficient and of relatively low cost when compared to the original Chihara *et al*. (1970) [15] and Mizuno (1999) [62] methods (Table 3).

II-3-2 β-D-glucans

The basic β-D-glucan is a repeating structure with the D-glucose nits oined together in linear chains by beta-bonds (β). These can extend from carbon 1 of one saccharide ring to carbon 3 of the next (β1-3), from carbon 1 to carbon 4 (β1-4) or from carbon 1 to carbon 6 (β1-6). Mostly there is a main chain which is either β1-3, β1-4 or mixed β1-3, β1-4 with β1-6 side chains. The basic repeating structure of a β1-3 glucan with β1-6 side chains is shown in Figs, 6 and 7.

Not all β-D-glucans contained in fungi exhibit anti-tumor acitivy. The extent of occurrence of this activity seems to be influenced by solubility in water, size of molecules, and the β-(1-6)-bonding system in the β-(1-3) major chain. Some of the water insoluble β-glucans are soluble in dilute alkali and then can show marked anti-tumor activity [3].

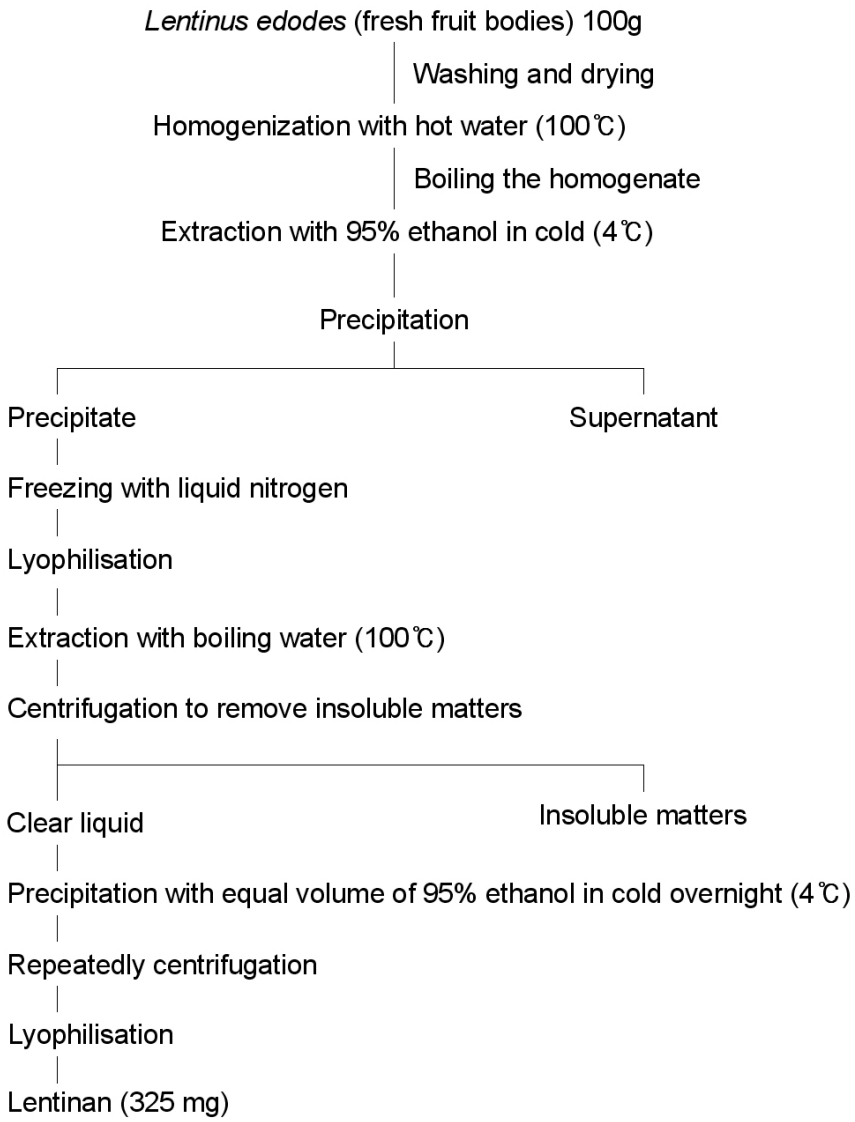

Fig. 5. New method for extracting lentinan from *Lentinus edodes*

Tabel 3. Comparison of two methods of preparation of β-D-glucan from *Lentinus edodes*

Characteristics of methods	*Method of extracting lentinan*	
	Chihara's method	*New biochemical method*
Number of days taken to prepare extract	14	5
Requirement of sophisticated equipment or rarely used chemicals	Many	None except liquid nitrogen
Cost of preparation	High	Low
Total yields from 100g of fresh mushroom	4 mg	325 mg
Percentage concentration of lentinan in extract produced (%)	96.03	87.50
Purity obtained	99.23	87.65

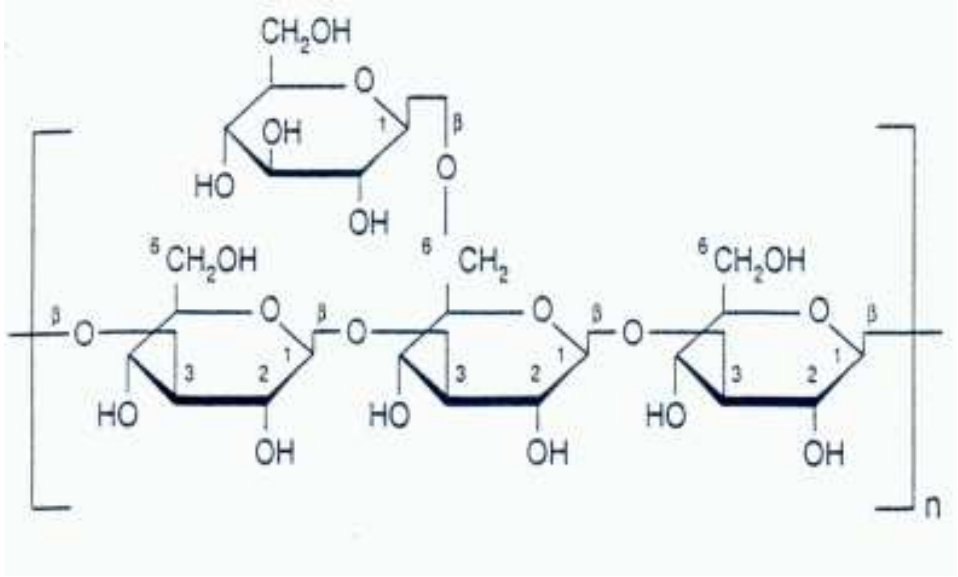

Fig. 6. Primary molecular diagram of mushroom β-D-glucan

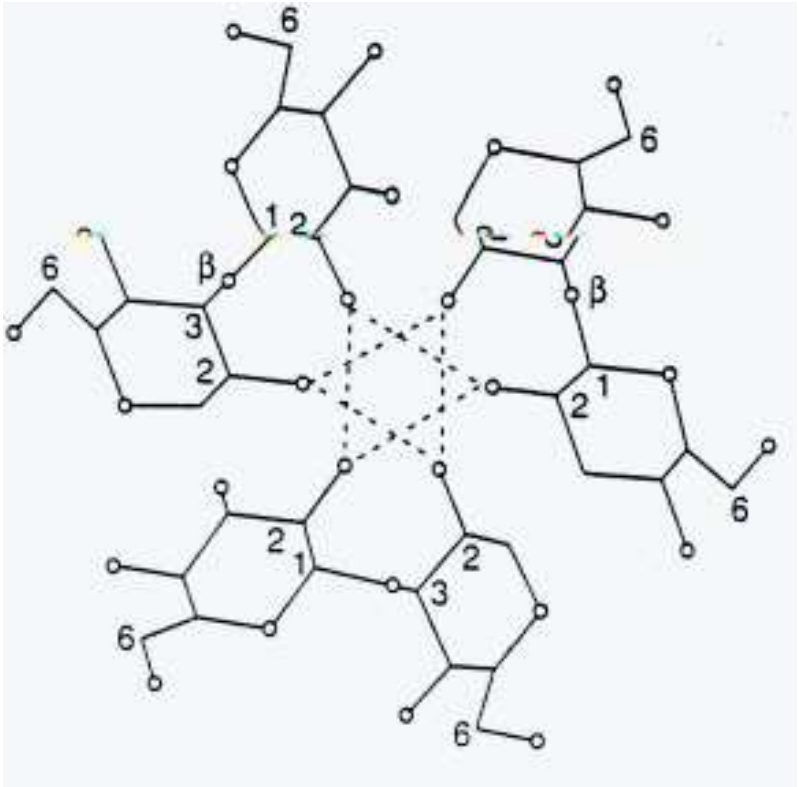

Fig. 7. Molecular model of the right-handed triple spiral helix of anti-tumor active β-D-glucan

Individual species-derived β-D-glucans have unique molecular structures [65] and it has been surmised that the higher ordered structures (triple helices) of high molecular weight β-D-glucans could be responsible for the considerable immunomodulatory activity [56]. Only higher molecular weight molecules apparently form triple helical structures which are stabilised by the β -D-glucopyranosly branch units [74]. There is good evidence to propose that both Lentinan and Schizophyllam are active only when they exist in a single helical structure [74].

Where polysaccharides are produced by fermentation processes it is much easier to then harvest at optimum production points as is already practised in other fermentations such as with anti-biotics.

II-3-3 Heteropolysaccharides and glycoproteins

While water-soluble β-D-glucans are widely distributed in mushroom species, many species also contain β-D-glucans with heterosaccharide chains of xylose, mannose, galactose and uronic acid which can be extracted by salt and alkali treatments. Other species can contain polysaccharide-peptides or glycoproteins which are polypeptide chains or small proteins to which polysaccharide β -D-glucan chains are stably attached [4] (Fig. 8).

Hot water extracts from *Grifola frondosa*, the Maitake mushroom, contain the D-fraction which appears to be a highly active anti-cancer agent for both animals and humans [37,57].

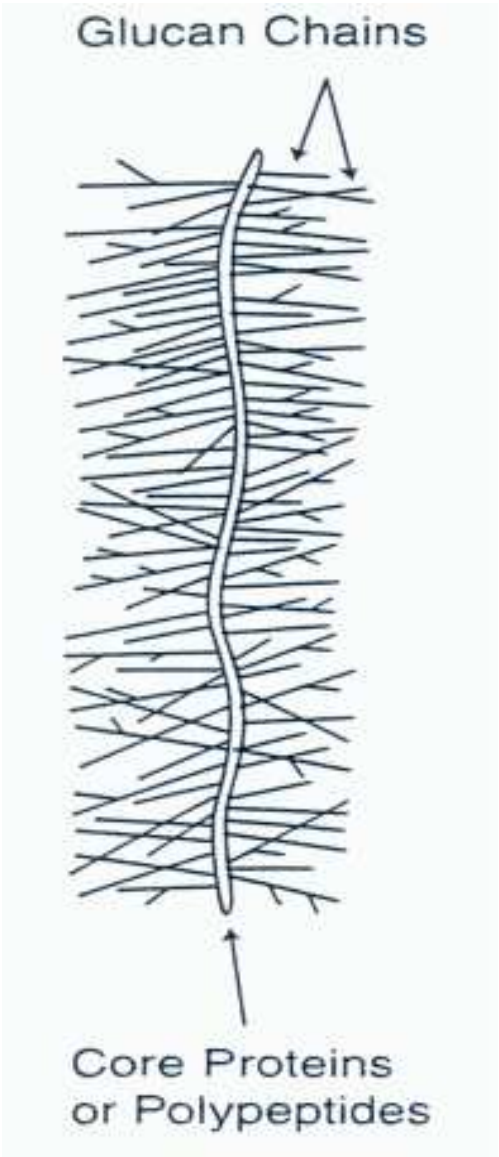

Fig. 8. The molecular plan of a mushroom proteoglycan

The D-Fraction is obtained from the hot water crude extract by deproteination. Maitake D-Fraction contains mainly β-D-glucan with 1-6 main chains and 1-4 branchings together with the more common 1-3 main chains and 1-6 branching.

