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Studies on cultivation and biological activities of *Pleurotus nebrodensis* inzenga

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Abstract–Environmental factors affecting mycelial growth and exo-polysaccharide production from *Pleurotus nebrodensis* Inzenga (PN) and biological activities of PN extracts *in vitro* were studied. The culture conditions for effective mycelial growth and exo-polysaccharide production were found to be 25 °C, 5% of inoculum size, and an initial pH from 6.5 to 7.0. When 5% of glucose was used, the maximum mycelial growth and exo-polysaccharide concentrations were 8.3 and 3.07 g/L, respectively. Among the various nitrogen sources, the mycelial growth and exo-polysaccharide production were very strong when polypeptone was used. In the case of the minerals sources, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ were found to best support for mycelial growth and exo-polysaccharide production. Under optimal conditions and methods, the maximum mycelial growth and exo-polysaccharide production were obtained after 10 days of culture, 12.84 and 4.85 g/L, respectively, in a jar fermentor. The effects of the PN extracts on the viability of three human cancer cell lines and antioxidant activity were investigated *in vitro*. When cancer cells of the lung (A549), cervical region (HeLa) and colon (KM12C) were incubated at 6 mg/mL of the PN ethanol extracts, the viabilities of the HeLa and KM12C cells were slightly decreased. However, the growth of the A549 cells was remarkably inhibited when the PN ethanol extract was over 4 mg/mL. The antioxidant activity showed 46.2% at 40 μL, which was about 3.2 fold higher than that of the PN methanol extract.

Key words: *Pleurotus nebrodensis* Inzenga, Exo-polysaccharide, Anti-tumor, Antioxidant

INTRODUCTION

Mushrooms have long been gaining a great deal of interest in many areas of nutritional supplements and biopharmaceuticals. Particularly, *Pleurotus* sp. have been traditionally used as a health food source for the prevention of diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis etc. [Noda, 1998; Paulik et al., 1996; Bobek et al., 2001; Opletal et al., 1997; Oguri et al., 1996]. *Pleurotus* belongs to the Basidiomycetes fungus. *Pleurotus nebrodensis* Inzenga is a *Pleurotaceae. Pleurotus nebrodensis* Inzenga originated from the Gobi Desert in the Xinjiang Autonomous Region (China), which makes it more suitable for arid climates. This mushroom is called Western Paradise White *Garnoderma* (in Chinese). White in color and rich in nutrients, it is shaped like the traditional Chinese medicine, *Ganoderma*. A unique feature of the white *Ganoderma* mushroom is that it has a thick structure with thick layers. According to the examination report of the Chinese Food Examination Center, white *Ganoderma* contains 14.7% of protein and the content of vitamin C, D and E is several times higher than in other types of mushrooms [Huang, 1998]. Recently, the mycelial growth and exo-polysaccharide production by various fungal strains have been extensively researched for potential applications using environmental parameters as well as the medium composition. Although many researchers have attempted to obtain the optimal culture conditions for mycelial growth and exo-polysaccharide production using different fungal strains, the nutritional requirements and factors of the environment for the liquid cultures for the mycelial growth and exo-polysaccharide production from the liquid culture by *Pleurotus nebrodensis* Inzenga have not been demonstrated [Kim et al., 1994; Jung et al., 1997; Hashimoto and Takahashi, 1974; Chi et al., 1996; Park et al., 1994].

We previously investigated the possibility of artificial production of *Pleurotu ferulae* fruiting bodies in solid-state culture. Especially, when various ratios of garlic powder were used, yields of fruiting bodies were drastically higher than those of synthetic mixture without garlic powder in sawdust culture [Cha et al., 2004]. We also studied batch cultures for effective exo-polysaccharide production from liquid cultures of *Pleurotus ferulae* in a jar fermentor. Additionally, the logistic model to describe the mycelial growth and Leudecking-Piret model for exo-polysaccharide production were proposed. The model profile showed good agreement with the experimental results for mycelial growth and exo-polysaccharide production. The specific production rate using the modified media is higher than that of basal media [Choi et al., 2005].

In this paper, for efficient mycelial growth and exo-polysaccharide production from a liquid culture by *Pleurotus nebrodensis* Inzenga using a jar fermentor, first, the factors of the environment such as temperature, inoculum size, initial pH, carbon and nitrogen sources, and minerals sources were investigated in a flask. Second, using the optimum results, a scale-up was performed in a jar fermentor. Additionally, the anti-tumor and antioxidant effects of the *Pleurotus nebrodensis* extract were also investigated *in vitro*.