Ganoderma lucidum, the Reishi mushroom, contains β-D-glucan in hot water extracts together with glucuronoglucan, xyloglucan, unannoglucan, xylomannoglucan and other active heteroglucans and protein complexes. Purifications involve using salts, alkali and DMSO [60].

II-3-4 Active hexose correlated compounds (AHCC)

This is a proprietary extract prepared from the co-cultivatio of several Basidiomycete mushrooms including *Lentinus edodes*, *Trametes versicolor* and *Schizophyllum commune* grown on rice [27]. However, there is no data available on the exact species complement or on methods of preparation. It is apparently a hot water extract following enzyme treatment, and the extract contains polysaccharide, amino acids and minerals and is orally bio-available. The glucans present are stated to have low molecular weight, c. 5,000 daltons and are α-1-3 type. These details are surprising since typically low molecular weight material is normally inactive and α-glucans have minimal immuno-potentiating activity.

However, ther have been limited studies and reports suggesting an interesting level of efficacy against hepatocellular carcinoma [42]. Ghoneum (1998) [26] found that a derivative, arabinoxylane, derived from this fermentation increased human NK activity by a factor of 5 over two months.

III-3-5 Dietary fiber

High molecular weight compounds excreted without digestion and absorption by humans are called dietary fibers. Mushrooms contain dietary fibers belonging to β-glucans, chitin and heteropolysaccharides (pectinous substances, hemicellulose, olyuronides etc), making up as much as 10-50% in the dry matter.

Much of the active polysaccharides, water soluble or insoluble, isolated from mushrooms, can be classified as dietary fibers (i.e. β-glucan, xyloglucan, heteroglucan, chitinous substance) and their protein complexes. Many of these compounds ave carcinostatic activity and by physicochemical interactions they will absorb possible carcinogenic substances and hasten their excretion from the intestine. Thus, mushrooms in general may have an important preventative action for colorectal carcinoma [59].

While most attention has been given to studies demonstration the medicinal effects of the polysaccharides from single mushroom species, several studies are suggesting that the human and murine immune systems can be given greater stimulation by using mixtures of polysaccharides from several proven medicinal mushrooms [27,78,92]. A complementary effect of each mushroom component on enhancing immunological function can be expected from mixed medicinal mushrooms extracts.

II-3-6 Terpenoids

Certain terpenoids and their derivatives have been isolated from mushroom species from the Polyporales and Ganodermatales and have been shown to be cytotoxic. At least 100 different triterpenoids have been identified from fruiting bodies ad mycelium of *Ganoderma lucidum* and *G. aplanatum* and include ganoderic, ganoderenic, lucidenic acids- and several ganoderals (for references see Wasser and Weis, 1999b). A cytotoxic tricyclic sesquiterpene, illudin, isolated from *Omphalotus olearius* and *Lampterimyces japanicus* shows interesting anti-cancer properties. Furthermore, the semisynthetic illudin agalog, 6-hydroxy-methylcylfulvene (HMAF) has inensity profiles of a tumour growth inhibitor. HMAF is undergoing phase I human clinical trials and could well be a promising new anti-cancer drug [95].

II-4 Anti-oxidant, anti-inflammatory, free radical scavenging activities and the ageing process

A wide variety of pathological damage, such as DNA, carcinogenesis and cellular degeneration, related to the ageing process and ageing itself can be caused by reactive oxygen species (ROS) produced by sunlight, ultraviolet and ionising radiation, chemical reactions and metabolic processes. Furthermore, there is a vast accumulation of studies that implicate oxygen derived free radicals such as superoxide, hydroxyl radicals and high energy oxidants such as peroxy nitrite as mediators of inflammation, shock and ischemia/reperfusion injury [19]. There is also growing evidence to show that production of ROS at the site of inflammation can contribute to tissue damage [76]. Interventions against ROS could exert beneficial effects on inflammation and shock [29]. Several mushroom species have been studied for anti-inflammatory and anti-oxidant activities [89] and patents have been established for these usages [95].

Extracts of *G. lucidum* can apparently remove the hyperoxide radical believed to be a main factor in the human ageing process [55], and the ageing mouse model [67]. In a clinical trial with 30 elderly people *Ganoderma lucidum* extract (GLE) was given oral 1.5 g 3 times daily for 30 days. Interleukin-2 and interferon (IFN) production by peripheral mononuclear cells (PBMC) ad NK cell activity in vitro were respectively measured. Production of IL-2 and IFN were significantly increased after GLE treatment. Such results could suggest that GLE is a possible treatment to raise the cellular immunological activity in ageing

people [86,87].

A *Ganoderma lucidum* polysaccharide GLB7 decreased the production of oxygen free radicals and antagonised the respiratory bursh induced by PMA in murine peritoneal macrophages [54]. Such observations could imply that the polysaccharide-induced inhibition of oxygen free radicals in murine peritoneal macrophages play an important role in the anti-ageing effect of *Ganoderma* extracts.

PSK in a cell-free system consisting of hypoxanthine-xanthine oxidase rapidly quenched the superoxide radical, a property not shared by Schizophyllan [75]. PSK further repressed the mimetic activity of superoxide dismutase (SOD) and promoted oxidative stress relief for cancer-bearing hosts [47]. PSK also gave protection to macrophages from lipoperoxide accumulation and foam cell formation created by oxidatively modified low-density lipoprotein [100]. This protection is believed to be due to the induction of gene expression of anti-oxidative enzymes [13].

PSP shows similar scavenging effects on superoxide and hydroxyl radicals [34]. Significant superoxide and hydroxyl radical scavenging activities have been demonstrated for several mushroom anti-tumor polysaccharide [55].

CHAPTER III

Materials and Methods

III-1 Liquid cultivation of *Morchella esculenta*

III-1-1 Strain

Morchella esculenta was obtained from Manyang Edible Fungi Research Institute (Sichuan, China). Cultures were maintained on PDA (potato dextrose agar) plate. Plates were inoculated and incubated at 25℃ for 7 days, and then stored at 4℃.

III-1-2 Inoculum preparation

Morchella esculenta was initially grown on medium PDA (potatoes 4.0 g/L, dextrose 20.0 g/L and agar 15.0 g/L) in a petri-dish, and then transferred into the seed culture medium PDB (potato starch 4.0 g/L and dextrose 20.0 g/L) by punching out from the 5×5 mm of the agar plate culture with a sterilized cork borer. The seed culture was grown in a 300 mL flask containing 100 mL of

seed culture medium at 25℃ on a rotary shaker at 100 rpm for 7-8 days, and then homogenized at 10,000 rpm for 30 sec. The 5% (v/v) of mycelium homogenate was used as a inoculum.

III-1-3 Culture condition

III-1-3-1 Flask culture

Flask cultures were carried out in 300 mL flasks containing 50 mL of basal medium. The basal medium was made up of the following table 4 components (in g/L). Medium were sterilized at 121℃ for 15 min. The pH was adjusted to the desired value by addition of either 1N HCl or NaOH. The flasks were cultivated on a rotary shaker (Model HB-201SL) under specific conditions for 8 or 12 days. All experiments were carried out in triplicate to ensure the trends observed were reproductivity.

III-1-3-2 Jar fermentation

The fermentation medium was inoculated with 5% (v/v) of the mycelium homogenate and then cultivated at 25℃ in a 5-L jar fermenter (Korea Fermenter Co., Korea) under specific conditions for 8 or 12 days. The temperature, pH and working volume were maintained at 25℃, pH 6.5, and 2 L, respectively.

Table 4. Composition of media used in this study

Medium	*Composition (g/L)*
PDB	Potato Starch 4.0, Dextrose 20
YSB	Yeast extract 4, Starch 10, $MgSO_4 \cdot 7H_2O$ 1.5, KH_2PO_4 1.0
MGB	Malt extract 10, Glucose 10, $MgSO_4 \cdot 7H_2O$ 1.5, KH_2PO_4 1.0
GPB	Glucose 10, Peptone 2, $MgSO_4 \cdot 7H_2O$ 1.0, KH_2PO_4 1.0
BG	Beef extract 10, Glucose 10, $MgSO_4 \cdot 7H_2O$ 1.5, KH_2PO_4 1.0
Czapek	Sucrose 10, $NaNO_3$ 2, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.5, KCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.01

Based on the flask cultures, a batch culture using optimally designed medium with a Jar fermenter was carried out for 12 days. The culture conditions for a jar fermentation are as follows; temperature 25℃, agitation speed 200 rpm, and aeration rate 1.5 v.v.m.. The pH of the fermentation was controlled at 6.5. All experiments were carried out in triplicate to ensure reproductivity.

III-1-4 Analytical methods

Mycelial growth in a solid medium PDA (potatoes 4.0 g/L, dextrose 20.0 g/L and agar 15.0 g/L) on the effect of temperature was evaluated by visual measurement of the average increase of the fungal colony along two perpendicular diameters [25].

Dry weights of mycelium were obtained by centrifuging samples at 3,000 rpm for 15 min, washing the sediment three times with distilled water, and drying to constant weight. All supernatants were collected, and then the crude exo-polysaccharide was precipitated with the addition of 4 volumes of 95% ethanol. The precipitated exo-polysaccharide was collected by centrifugation at 3,000 rpm for 10 min and then dried to remove residual ethanol at 60℃ (Fig. 9.).

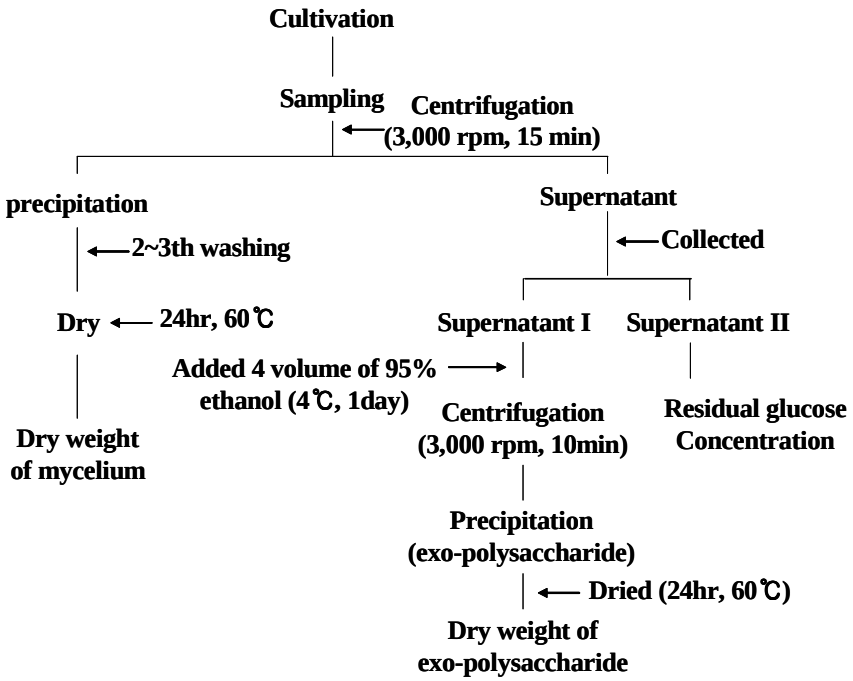

Fig. 9. Experimental procedure in this study

III-2 Effects of anti-cancer and anti-oxidant from *Pleurotus nebrodensis* Inzenga

III-2-1 Materials

Pleurotus nebrodensis Inzenga fruiting bodies (PNF) were obtained from Culture Ground Kaya-Backsong (Chungnam, Korea). PNF obtained was washed four times with distilled water, and dried with drying oven at 60℃ for 1 day and then powdered using Wiley Mill having particle size 300 mesh.