MATERIALS AND METHODS

1. Strain and Cultures

Pleurotus nebrodensis Inzenga was obtained from the culture ground of Kaya-Backsong (Chungnam, Korea). Cultures were maintained on Potato dextrose agar (PDA) plates. Plates were incubated

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at 25 °C for 7 days, and then stored at 4 °C. *Pleurotus nebrodensis* Inzenga was initially grown on a PDA medium in a petri dish, and then transferred into the seed medium containing malt extract 10 g/*l*, yeast extract 4 g/*l*, and glucose 4 g/*l* by punching out from the agar plate culture with a sterilized cork borer. The seeds were grown in a 300 ml flask containing 100 ml of the seed medium at 25 °C on a rotary shaker at 100 rpm for 7-8 days, and then homogenized at 10,000 rpm for 30 sec. Flask cultures for exo-polysaccharide production were performed in 300 mL flasks containing 50 ml of the basal medium on a rotary shaker (Model HB-201SL) under specific conditions. All the media were sterilized at 121 °C for 15 min. The pH was controlled to the desired value by the addition of either 1 N HCl or NaOH. The culture medium was inoculated with 5% (v/v) of the mycelial homogenate, and then cultivated at 25 °C in a 5-L jar fermentor (Korea Fermentor Co., Korea) under specific conditions. All the experiments were performed in triplicate to ensure replication.

2. Analysis

The mycelial growth was obtained by centrifuging samples at 3,000 rpm for 15 min, washing the sediment three times with distilled water, and then drying to a constant weight. All supernatants were collected, and then the crude exo-polysaccharide was precipitated with the addition of four volumes of 95% ethanol. The precipitated exo-polysaccharide was collected by centrifugation at 3,000 rpm for 10 min, and then dried to remove the residual ethanol at 60 °C. The residual glucose concentration was determined by the di-nitrosalicylic acid method.

3. Materials

Pleurotus nebrodensis Inzenga fruiting bodies (PNF) were obtained from solid culture by using a YMGA medium containing malt extract 10 g/L, glucose 4 g/L, yeast extract 4 g/L and agar 20 g/L. The PNF was washed four times with distilled water, and dried with a drying oven at 60 °C for one day, and then powdered using Wiley Mill having a particle size of 300 mesh.

4. Extract Preparation of *Pleurotus nebrodensis* Inzenga

About 500 g of dry powdered PNF was extracted with 1,000 mL of 95% ethanol or methanol using a 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 the 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 1,000 mL of hot distilled water with a soxhlet apparatus at 100 °C for 2-3 h. After standing overnight at 4 °C, the solvent was centrifuged and the supernatants were evaporated and lyophilized, and the solid mass with a yield of 12-13% was used as hot water extracts of *Pleurotus nebrodensis* Inzenga.

5. Cancer Cells and Culture

Lines of human cancer cells from the lung (A549), cervical region (HeLa) and colon (KM12C) were obtained from the 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 °C in a humidified atmosphere with 5% CO_2 and were subcultured twice a week. Cells were incubated in a CO_2 incubator at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for varying periods with or without *Pleurotus nebrodensis* Inzenga extracts and different concentrations of *Pleurotus nebrodensis* Inzenga extracts.

6. Anti-tumor Activity

Anti-tumor activity was determined by the viability of the cells using the MTT assay. The MTT assay is based on the optical measurement of a dye formazan from the MTT by mitochondria dehydrogenase. Human cancer cells were cultured on RPMI-1640 containing 10% FBS with 2×10^4 cells per well added to 96-well microtiter plates. After the addition of various concentrations of *P. ferulae* extracts into each well, the 96-well plate was maintained in a CO_2 incubator (37 °C) for 2 days. After the cultivation was complete, the RPMI-1640 was removed, and 50 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and 200 μl of fresh RPMI-1640 were added to the 96-cell plate. Again, the plate was maintained in a CO_2 incubator for 4 hr to allow for formazan formation. The quantity of formazan produced can be regarded as an indicator of the cell density or viability. After the formazan was dissolved in 150 μl of DMSO (dimethyl sulfoxide), the absorbance at 540 nm was measured with a Microplate Autoreader (Labsystem Multiscan Multisoft, Finland). The results gathered were presented as a percentage of the control values. The control values were determined from cultures with cancer cells grown in the medium without any *Pleurotus nebrodensis* Inzenga extracts and were considered as 100%.

7. Antioxidant Activity

Antioxidant activity was measured with TBA (thiobarbituric acid). Rats (300 g) were anaesthetized with ether. Their brains were rapidly excised and placed immediately in ice-cold saline to wash them free of blood. The brain matter was then homogenized in a 0.1 M Tris-HCl buffer (pH 7.4) using a homogenizer. After homogenization, the lipids were 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 °C. 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 °C. The solution was centrifuged at 12,000 rpm for 10 minutes. The TBA value was determined by reading the absorbance at 532 nm. The inhibition activity was determined against the TBA value of the control when only $FeCl_3$ was used.

RESULTS AND DISCUSSION

1. Effect of Temperature on Mycelial Growth

The effect of temperature on mycelial growth of *Pleurotus nebrodensis* Inzenga was investigated on a petri dish containing a solid medium (YMGA) for 10 days. The culture temperature was controlled at 20, 25, and 30 °C. The results are shown in Fig. 1. The optimal temperature for mycelial growth was discovered to be 25 °C. A similar phenomenon was also observed in *Pleurotus eryngii* [Zadrazil, 1974], *Lentinus lepideus*, and *Naematoloma Sublateritiu* [Kim et al., 1994]. But in the case of *Pleurotus ostreatus*, *Pleurotus fiorida* [Zadrazil, 1974], *Phellinus igniarius* [Jung et al., 1997], and *Ganoderma lucidum* [Hong et al., 1986], the optimal temperature was 30 °C.

2. Effect of Inoculum Sizes on Mycelial Growth and Exo-poly-

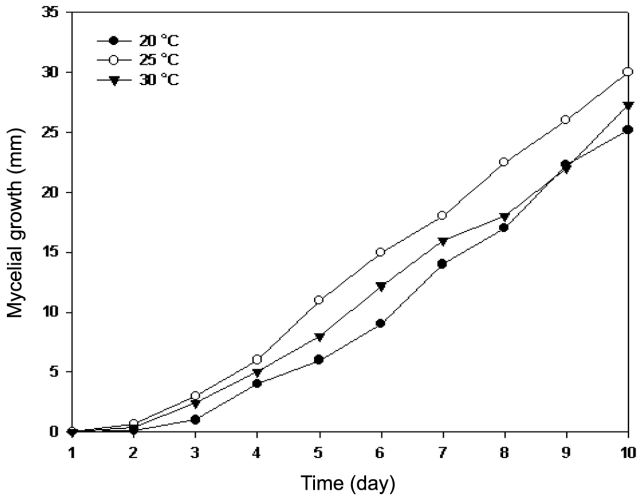

Fig. 1. Effects of temperature on the mycelial growth of *Pleurotus nebrodensis* Inzenga on YMGA medium.

Table 1. Effects of inoculum volume on mycelial growth and exo-polysaccharide production

Inoculum size (%)	Mycelial growth (g/L)	Exo-polysaccharide (g/L)
1	2.87	1.01
3	4.04	1.07
5	7.52	1.37
7	7.01	1.08
10	6.05	0.84

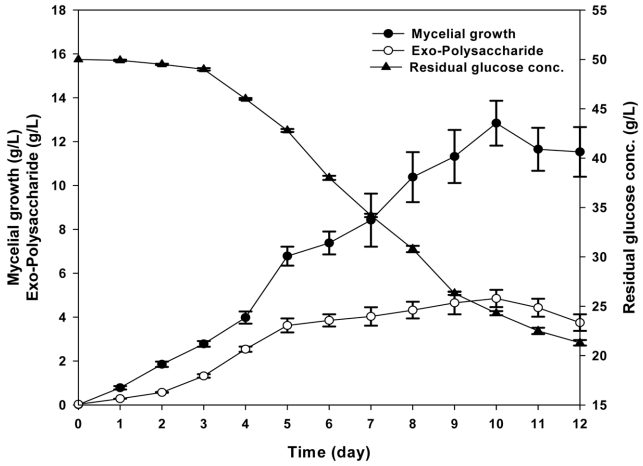

Fig. 2. Typical time course of mycelial growth and exo-polysaccharide production in a jar fermentor.