III-2-2 Extract preparation of *Pleurotus nebrodensis* Inzenga

About 500 g of dry powdered PNF was extracted with 1000 mL of 95% ethanol or methanol using soxhlet apparatus at room temperature for 8-10 h. The extracts were evaporated in a rotavapor. The residue was dissolved in distilled water, and the solvent was evaporated and lyophilized, and solid mass with a yield of 3-5% was used as ethanol extracts of *Pleurotus nebrodensis* Inzenga. About 100 g of dry powdered PNF was extracted with 1000 mL of hot distilled water using soxhlet apparatus at 100℃ for 2-3 h. After standing overnight at 4℃, the solvent was centrifuged and the supernatants were evaporated and lyophilized, and solid mass with a yield of 12-13% was used as hot water extracts of *Pleurotus nebrodensis* Inzenga.

III-2-3 Cells and culture

Human lung cancer cell (A549), cervical cancer cell (HeLa) and colon cancer cell (KM12C) lines were obtained from Korean Cell Culture Bank and cultured in RPMI-1640 medium (GIBCO RBL) supplemented with 10% (v/v) of fetal bovine serum, 100 U/mL of streptomycin, and 100 U/mL of penicillin. Cells were maintained at 37℃ in humidified atmosphere with 5% CO_2 and were subcultured twice a week. Cells were incubated in CO_2 incubator at 37℃ in humidified atmosphere of 95% air and 5% CO_2 for varying periods of time with or without *Pleurotus nebrodensis* Inzenga extracts and different concentrations of *Pleurotus nebrodensis* Inzenga extracts.

III-2-4 Cytotoxicity assay

The viability of the cells was determined by MTT assay [84]. MTT assay is based on the optical measurement of a dye formazan from MTT by mitocondrial dehydrogenase. Human cancer cells were cultured on RPMI-1640 containing 10% FBS and then 2×10^4 cells per well were added on 96-well microtiter plates. After addition of various concentrations of *Pleurotus nebrodensis* Inzenga extracts into each well, the 96-well plate was maintained at CO_2 incubator (37℃) for 2 days. After the cultivation was completed and RPMI-1640 was removed, 50 ㎕ of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and 200 ㎕ of fresh RPMI-1640 was added on a 96-well plate. Again, the plate was

maintained at CO_2 incubator for 4 h to give formazan formation. The quantity of formazan produced can be regarded as an indicator of cell density or viability. After dissolving the formazan in 150 μL of DMSO (dimethyl sulfoxide), the absorbance at 540 nm was measured with Microplate Autoreader (Labsystem Multiscan Multisoft, Finland). The results obtained were presented as percent of control values. The control values were determined from cultures with cancer cells growth in the medium without any *Pleurotus nebrodensis* Inzenga extracts and considered as 100%. Subsequently the growth rates of cancer cells treated with hot water or ethanol extracts were calculated the percent of control value and plotted.

III-2-5 Radical scavenging activity test by TBA method

Anti-oxidant activity was measured with TBA (thiobarbituric acid) by the Wong *et. al.*, method.

Rats (300 g) were anaesthetized with ether and the brain was quickly excised, placed immediately in ice-cold saline to wash it free from blood and homogenized in 0.1 M tris-HCl buffer (pH 7.4) using homogenizer. After homogenization, lipid was separated by centrifugation at 3,000 rpm for 10 min. Each sample was added to a solution mixture of hot water or methanol extracts (10, 20, 40 μL) and 100 μL of $FeCl_3$ (10 mM), respectively. The mixed solution was shaken in a water bath for 24 hours at 37℃. The solution was then mixed with 0.3 mL TCA (trichloroacetic acid) and 0.5 mL of 1% TBA (thiobarbituric

acid), and boiled in the water bath for 15 minutes at 100℃. The solution was centrifuged at 12,000 rpm for 10 minutes and the TBA value was determined by reading the absorbance at 532 nm. The inhibition activity was calculated against the TBA value of the control when added $FeCl_3$ only.

CHAPTER IV

Results and Discussion

IV-1 Flask culture

IV-1-1 Effect of temperature

The effect of temperature on mycelial growth of *Morchella esculenta* was investigated using PDA medium with 7 days cultivation. The culture temperature was controlled at 20, 25, 30 and 35℃. The results were presented in Fig. 10. Mycelial growth was the highest at the optimal temperature 25℃.

IV-1-2 Selection of basal medium

The effects of media composition on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* were determined with several media.

Batch cultures were carried out in 300 mL flasks containing 50 mL of the 6 different media (PDB, YSB, NGB, GPB, BG and Czapek) for 8 days. The mycelial growth and exo-polysaccharide concentrations were shown in Fig. 11.

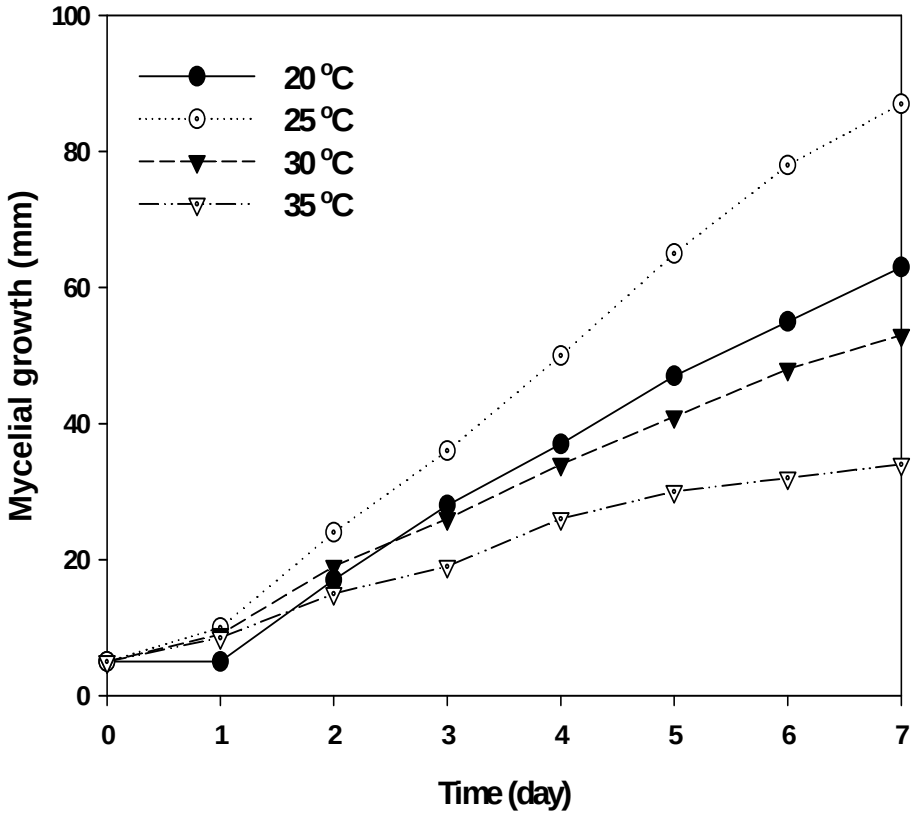

Fig. 10. Effect of temperature on the mycelial growth of *Morchella esculenta* in PDA medium

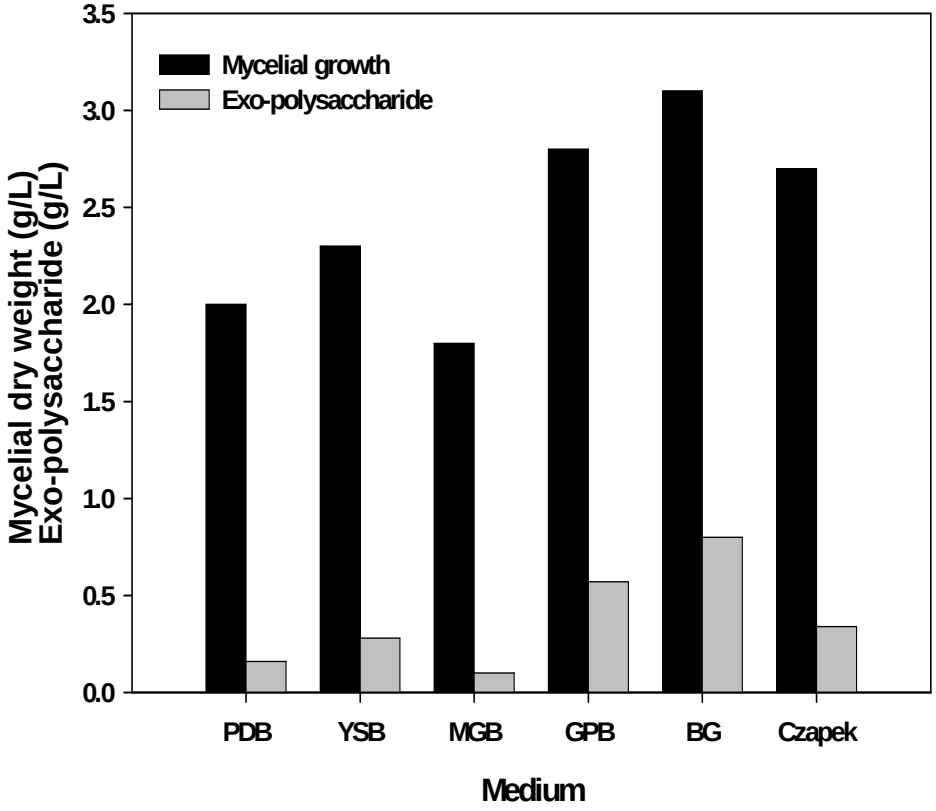

Fig. 11. Effect of medial composition on the mycelial growth and exo-polysaccharide of *Morchella esculenta* on several medium

Among 6 different media, the mycelial growth and exo-polysaccharide production were very good with BG, GPB and Czapek media. The highest mycelial growth and exo-polysaccharide production were obtained with a BG medium and they were 3.1 g/L and 0.8 g/L, respectively.

Therefore, it was concluded that the best medium for both mycelial growth and exo-polysaccharide production was BG medium.

IV-1-3 Effect of initial pH

To determine the effect of initial pH on mycelial growth and exo-polysaccharide production, *Morchella esculenta* was cultivated in a basal medium (BG) with different initial pHs (4.0-8.0) in a flask culture. The mycelial and exo-polysaccharide concentrations were presented in Fig. 12. Mycelial growth and exo-polysaccharide production were increased when the initial pH was 6.0-6.5. The optimal initial pH for mycelial growth was pH 6.5. At the initial pH 6.5, the maximum mycelial and exo-polysaccharide concentration were 3.56 g/L and 0.85 g/L, respectively.

Hashimoto and Takahasi [30] reported that the optimal pH for *Pleurotus ostreatus* was 6.2-6.5 and Wolport [94] reported that the optimal pH ranges for mycelial growth of basidiomycetes were 4.0-7.0. The optimal pH for mycelial growth and exo-polysaccharide production from *Morchellla esculenta* was 6.5.

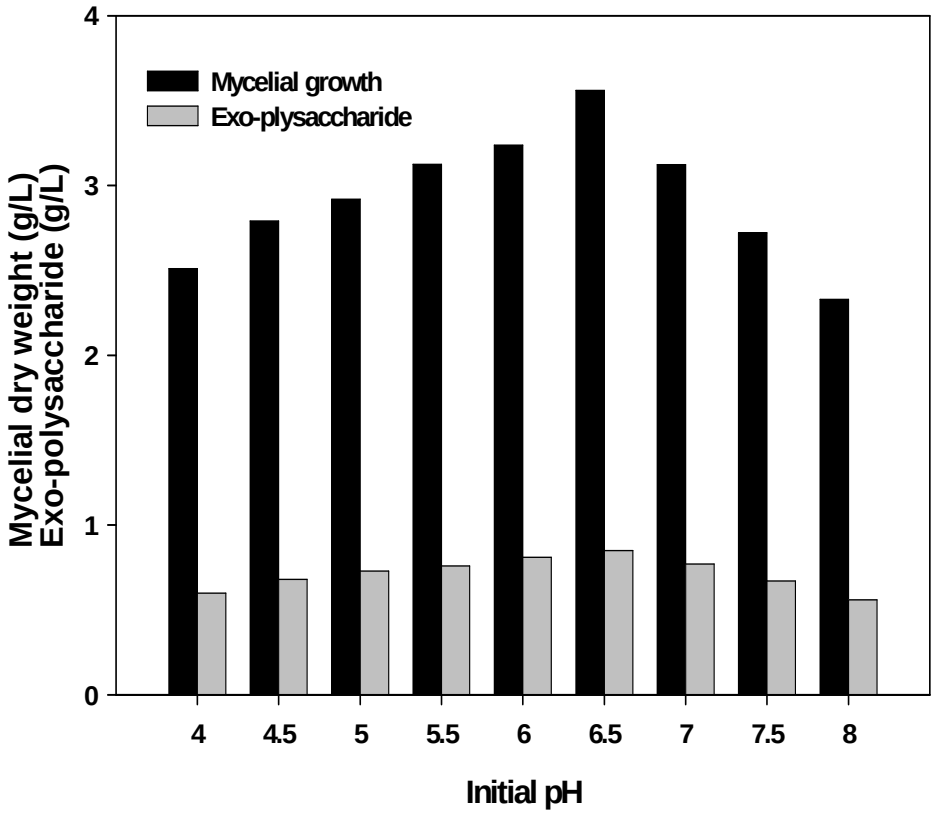

Fig. 12. Effect of initial pH on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in BG medium at 25℃ for 7 days

IV-1-4 Effect of surface aeration

Effect of surface aeration on mycelial growth was studied with various culture volumes of basal medium in 300 mL flasks which were shaken in a rotary shaker at 100 rpm for 8 days. The working culture volumes of 50, 100, 150 and 200 mL were tested. The mycelial concentration was measured and shown in Fig. 13. The flask with 50 mL of BG medium showed the highest mycelial growth (3.67 g/L) and exo-polysaccharide production(0.9 g/L), while the volume of basal medium higher than or lower than 50 mL showed lower mycelial growth and exo-polysaccharide production. These results indicate that adequate surface aeration is essential for both mycelial growth and exo-polysaccharide production.

IV-1-5 Effect of shaking speed

The viscosity of the culture media increased substantially due to mycelial growth and the accumulation of exo-polysaccharide. Efficient mixing for increasing mass transfer would be vital to enhance mycelial growth and polysaccharide synthesis. The effect of shaking speed on mycelial growth and exo-polysaccharide production was investigated using shaking incubator with 50-200 rpm. Mycelial culture was carried out in a 300 mL flask containing 50 mL BG medium at 25℃ and initial pH 6.5 for 8 days. The results were shown in Fig. 14 and the highest mycelial growth (3.94 g/L) and exo-polysaccharide

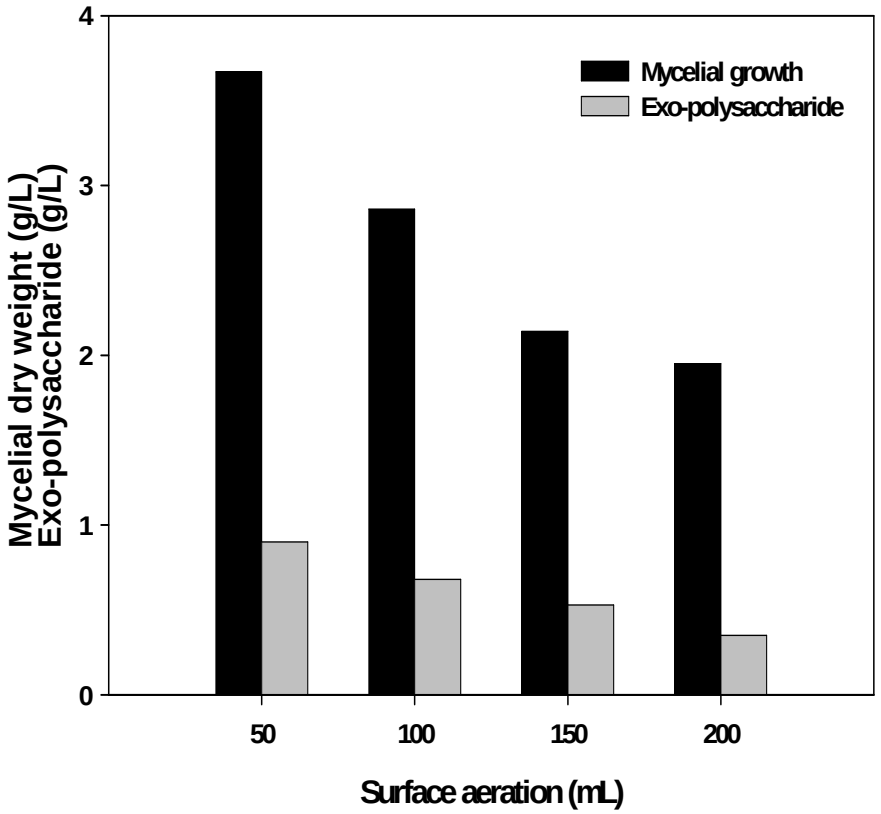

Fig. 13. Effect of surface aeration on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in BG medium at 25℃ for 7days

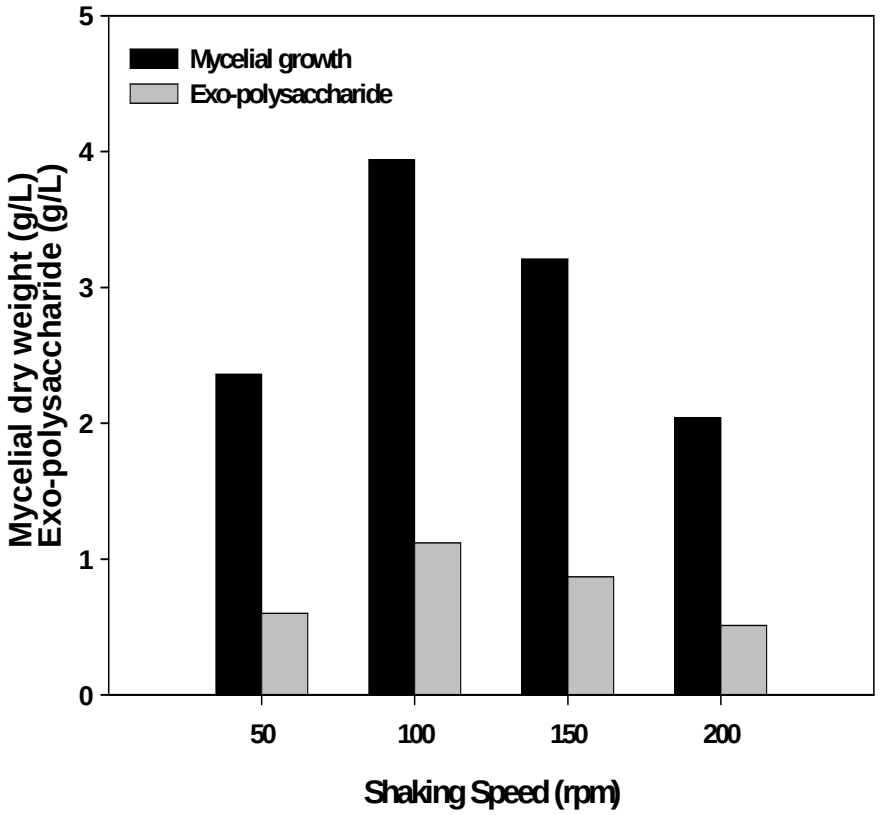

Fig. 14. Effect of shaking speed on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in BG medium at 25℃ for 7days

production (1.12 g/L) was achieved at 100 rpm. It was believed that higher shaking speed would be favorable to the mycelial growth and exo-polysaccharide production with increased mass transfer rate. However, when the shaking speed was higher than 100 rpm, the mycelial growth and exo-polysaccharide production decreased and this could be due to a detrimental effect of increased shear stress on the mycelium.

IV-1-6 Flask culture using optimal culture condition

The mycelial and exo-polysaccharide concentrations cultured in a flask culture were measured and shown in Fig. 15. The mycelia were grown under optimal culture conditions for 12 days. The optimal culture conditions for a flask culture are as follows; temperature 25℃, shaking speed 100 rpm and initial pH 6.5. Fig. 15 shows the change of mycelial growth and exo-polysaccharide concentrations.

In a flask culture, it took about 9 days to reach maximum mycelial concentration and the cells were grown rapidly between 3 and 8 days. The maximum mycelial concentration and exo-polysaccharide production was 3.98 g/L and 1.10 g/L, respectively, after 9 days.

IV-1-7 Nutritional requirement

IV1-7-1 The effects of carbon source

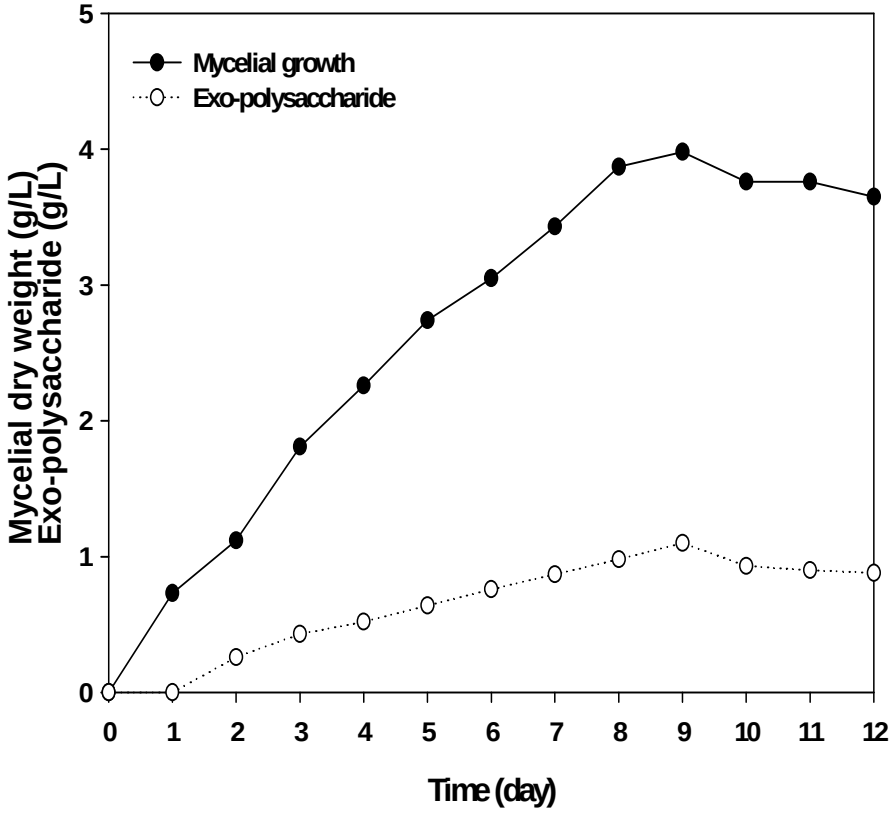

Fig. 15. Changes of mycelial growth and exo-polysaccharide production with flask culture at 25℃ and initial pH 6.5

To find a suitable carbon source for the mycelial growth and exo-polysaccharide production in *Morchella esculenta*, cells were cultivated in the medium containing various carbon sources, and each caron source was added to the BG medium at a concentration level of 1.0% (w/v) for 8 days. Among a carbon sources were tested, fructose, sucrose, starch and glucose were favorable for mycelial growth and exo-polysaccharide production (table 5).

With the addition of fructose, the highest mycelial growth (4.2 g/L) and exo-polysaccharide production (1.2 g/L) were observed. Kim *et al*.[46] reported that the addition of glucose and fructose resulted in maximum mycelial growth of *Flammulina velutipes* and the addition of sucrose resulted in highly ACE inhibitory effect. The pappern of exo-polysaccharide production was consistent with mycelial growth in *Morchella esculenta*. Park *et al*.[69] reported that good mycelial growth was closely related to the protein-bound polysaccharide production.

The effect of fructose concentration on the mycelial growth and exo-polysaccharide production was added for a glucose concentration ranged from 1 to 10% (w/v) in flask cultures. As shown in Fig. 16, the highest mycelial growth and exo-polysaccharide production of *Morchella esculenta* were obtained with a fructose concentration of 5% (w/v). When fructose concentration lower than or higher than 5% (w/v), mycelial growth and exo-polysaccharide production decreased. Therefore, it was concluded that the optimum fructose concentration for both mycelial growth and exo-polysaccharide production was 5% (w/v).