saccharide production

In order to determine the optimal inoculum size for mycelial growth and exo-polysaccharide production, *Pleurotus nebrodensis* Inzenga was cultured with various inoculum sizes in a flask at 25 °C for seven days. Five inoculum sizes were tested: 1, 3, 5, 7, and 10%. The results are shown in Fig. 2. The mycelial growth and exo-polysaccharide production were affected by the inoculum sizes. Especially, when 5% of the inoculum size was used, the maximum mycelial growth and exo-polysaccharide production were obtained, 7.52 and 1.37 g/L, respectively. However, in the case of *Agaricus blazei*, the optimal inoculum size was 10-20% [Kim et al., 2004]. Therefore, 5% of the inoculum size was used for the subsequent experiments.

Table 2. Effects of initial pH on mycelial growth and exo-polysaccharide production

pH	Mycelial growth (g/L)	Exo-polysaccharide (g/L)
4.0	1.84	0.59
4.5	2.21	0.60
5.0	3.41	0.76
5.5	4.75	0.86
6.0	5.58	1.79
6.5	6.35	2.07
7.0	5.95	2.09
7.5	5.55	1.85
8.0	5.10	1.58

3. Effect of Initial pH on Mycelial Growth and Exo-polysaccharide Production

To investigate the effects of initial pH on mycelial growth and exo-polysaccharide production, *Pleurotus nebrodensis* Inzenga was cultured in a basal medium with various initial pHs (4.0-8.0) in flasks. The results are shown in Table 2. When the initial pH ranged from 4.0 to 5.5, or above 7.5, the mycelial growth and exo-polysaccharide production were decreased. However, the optimal initial pH for mycelial growth and exo-polysaccharide production ranged from 6.5 to 7.0. At the initial pH 6.5-7.0, the mycelial growth and exo-polysaccharide concentrations were 5.95-6.35 and 2.07-2.09 g/L, respectively. These results were similar to the results obtained by *Ganoderma lucidum* when fatty acids were used as the carbon source [Yang et al., 2000]. On the other hand, the optimum pH for mycelial growth and thioproline production in sawdust-based cultures of *Lentinus edodes* [Ohga, 1999] and for polysaccharide production by *Ganoderma lucidum* [Yang and Liau, 1998], *Lentinus lepideus* [Kim et al., 1994], *and Poria cocos* [Hong and Lee, 1990] was 4.0 and for exo-biopolymer production by *Paecilomyces japonica*, 5.0 [Bae et al., 2000].

4. Effect of Various Carbon Sources on Mycelial Growth and Exo-polysaccharide Production

Table 3. Effects of various carbon sources on mycelial growth and exo-polysaccharide production

Carbon sources	Mycelial growth (g/L)	Exo-polysaccharide (g/L)
Control (none)	2.89	0.77
Glucose	6.48	2.64
Mannose	4.59	1.20
Galactose	2.86	0.59
Fructose	5.68	1.39
Arabinose	2.25	0.64
Xylose	3.54	0.72
Maltose	5.27	1.29
Lactose	3.01	0.60
Sucrose	3.05	0.53
Manitol	3.62	0.56

For selecting the best carbon source for mycelial growth and exo-polysaccharide production, glucose, mannose, galactose, fructose, arabinose, xylose, maltose, lactose, sucrose, and manitol were investigated. Each carbon source (2%) was added to the basal medium and tested in flasks. The results are shown in Table 3. When glucose, fructose, or maltose was used, the mycelial growth and exo-polysaccharide production were favorable. In particular, when glucose was used, the maximum mycelial growth and exo-polysaccharide concentrations were obtained, 6.48 and 2.64 g/L, respectively. However, the mycelial growth and exo-polysaccharide production were poor when galactose or arabinose was used. The pattern of exo-polysaccharide production was consistent with the mycelial growth of *Pleurotus nebrodensis Inzenga*. The results showed that the strong mycelial growth was closely related to the polysaccharide production.

In determining the optimal concentration of glucose for the mycelial growth and exo-polysaccharide production, a range of 1 to 10% was investigated. When 5% glucose was used, the maximum mycelial growth and exo-polysaccharide concentration were reached, 8.3 and 3.07 g/L, respectively (data not shown). When the glucose concentration was lower or higher than 5%, the mycelial growth and exo-polysaccharide production were decreased (data not shown). Therefore, it was concluded that the optimum glucose concentration for both mycelial growth and exo-polysaccharide production was 5%.

5. Effects of Various Nitrogen Sources on Mycelial Growth and Exo-polysaccharide Production

To investigate the effects of nitrogen sources on the mycelial growth and exo-polysaccharide production, batch cultures were performed in flasks containing the basal medium with 0.6% of various nitrogen sources and 5% glucose. The results are shown in Table 4. Among the 12 different nitrogen sources, the mycelial growth and exo-polysaccharide production were very strong when polypeptone was used. However, peptone, malt extract, and tryptone as organic nitrogen sources were not found to be suitable for mycelial growth and exo-polysaccharide production. Especially, with the increase in the polypeptone concentration up to 1.0%, the mycelial growth and exo-polysaccharide production were increased. Above 1% polypeptone, there was a slight decrease (data not shown). Therefore, the optimum concentration of polypeptone was 1.0%.

Table 4. Effects of nitrogen sources on mycelial growth and exo-polysaccharide production

Nitrogen sources	Mycelial growth (g/L)	Exo-polysaccharide (g/L)
Control (none)	1.14	0.49
Ca $(NO_3)_2$	2.20	0.81
$NaNO_3$	0.01	N.D.
$(NH_4)_2SO_4$	0.75	N.D.
NH_4NO_3	0.71	N.D.
$NH_4H_2PO_4$	3.68	1.07
$(NH_4)_2HPO4$	3.01	1.02
KNO_3	1.09	0.36
Malt extract	2.22	1.44
Peptone	3.04	1.59
Tryptone	4.65	1.79
Yeast extract	6.19	2.45
Polypeptone	6.84	3.55

Table 5. Effects of mineral sources on growth and exo-polysaccharide production

Mineral sources	Mycelial growth (g/L)	Exo-polysaccharide (g/L)
Control (none)	7.15	1.90
$MgSO_4$	7.84	2.51
$CaCl_2 \cdot 2H_2O$	8.30	0.54
$ZnSO_4 \cdot 7H_2O$	0.16	N.D.
$MgCl_2$	8.57	1.48
$MgSO_4 \cdot 7H_2O$	8.69	3.34
Na_2HPO_4	8.32	1.27
$FeSO_4 \cdot 7H_2O$	8.64	1.02
K_2HPO_4	9.92	3.75

6. Effects of Mineral Sources on Mycelial Growth and Polysaccharide Production

The influence of mineral sources on mycelial growth and exo-polysaccharide production in a medium containing 5% of glucose and 1.0% of polypeptone was investigated in flasks. The mineral source was added to the medium at a concentration of 0.15%. The results are shown in Table 5. K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ were found to be the optimal mineral sources for mycelial growth. These mineral ions are also recognized as a favorable bioelement for mycelial growth and exo-polysaccharide production. The best results for the mycelial growth and exo-polysaccharide production in the medium containing K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ was also similar to the effects of various inorganic salts on enzyme production by *P. ferulae* (data not shown). Phosphate has been used as a buffering reagent. Further, potassium is an important mineral for/involving cell structure. However, the magnesium cation can stimulate biosynthesis of the fungal cell wall and affect its permeability. When $MgCl_2$, $CaCl_2 \cdot 2H_2O$, KH_2PO_2, $MgSO_4$, Na_2HPO_2 or $FeSO_4 \cdot 7H_2O$ was used, the mycelial growth was similar to that of K_2HPO_4 or $MgSO_4 \cdot 7H_2O$, but the exo-polysaccharide production was significantly decreased. However, in the case of $ZnSO_4 \cdot 7H_2O$, the mycelial growth and exo-polysaccharide production were very poor.

7. Fermentation under Optimal Culture Conditions

The optimal culture conditions in a jar fermentor were as follows: temperature 25 °C, agitation speed 150 rpm, and aeration rate 0.5 vvm. The pH of the fermentor was controlled at 6.5. Based on optimal conditions and the optimized medium, a batch culture in a jar fermentor was performed for 12 days. Fig. 2 shows the change of *Pleurotus nebrodensis* Inzenga mycelial growth, exo-polysaccharide production, and residual glucose concentrations. The mycelial growth and the exo-polysaccharide production were increased with the increase of culture time up to 10 days. However, the decrease began after 11 days of culture. The maximum mycelial growth and exo-polysaccharide production were obtained after 10 days, 12.84 and 4.85 g/L, respectively. The glucose consumption was rapidly increased with the increase of culture time after three days. The residual glucose concentration was 21.3 g/L after 12 days of culture.