Table 5. Effect of various carbon sources on the mycelial growth and exo-polysaccharide production of *Morchella esculenta*

Carbon sources	Mycelial dry weight (g/L)	Exo-Polysaccharide (g/L)
Dextrin	2.1	0.41
Fructose	4.2	1.20
Glucose	3.0	0.70
Lactose	2.1	0.45
Maltose	2.8	0.58
Mannitol	2.5	0.53
Starch	3.1	0.73
Sucrose	3.3	0.81

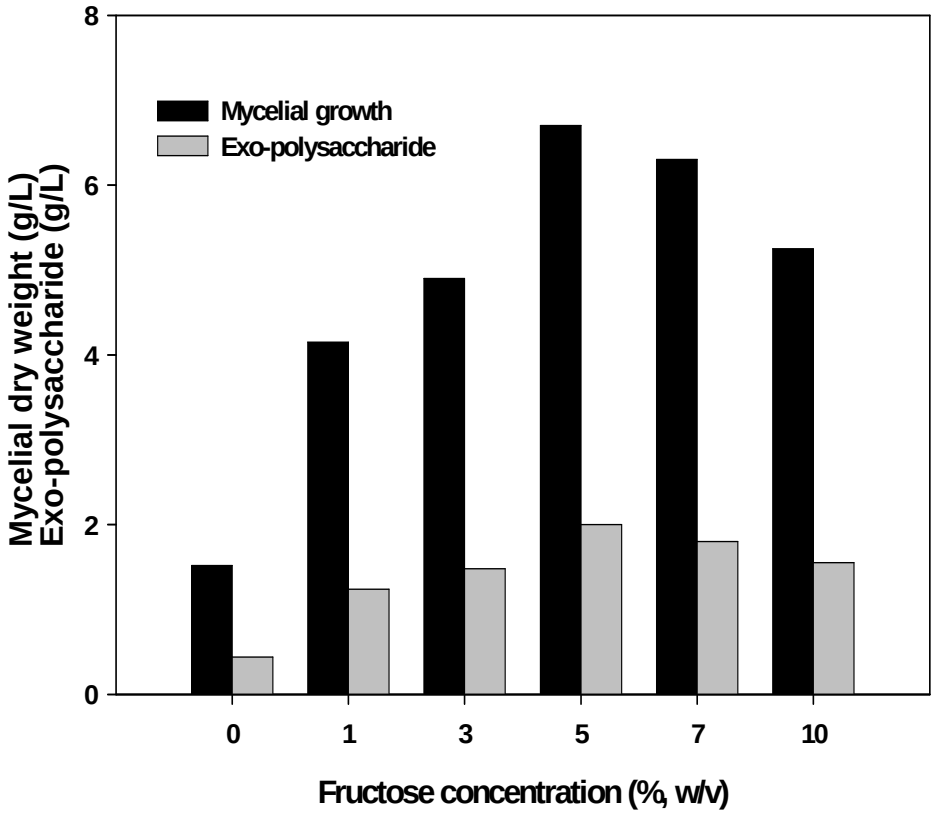

Fig. 16. Effect of fructose concentration on the mycelial growth and exo-polysahccharide production of *Morchella esculenta*

IV-1-7-2 The effects of nitrogen sources

To investigate the effects of nitrogen sources on the mycelial growth and exo-polysaccharide production, batch cultures were carried out in 300 mL flasks containing 50 mL of the BG medium with 1.0% (w/v) of final concentration of various nitrogen sources and 5.0% (w/v) of fructose. The mycelial growth and exo-polysaccharide production were shown in table 6.

Among 6 different nitrogen sources were added to the medium with various combinations, the mycelial growth and exo-polysaccharide production were very good with peptone plus NH_4Cl, yeast extract plus beef extract, peptone plus $NaNO_3$ and peptone plus beef extract group. Also, the effect of combinations of malt extract on the mycelial growth and exo-polysaccharide production were very poor. With combination of peptone and inorganic nitrogen sources, mycelial growth and exo-polysaccharide production were significantly increased. These results suggest that peptone and NH_4Cl might contain necessary components in the mycelial growth and exo-polysaccharide production.

Optimum initial concentration of peptone plus NH_4Cl ration [(w/v) of 1:1] for mycelial growth and exo-polysaccharide production were determined in a flask culture. We added peptone plus NH_4Cl with various concentration 0.5 to 5.0% (w/v) in a media containing 5% (w/v) of fructose and checked mycelial growth and exo-polysaccharide production for 8 days. As shown in Fig. 17, the highest mycelial growth and exo-polysaccharide production by *Morchella esculenta* were observed with a 4% (w/v) peptone plus NH_4Cl and they were 7.52 g/L and 2.74

Table 6. Effect of various nitrogen sources on the mycelial growth and exo-polysaccharide production of *Morchella esculenta*

Nitrogen sources	Mycelial dry weight (g/L)	Exo-Polysaccharide (g/L)
Yeast extract	6.17	2.20
Malt extract	1.52	0.55
Peptone	4.18	1.52
Beef extract	5.43	2.00
Yeast extract + Malt extract	4.95	1.73
Yeast extract + Peptone	6.02	2.15
Yeast extract + Beef extract	6.73	2.41
Malt extract + Peptone	4.73	1.81
Malt extract + Beef extract	3.02	1.14
Peptone + Beef extract	6.32	2.27
Yeast extract + NH_4Cl	5.39	1.96
Yeast extract + $NaNO_3$	6.21	2.23
Malt extract + NH_4Cl	0.97	0.22
Malt extract + $NaNO_3$	0.83	0.30
Peptone + NH_4Cl	6.91	2.58
Peptone + $NaNO_3$	6.67	2.43
Beef extract + NH_4Cl	4.52	1.60
Beef extract + $NaNO_3$	3.25	1.27

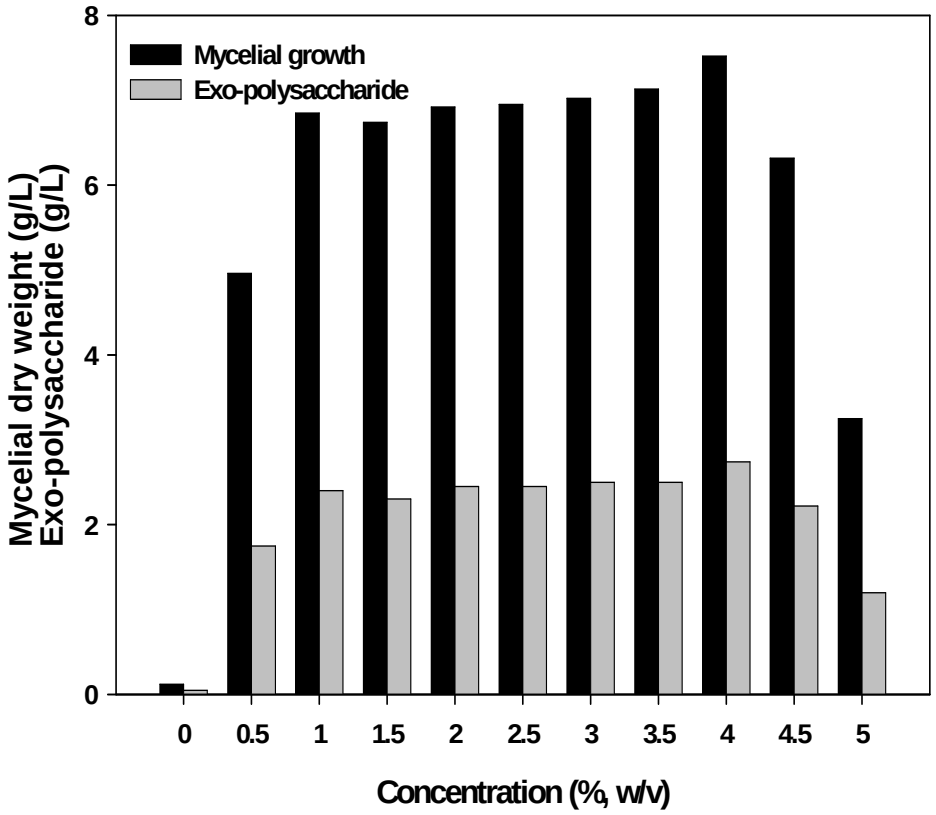

Fig. 17. Effect of peptone and NH_4Cl [ratio (w/w) of 1:1] on the mycelial growth and exo-polysaccharide production of *Morchella esculenta*

g/L, respectively.

The mycelial growth and exo-polysaccharide production increased with the increase in the peptone plus NH_4Cl concentration up to 4.0% (w/v) and then slightly decreased at the higher peptone plus NH_4Cl concentration. Therefore, the optimum concentration of peptone plus NH_4Cl [ratio (w/v) of 1:1] was 4.0% (w/v).

IV-1-7-3 The effects of mineral sources

The influence of mineral sources on the mycelial growth and exo-polysachcaride production in a medium containing 5% (w/v) of fructose and 4% (w/v) of peptone plus NH_4Cl [ratio (w/v) of 1:1] was investigated for 8 days cultivation. Mineral source was added to the medium at a concentration of 0.15% (w/v). As shown in table 7, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 were found to be the best mineral sources for mycelial growth and exo-polysaccharide production. The best result of the mycelial growth and exo-polysaccharide production in the medium containing K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ was similar to a effects of various inorganic salts on enzyme production by *Aspergillus japonicus* [12].

In general, phosphate have been used as a buffering reagent and potassium is important minerals involving the cell structure; magnesium cation may stimulate biosynthesis of the fungal cell and affect its permeability. When $ZnSO_4 \cdot 7H_2O$, $MgCl_2$, $MgSO_4$ and Na_2HPO_4 were used, mycelial growth and exo-polysaccharide production were slightly decreased than those of control. Therefore, the optimum

Table 7. Effect of mineral sources on the mycelial growth and exo-polysaccharide production of *Morchella esculenta*

Mineral source	Mycelial dry weight (g/L)	Exo-polysaccharide (g/L)
Control(none)	7.53	2.78
$MgSO_4$	7.21	2.65
$CaCl_2 \cdot 2H_2O$	7.82	0.75
$ZnSO_4 \cdot 7H_2O$	2.43	0.14
$MgCl_2$	6.57	1.35
$MgSO_4 \cdot 7H_2O$	10.2	3.52
Na_2HPO_4	7.36	2.01
$FeSO_4 \cdot 7H_2O$	8.44	3.22
K_2HPO_4	11.5	3.83
KH_2PO_4	8.96	2.97

mineral sources of *Morhcella esculenta* were K_2HPO_4 and $MgSO_4 \cdot 7H_2O$.

IV-1-8 Jar fermentation

IV-1-8-1 The effect of agitation speed

The effect of agitation speed on the mycelial growth and exo-polysaccharide production was carried out in a 5 L jar fermenter at different agitation speeds for 7 days. Aeration and pH were controlled 0.5 v.v.m. and 6.5, respectively. Fig. 18-1 ~ 18-3 shows the time profile of mycelial growth and exo-polysaccharide production at different agitation speeds. The agitation speed was controlled from 100 to 300 rpm and it significantly affected mycelial growth and exo-polysaccharide production. When agitation speed was increased above 200 rpm, mycelial growth and exo-polysaccharide production decreased. It was due to higher shear stress induced by the impeller agitation, which had an negative effects on growth of mycelia. Maximum mycelial and exo-polysaccharide concentrations were achieved at the agitation speed of 200 rpm, and they were 7.36 g/L and 2.92 g/L, respectively.