8. Anti-tumor Effect of *Pleurotus nebrodensis* Inzenga Extract

The effects of *Pleurotus nebrodensis* Inzenga (PN) extracts on the viability of human cancer cell lines *in vitro* were demonstrated. When cancer cells of the lung (A549), cervical region (HeLa) and

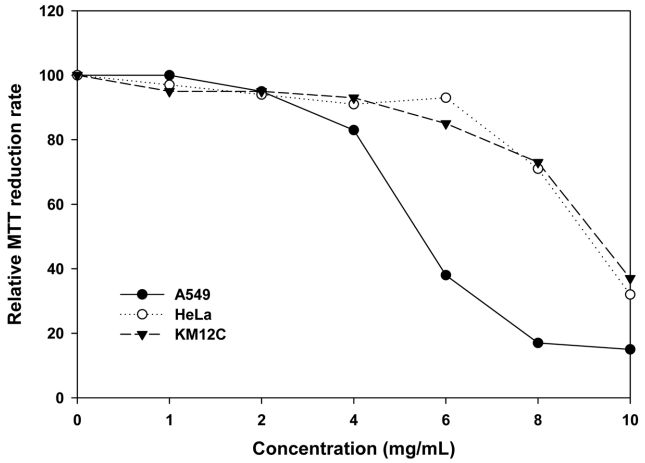

Fig. 3. The cytotoxic effects of hot water extracts from *Pleurotus nebrodensis* Inzenga on A549, HeLa and KM12C cancer cell.

colon (KM12C) were incubated with various concentrations of PN extracts, which separated hot water, the viability of the three cancer cell lines was measured by an MTT assay. The results are shown in Fig. 3. The HeLa and KM12C cells were slightly sensitive to the PN hot water extracts, the inhibition rate of HeLa and KM12C was 71 and 73%, respectively, at 8 mg/mL of PN extracts. Also, when the A549 cells were incubated at concentrations lower than 4 mg/mL of the PN hot water extracts, a slight growth inhibition rate (less than 83%) was observed. However, 6 mg/mL of the PN hot water extracts significantly inhibited to the A549 cells, and cell viability decreased to 38%.

When A549, HeLa and KM12C cells were incubated with 6 mg/mL of the PN ethanol extracts, the viability of HeLa and KM12C cells was slightly decreased, while the growth of A549 cells was inhibited at concentrations over 4 mg/mL of the PN ethanol extracts as shown in Fig. 4. The differences of cytotoxic effects of the PN ethanol and hot water extracts were significant. These results suggested that the PN ethanol extracts have cytotoxic substances, the activities of which are more potent than those of the PN hot water extracts, and the A549 cells were the most sensitive to the cytotoxic effects of the PN ethanol extracts. Thus, the cytotoxic substance containing the PN ethanol extracts should be characterized and identified for the development of anticancer agents.

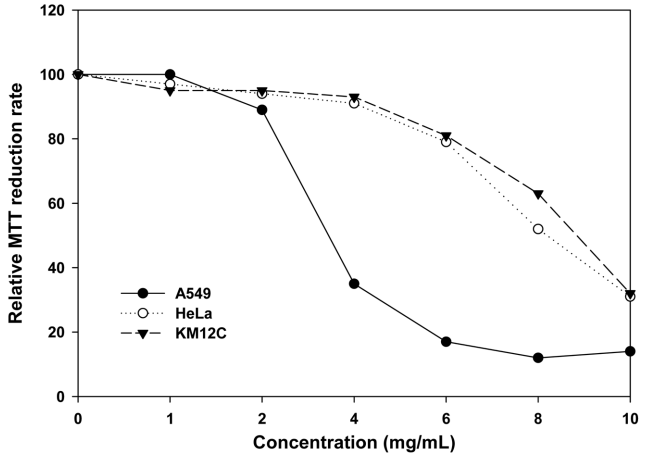

Fig. 4. The cytotoxic effects of ethanol extracts from *Pleurotus nebrodensis* Inzenga on A549, HeLa and KM12C cancer cell.