IV-1-8-2 The effect of aeration rate

The response of *Morchella esculenta* to intermediate levels of dissolved oxygen was investigated using jar fermenter. Fig. 19-1 ~ 19-2 showed the time profiles

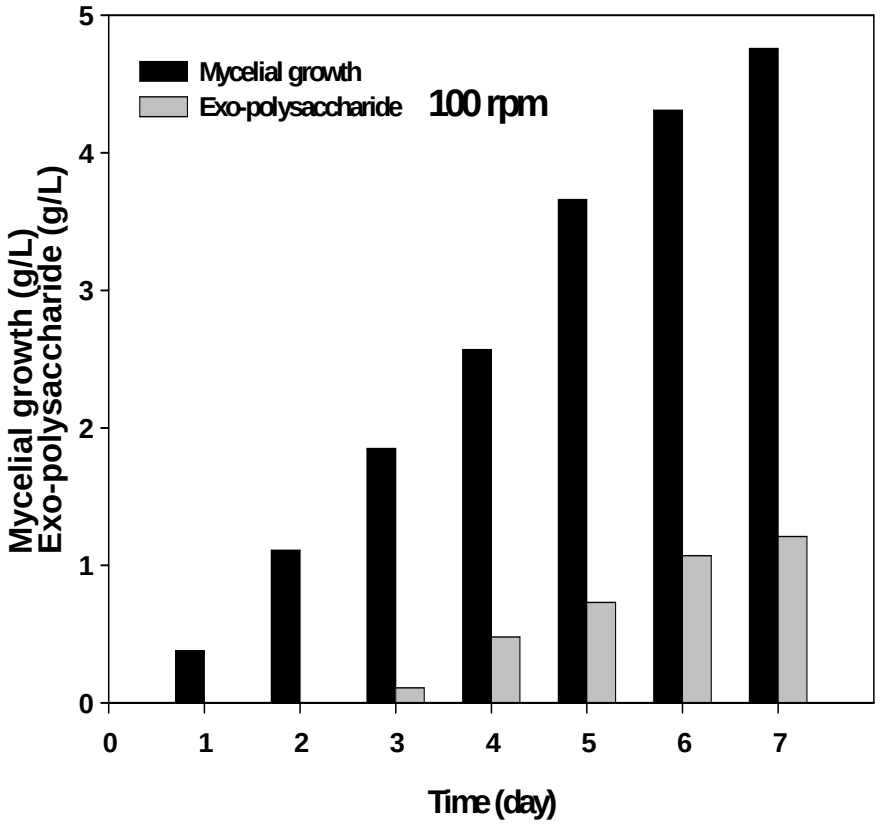

Fig. 18-1. Effect of agitation speed on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in jar fermentation at 25℃, pH 6.5 and 0.5 v.v.m.

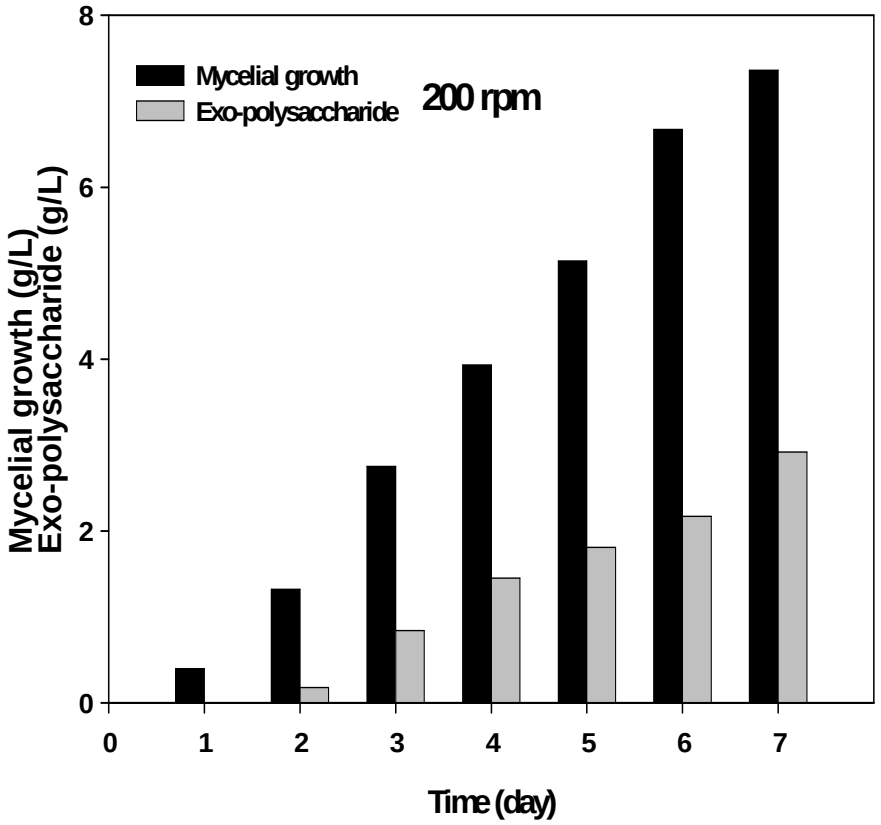

Fig. 18-2. Effect of agitation speed on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in jar fermentation at 25℃, pH 6.5 and 0.5 v.v.m.

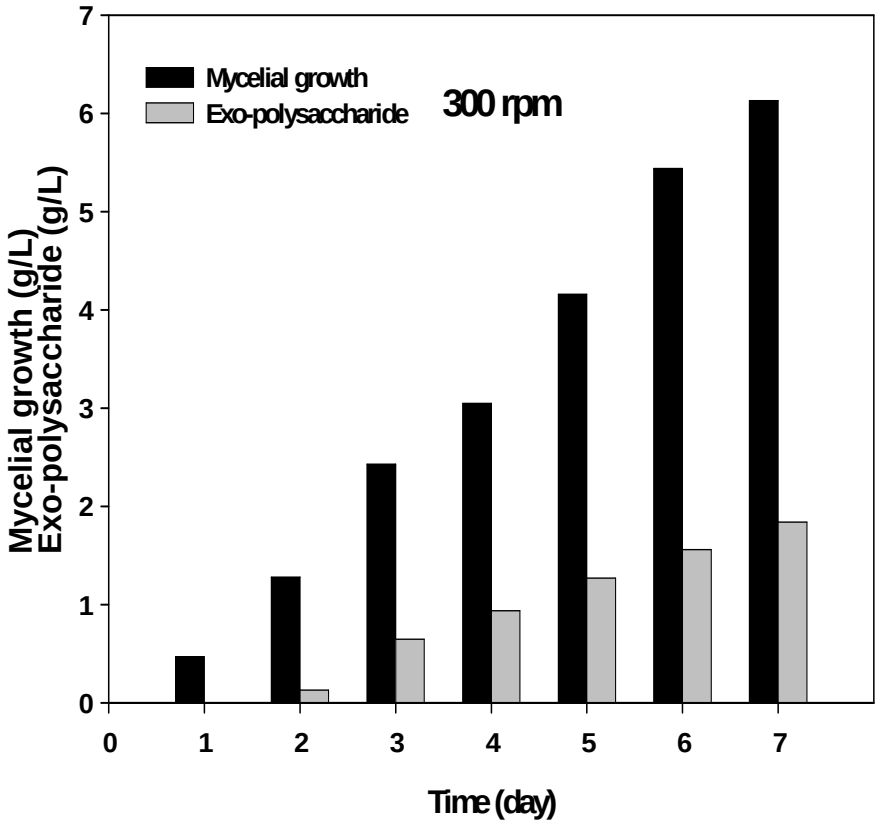

Fig. 18-3. Effect of agitation speed on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in jar fermentation at 25℃, pH 6.5 and 0.5 v.v.m.

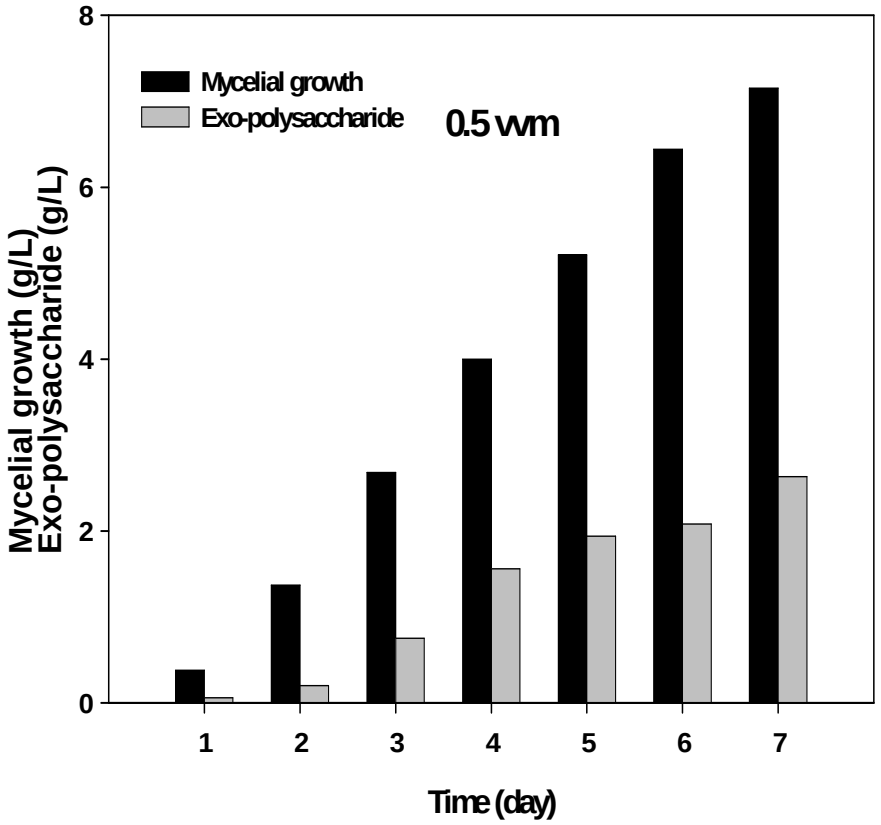

Fig. 19-1. Effect of aeration on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in jar fermentation at 25℃, pH 6.5 and 200 rpm

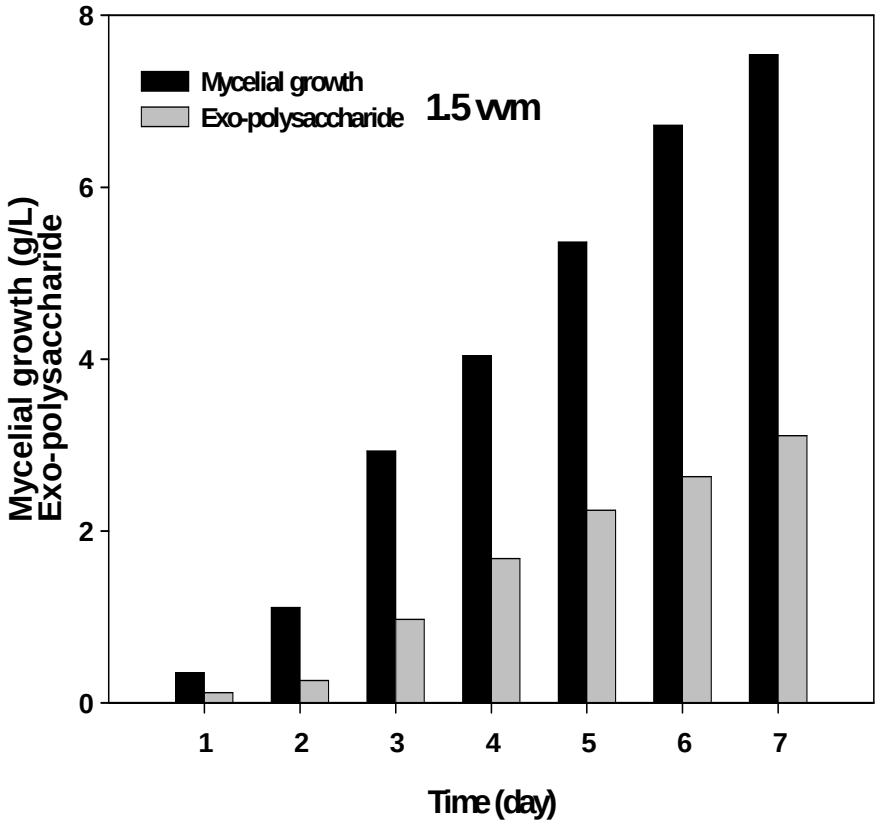

Fig. 19-2. Effect of aeration on the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in jar fermentation at 25℃, pH 6.5 and 200 rpm

of mycelial growth and exo-polysaccharide production in a jar fermentation at the different aeration rates of 0.5 and 1.5 v.v.m.. Agitation speed, pH, temperature were 200 rpm, 6.5 and 25℃, respectively.

Maximum mycelial growth and exo-polysaccharide production wee observed at the 1.5 v.v.m. of aeration rate and they were 7.54 g/L and 3.11 g/L, respectively. The increase in aeration rate results in the increase in oxygen transfer rate, which lead to an increase in mycelial growth and exo-polysaccharide production.

In the jar fermenter, the fermentations were subjected to oxygen limiting conditions. It should be noted that the limitation of oxygen transfer during the fermentation was also due to the increase of viscosity of liquid resulting from the accumulation of extra-cellular polysaccharide. Even though, the oxygen transfer co-efficient was not measured during this study, this parameter should be decrease significantly during the course of the fermentation.

IV-1-8-3 Comparison of basal medium and optimal medium in a jar fermentation

The mycelial growth and exo-polysaccharide concentrations cultured in a 5 L jar fermenter with a basal medium were measured and shown in Fig. 20. The optimal culture conditions for a jar fermentation are following conditions; temperature 25℃, agitation speed 200 rpm and aeration rate 1.5 v.v.m.. The pH of the fermenter was controlled at 6.5. The maximum mycelial growth and exo-polysaccharide production were obtained after 10 days and they were 8.17

g/L and 3.54 g/L, respectively (Fig. 21).

Based on the flask culture, a batch culture using optimally designed medium (5% of fructose, 4% of peptone plus NH_4Cl [ratio (w/v) of 1:1], 1% of K_2HPO_4 and 1.5% of $MgSO_4 \cdot 7H_2O$, w/v) with a jar fermenter was carried out for 12 days. Fig. 21 shows the change of mycelial growth and exo-polysaccharide concentrations. The mycelial growth and exo-polysaccharide concentrations were the higher after 11 days fermentation and they were 10.2 g/L and 5.24 g/L, respectively.

The exo-polysaccharide concentrations were rapidly increased in paralled with the growth rate of *Morchella esculenta* and the product formation is associated with mycelial growth. The yields of mycelial growth and exo-polysaccharide production of jar fermentation with optimal medium were increased 20% and 32%, respectively, than those of basal medium.

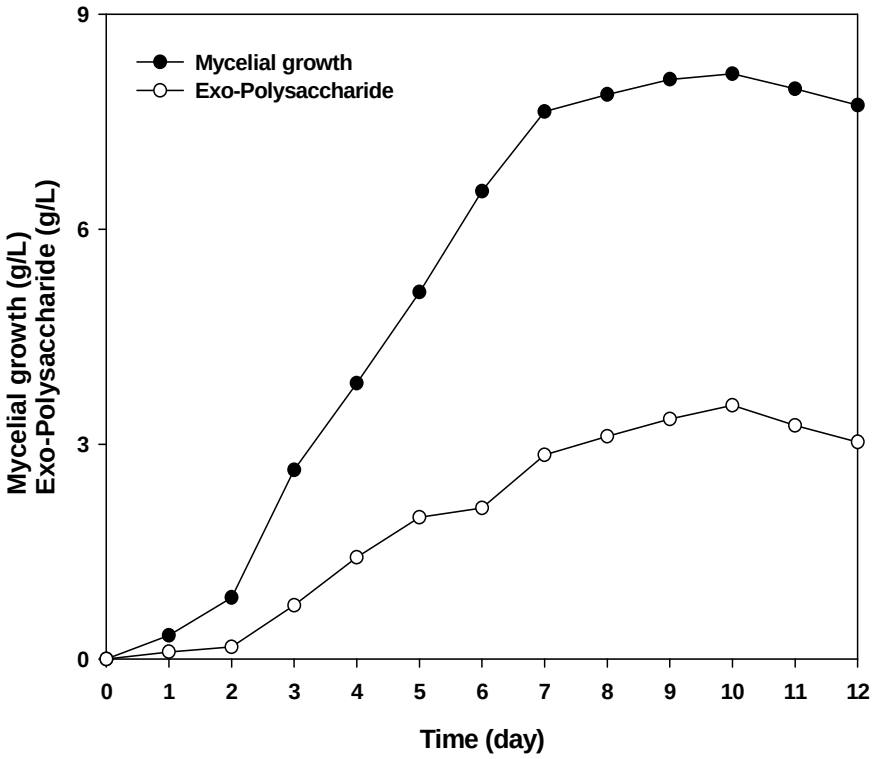

Fig. 20. Change of *Morchella esculenta* growth and exo-polysaccharide production with jar fermentation at 25℃, pH 6.5, 1.5 v.v.m. and 200 rpm

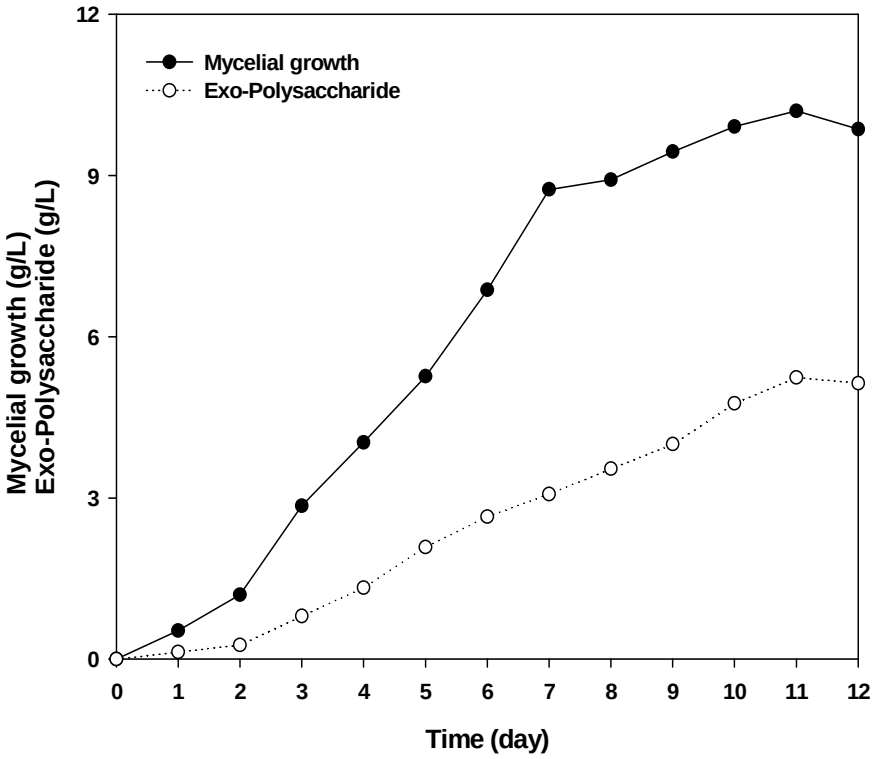

Fig. 21. Change of *Morchella esculenta* growth and exo-polysaccharide production with jar fermentation under optimal medium

IV-2 Anti-cancer and anti-oxidant activities of *Pleurotus nebrodensis* Inzenga extracts

IV-2-1 Cytotoxicity of *Pleurotus nebrodensis* Inzenga extracts on the human cancer cell lines

The effects of *Pleurotus nebrodensis* Inzenga (PN) extracts on the viability of human cancer cell lines *in vitro* system were demonstrated. When A549, HeLa and KM12C cancer cells were incubated with various concentrations of PN extracts which separated hot water, the viability of 3 cancer cell lines was measured by MTT assay and shown in Fig. 22.

Cervical cancer cells (HeLa) and colon cancer cells (KM12C) were slightly sensitive to PN hot water extracts, slight inhibition rate of HeLa and KM12C were 71% and 73%, respectively, at the treatment of 8 mg/mL. Also, when lung cancer cells (A549) were incubated lower than 4 mg/mL of PN hot water extracts, slight growth inhibition rate (less than 83%) was observed. However, treatment of 6 mg/mL of PN hot water extracts was significantly inhibited to the A549 cells and cell viable decreased to 38%.

When A549, HeLa and KM12C cells were incubated with 6 mg/mL ethanol extracts of PN, the viability of HeLa and KM12C cells were slightly decreased, while the growth of A549 cells was inhibited at concentrations over 4 mg/mL ethanol extracts of PN (Fig. 23).

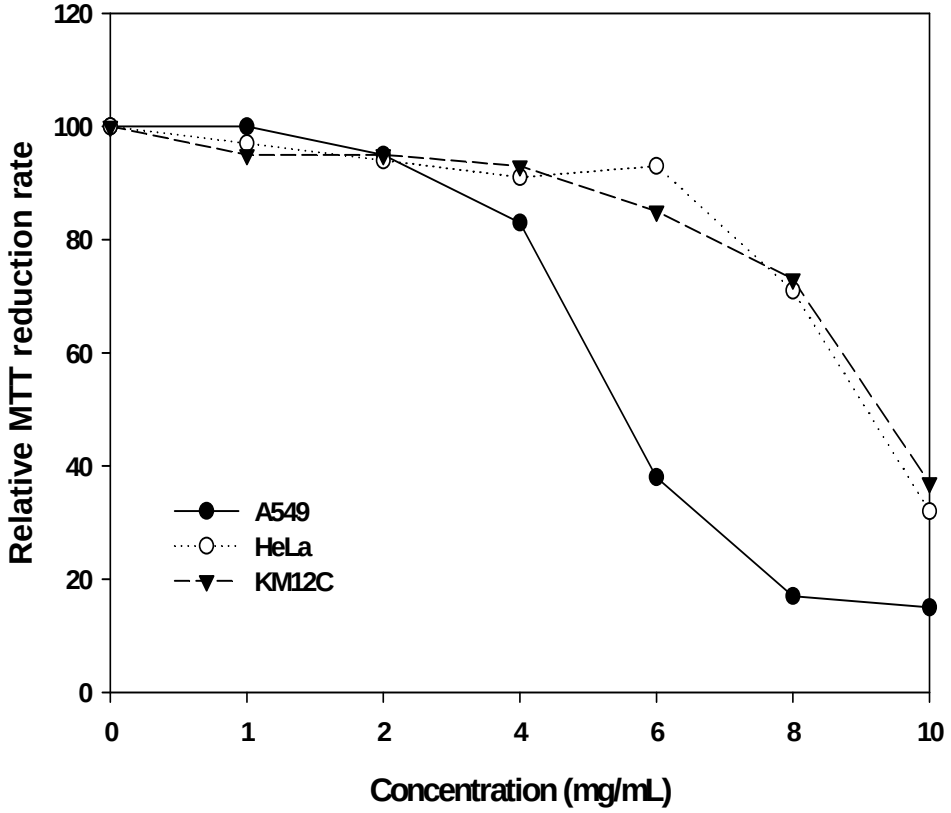

Fig. 22. The cytotoxic effects of water extracts from *Pleurotus nebrodensis* Inzenga on A549, HeLa and KM12C cancer cell