Table 6. Antioxidant activity of hot water and methanol extract from *Pleurotus nebrodensis* Inzenga

Substances	Antioxidant activity (%)
$FeCl_3$ (10 mM)	0
$FeCl_3$+BHA (40 ug/mL)	38.1
PN methanol extract (10 μL)	0
PN methanol extract (20 μL)	8.7
PN methanol extract (40 μL)	14.6
PN hot water extract (10 μL)	23.1
PN hot water extract (20 μL)	30.6
PN hot water extract (40 μL)	46.2

9. Antioxidant Effect of *Pleurotus nebrodensis* Inzenga Extract

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals and other reactive oxygen species, which 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 arteriosclerosis, diabetes, cancer and cirrhosis [Halliwell and Gutteridge, 1984]. Table 6 illustrates the potential antioxidant activity of *Pleurotus nebrodensis* Inzenga based on the TBA method. When the methanol extracts were used, the antioxidant activity of PN showed low activity (0-14.6%) with various concentrations. On the other hand, in the case of the hot water extracts, the maximum antioxidant activity showed 46.2% at 40 μL of PN hot water extracts, which was about 3.2 fold higher than that of the PN methanol extract at the same concentration.

CONCLUSIONS

To develop healthy, functional food materials using mushrooms, we investigated the factors affecting mycelial growth and exo-polysaccharide production during the culture of *Pleurotus nebrodensis* Inzenga in a flask and a jar fermentor, and also evaluated the effects of *Pleurotus nebrodensis* Inzenga extracts on viability of three human cancer cell lines and antioxidant activities *in vitro*. The optimal temperature for mycelial growth was found to be 25 °C. The mycelial growth and exo-polysaccharide production were affected by the inoculum sizes. Especially, when 5% of inoculum size was used, the maximum mycelial growth and exo-polysaccharide production were obtained. The optimal initial pH for mycelial growth and exo-polysaccharide production ranged from 6.5 to 7.0. When glucose, fructose, or maltose was used, the mycelial growth and exo-polysaccharide production were favorable. In particular, when 5% of glucose was used, the maximum mycelial growth and exo-polysaccharide concentrations were obtained, 8.3 and 3.07 g/L, respectively. These results indicated that a carbon source could be used to improve the exo-polysaccharide production in *Pleurotus nebrodensis Inzenga*. The nitrogen and carbon sources are two important factors affecting cell growth and exo-polysaccharide production. Among the 12 different nitrogen sources, polypeptone was found to be the best nitrogen source. These results suggested that polypeptone contains the necessary components for mycelial growth and exo-polysaccharide production. In the case of mineral sources, K_2HPO_4 and

$MgSO_4 \cdot 7H_2O$ were found to be superior for mycelial growth and exo-polysaccharide production.

Under optimal culture conditions and medium, a batch culture was performed in a jar fermentor. When the agitation speeds were controlled from 50 to 300 rpm, the mycelial growth and exo-polysaccharide production were significantly affected. Especially, when the agitation speed was increased above 150 rpm, the mycelial growth and exo-polysaccharide production decreased (data not shown). It was due to the high shear stress induced by the impeller agitation. The mycelial growth and the exo-polysaccharide production were increased with the increase of culture time up to 10 days. However, the decrease began after 11 days of culture. The maximum mycelial growth and exo-polysaccharide production were obtained after 10 days of culture, 12.84 and 4.85 g/L, respectively. These results indicated that exo-polysaccharide production increased in parallel with the mycelial growth and that the product formation was associated with mycelial growth.

The effects of *Pleurotus nebrodensis* Inzenga (PN) extracts using hot water and methanol on the viability of three human cancer cell lines *in vitro* were demonstrated. The PN ethanol extracts have cytotoxic substances, the activities of which are more potent than those of the PN hot water extracts, and A549 cells were most sensitive to the cytotoxic effects of the PN ethanol extract. Thus, a cytotoxic substance containing the PN ethanol extracts should be characterized and identified for the development of anticancer agents. The oxidant activities of the PN methanol and hot water extract were increased with the increase of the extract concentrations. Especially, when 40 μL of PN hot water extract was used, the maximum antioxidant activity was obtained, 46.2%, which was similar to that of BHA or about 3.2 fold higher than that of the PN methanol extract at same concentration. These antioxidant activities of PN would provide a pharmacological background for its use in folk medicine.

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