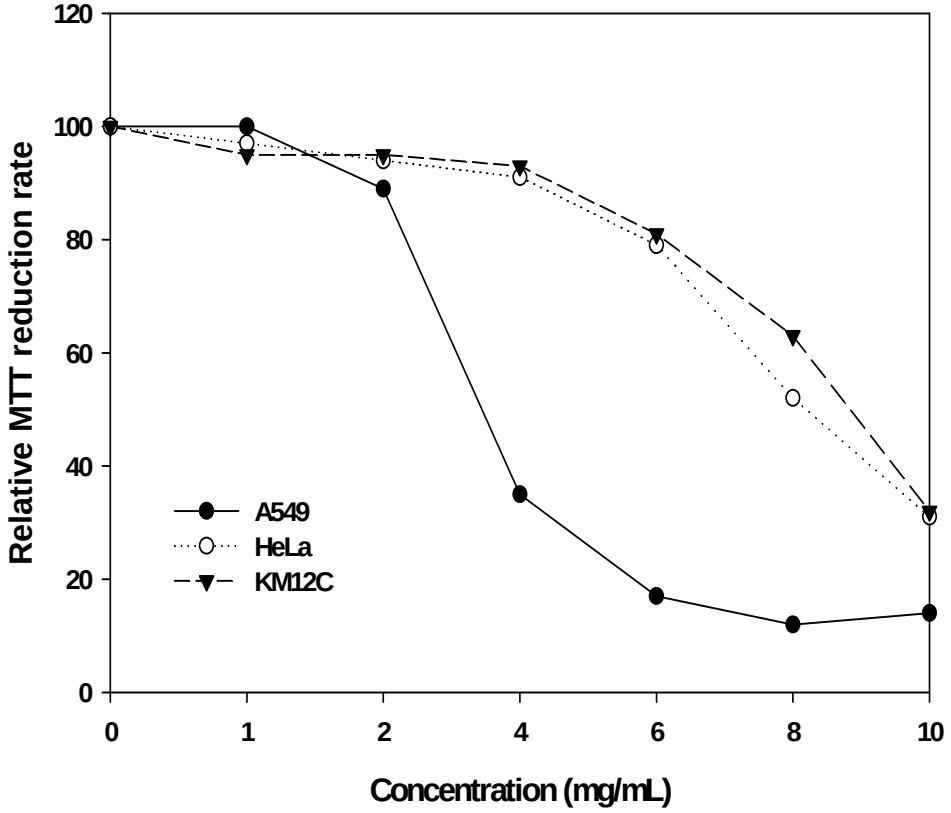

Fig. 23. The cytotoxic effects of ethanol extracts from *Pleurotus nebrodensis* Inzenga on A549, HeLa and KM12C cancer cell

The differences of cytotoxic effects of ethanol and hot water extracts of PN were significant. These results suggest that ethanol extracts of PN have cytotoxic substances, which activities are more potent then those of hot water extracts of PN and A549 cells were most sensitive to the cytotoxic effects of ethanol extract of PN.

Thus, cytotoxic substance containing in ethanol extracts of PN should be characterized and identified for the development of anti-cancer agents.

IV-2-2 Anti-oxidant activity of *Pleurotus nebrodensis* Inzenga

oxidation is essential to many living organics for the production of energy to fuel biological processes. However, oxygen-centored free radicals and other reactive oxygen species, that are continuously produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis [28].

Although almost all organisms possesses anti-oxidant supplements, or foods containing anti-oxidants, may be used to help the human body reduce oxidative damage [98]. Because the thiobarbituric acid (TBA) method is simple and sensitive, and it has a higher correlation with sensory evaluation results as compared with other methods, it is the most widely used method for measuring the development of lipid oxidation [70].

In this study, the potential anti-oxidant activity of *Pleurotus nebrodensis*

Inzenga (PN) has been assessed based on the TBA method.

Using the TBA method, the methanol extracts from PN showed low (0-14.6%) with various concentrations. In the care of water extracts, however, high anti-oxidant activity showed 37.43% at 40 μL (table 8 and Fig. 24). Obviously, the anti-oxidant activities of PN the two extracts with regard to the anti-oxidant activities of methanol and water extracts, the water extracts were better than methanol extracts. These anti-oxidant activities of PN would provide on of pharmacological backgrounds for its use in folk medicine.

Table 8. Anti-oxidant activity of hot water and methanol extract from *Pleurotus nebrodensis* Inzenga. The values are mean ± SD, n=3

	1	2	3	AVR	STDEV	MDA %
NA (supernatant of rat's brain)	0.053	0.036	0.035	0.041	0.01	
1. $FeCl_3$ (10 mM)	0.496	0.506	0.512	0.505	0.01	100
2. $FeCl_3$+BHA (50 ug/mL)	0.08	0.077	0.081	0.079	0.00	15.71
3. PM 10 μL	0.508	0.567	0.492	0.522	0.04	100
4. PM 20 μL	0.412	0.482	0.491	0.462	0.04	91.42
5. PM 40 μL	0.461	0.43	0.403	0.431	0.03	85.41
6. PW 10 μL	0.423	0.364	0.378	0.388	0.03	76.90
7. PW 20 μL	0.353	0.338	0.36	0.350	0.01	69.37
8. PW 40 μL	0.316	0.323	0.309	0.316	0.01	62.57

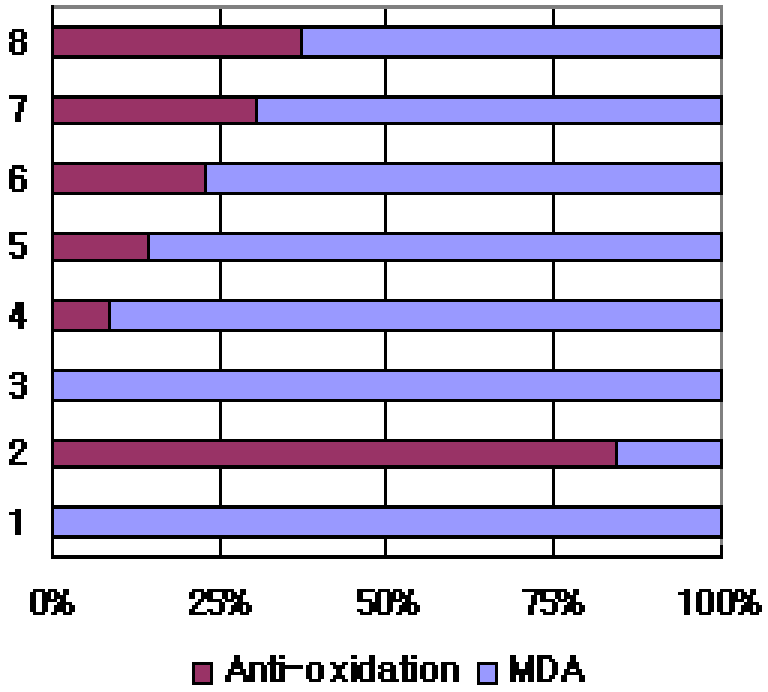

Fig. 24. Anti-oxidant activity of hot water and methanol extract from *Pleurotus nebrodensis* Inzenga

CHAPTER V

Conclusion

Morchella esculenta, a Ascomycotina fungus belonging to the genus Morchellaceae and the family Morchella, is low in calories, sodium, fat and cholesterol, while rich in protein, carbohydrate, fiber, vitamins and minerals. These nutritional properties make more a attractive food stuff than *Agaricus bisporous* in the many countries. *Pleurotus nebrodensis* Inzenga is shaped like the traditional Chinese medicine, *Ganoderma*, but its color white, and more importantly it is rich in nutrients. Especially, the content of polysaccharide in *Pleurotus nebrodensis* Inzenga reaches 19% and has the ability to strengthen the immune system. Although there have been many reports on different *Pleurotus* genus and *Morchella esculenta* in agricultural and genetic areas, there are no reports on cultivation and biological activities of *Pleurotus ferulae* and *Morchella esculenta* in Korea.

Therefore, the objectives of this work are to determine the optimal culture conditions in liquid-state fermentation for production of *Morchella esculenta*, and to investigate the effects of *Pleurotus nebrodensis* Inzenga various extracts on viability of human cancer cell lines and anti-oxidative activity for screening the substances contained in *Pleurotus nebrodensis* Inzenga.

Optimal culture conditions for the mycelial growth and exo-polysaccharide production of *Morchella esculenta* in flask cultures were determined. The optimal temperature and pH of *Morchella esculenta* were 25℃ and 6.5, respectively. Among various media tested, the best medium for both mycelial growth and exo-polysaccharide production was BG medium. The flask with 50 mL of BG medium at 200 rpm showed the highest mycelial growth and exo-polysaccharide production. Under optimal culture conditions, the maximum mycelial concentration and exo-polysaccharide production was 3.98 g/L and 1.10 g/L, respectively, after 9 days in a flask culture. The best carbon source was fructose with 5% (w/v) for mycelial and exo-polysaccharide production of *Morchella esculenta*. Among 6 different nitrogen sources were added to the medium with various combinations, the mycelial growth and exo-polysaccharide production were very good with peptone plus NH_4Cl, and the optimum concentration of peptone plus NH_4Cl [ratio (w/v) of 1:1] was 4.0% (w/v). Also, the optimum mineral sources of *Morhcella esculenta* were K_2HPO_4(1.5%, w/v) and $MgSO_4 \cdot 7H_2O$(1.0%, w/v). In conclusion, the optimal medium compositions for *Morchella esculenta* were % of fructose, 4% of peptone plus NH_4Cl [ratio (w/v) of 1:1], 1% of K_2HPO_4 and 1.5% of $MgSO_4 \cdot 7H_2O$(w/v). Jar fermentations were carried out to optimize the culture conditions for mycelial growth and exo-polysaccharide production of *Morchella esculenta.* The agitation speed was controlled from 100 to 300 rpm and it significantly affected mycelial growth and exo-polysaccharide production. It was due to higher shear stress induced by the impeller agitation, which had an negative effects on growth of mycelia. Maximum mycelial and exo-polysaccharide

concentrations were achieved at the agitation speed of 200 rpm, and they were 7.36 g/L and 2.92 g/L, respectively. In the case of aeration, maximum mycelial growth and exo-polysaccharide production wee achieved at the 1.5 v.v.m. of aeration rate and they were 7.54 g/L and 3.11 g/L, respectively. The increase in aeration rate results in the increase in oxygen transfer rate, which lead to an increase in mycelial growth and exo-polysaccharide production. Under optimal culture conditions, the maximum mycelial growth and exo-polysaccharide production in a 5 L jar fermenter with a basal medium were obtained after 10 days and they were 8.17 g/L and 3.54 g/L, respectively. By using optimally designed medium, the highest mycelial growth and exo-polysaccharide concentrations were the higher after 11 days fermentation and they were 10.2 g/L and 5.24 g/L, respectively. The yields of mycelial growth and exo-polysaccharide production of jar fermentation with optimal medium were increased 20% and 32%, respectively, than those of basal medium. The exo-polysaccharide formation is associated with mycelial growth of *Morchella esculenta*.

The effects of *Pleurotus nebrodensis* Inzenga (PN) extracts on the viability of three human cancer cell lines *in vitro* system were demonstrated. Cervical cancer cells (HeLa) and colon cancer cells (KM12C) were slightly sensitive to PN hot water extracts, slight inhibition rate of HeLa and KM12C were 71% and 73%, respectively, at the treatment of 8 mg/mL. Also, when lung cancer cells (A549) were incubated lower than 4 mg/mL of PN hot water extracts, slight growth inhibition rate (less than 83%) was observed. However, treatment of 6 mg/mL of

PN hot water extracts was significantly inhibited to the A549 cells and cell viable decreased to 38%. When A549, HeLa and KM12C cells were incubated with 6 mg/mL ethanol extracts of PN, the viability of HeLa and KM12C cells were slightly decreased, while the growth of A549 cells was inhibited at concentrations over 4 mg/mL ethanol extracts of PN These results suggest that ethanol extracts of PN have cytotoxic substances, which activities are more potent then those of hot water extracts of PN and A549 cells were most sensitive to the cytotoxic effects of ethanol extract of PN. Thus, cytotoxic substance containing in ethanol extracts of PN should be characterized and identified for the development of anti-cancer agents.

In this study, the potential anti-oxidant activity of *Pleurotus nebrodensis* Inzenga (PN) has been assessed based on the TBA method. Using the TBA method, the methanol extracts from PN showed low (0-14.6%) with various concentrations. In the case of hot water extracts, however, high anti-oxidant activity showed 37.43% at 40 μL. The anti-oxidant activities of PN the two extracts with regard to the anti-oxidant activities of methanol and hot water extracts, the hot water extracts were better than methanol extracts. These anti-oxidant activities of PN would provide one of pharmacological backgrounds for its use in folk medicine.

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