Korean J. Chem. Eng., **26**(4), 1075-1083 (2009)
DOI: 10.1007/s11814-009-0179-6

Production of polysaccharides by submerged mycelial culture of entomopathogenic fungus *Cordyceps takaomontana* and their apoptotic effects on human neuroblastoma cells

Sung Hak Lee, Hee Sun Hwang, and Jong Won Yun†

Department of Biotechnology, Daegu University, Gyeongsan, Gyeongbuk 712-714, Korea
(*Received 13 December 2008 • accepted 29 January 2009*)

Abstract−The effect of medium components and environmental factors on the production of mycelial biomass for the preparation of intracellular polysaccharides (IPS) and exopolysaccharides (EPS) by *Cordyceps takaomontan* was investigated in submerged cultures. The optimal culture condition was as follows (g/*l*): glucose 30, yeast extract 4, KH_2PO_4 0.46, K_2HPO_4 1, and $MgSO_4$ 0.5, and 28 °C and pH 8. When the fungus was cultivated under various agitation and aeration conditions in a 5-*l* stirred-tank fermenter, the maximum mycelial biomass (10.7 g/*l*) and EPS production (1.9 g/*l*) was obtained at 300 rpm and 2 vvm, respectively. The inhibitory effect of both IPS and EPS on the growth of SK-N-SH cells was studied by treating the cells with crude IPS and EPS at concentrations of 0.5, 1, and 2 mg/ml for 24, 48, 72 h, respectively. The maximum inhibitory effect on apoptosis of SK-N-SH cells (64.6%) was observed when IPS was treated at a concentration of 2 mg/ml, for 72 h. The apoptosis of SK-N-SH cells induced by IPS was evidenced by comet assay, where the number of the comet cells increased by the IPS treated. These findings suggest that IPS may therefore have therapeutic potential against neuroblastoma cells.

Key words: Apoptosis, *Cordyceps takaomontana*, Entomopathogenic Fungus, Polysaccharide, Submerged Culture

INTRODUCTION

The genus *Cordyceps* (Clavicipitaceae) is a group of entomopathogenic fungi that forms fruiting body in their insect hosts, and over 750 species have been known so far [1]. Among them, the species *C. takaomontana* (anamorph: *Peacilomyces tenuipes* [2,3]) has been used as either crude materials or biological active components extracted from fruit bodies by biotechnological methods [3]. However, only a few *Cordyceps* and bioactive substances from them have been exploited for commercial use and are currently not able to meet the market demand [4].

Although the artificial culture of *Cordyceps* has been solved in some specific solid media, the growth time is too long to satisfy economic efficiency [5]. Therefore, to provide opportunities for increasing economic benefit and practical use, numerous submerged culture systems for mycelial culture of *Cordyceps* have been developed [6-10].

It is a general opinion that the medicinal merits of cultured mycelia of these macrofungi are similarly effective as those found in the wild. In addition, submerged culture has potential advantages for higher mycelial production in a compact space and for a shorter incubation time with a lesser chance of contamination.

During the past decades, many different types of polysaccharides have been isolated from the fruit bodies of macrofungi, and their diverse biological activities were addressed with a special attention on anti-cancer effects [11-14]. Recently, constituent molecules of microfungi themselves and their secondary metabolites are known as bioactive compounds, including polysaccharides, glycoproteins, proteoglycans, terpenoids, fatty acids, proteins, lectins, etc. [15-19]. These compounds are found in fruiting bodies, mycelia and spores, and culture broth. Some beneficial substances with immunomodulating and antitumor activity have been isolated from medicinal macrofungi. For instance, Lentinan is known as a pure β-D-glucan derivative separated from fruiting body of *Lentinus edodes*, which stimulates the core immune mechanisms mediating destruction of tumor cells [19]. The polysaccharide isolated from the medicinal mushroom, *Phellinus linteus* is also a well-known antitumor agent that decreases IL-2 and TNF-α production in splenocytes, and enhances spontaneous cell apoptosis in macrophages and lympocytes stimulated with lipopolysaccharide *in vitro* [20].

To date, no reports have been documented concerning submerged culture of *C. takaomontana* and biological activities of its products from mycelial submerged culture. The objectives of the current study are 1) to optimize the liquid culture condition of *C. takaomontana* for the production of mycelial biomass and EPS, and 2) to study the apoptotic effects of IPS and EPS on human neuroblastoma (SK-N-SH) cells. To the best of our knowledge, this is the first report describing the production of intra- and extracellular polysaccharides possessing apoptotic effects on cancer cells.

MATERIALS AND METHODS

1. Microorganism and Media

C. takaomontana DG-07 was a culture collection of our laboratory isolated from a mountainous district in Korea. The stock culture was maintained on potato dextrose agar (PDA) slants and subcultured every month. Slants were incubated at 25 °C for 6 days and then stored at 4 °C.

2. Preparation of Inoculum and Flask Cultures

The fungus was initially grown on PDA medium in a petri dish and then transferred to the seed culture medium by punching out 8

†To whom correspondence should be addressed.
E-mail: jwyun@daegu.ac.kr

mm of the agar plate culture with a home-made cutter. The seed culture was grown in 250 ml flasks containing 50 ml of Mushroom Complete Medium (g/*l*) glucose 20, yeast extract 2, peptone 2, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 1, and KH_2PO_4 0.46, at 25 °C on a rotary shaker incubator at 150 rpm for five days. The flask culture experiments were performed in 250 ml flasks containing 50 ml of the media after inoculating with 4% (v/v) of the seed culture. All experiments were performed at least in triplicate.

3. Culture in Bioreactor

The culture medium for the fungus was inoculated with 4% (v/v) of the seed culture, and then cultivated for 8 days at 28 °C in a 5-*l* stirred-tank reactor (KoBioTech Co., Incheon, Korea). Unless otherwise specified, cultures were performed under the following conditions: temperature, 28 °C; aeration rate, 2 vvm; agitation speed, 150 rpm; initial pH, 8; working volume, 3 *l*. The influences of aeration rate and agitation speed were examined within the ranges from 0.5 to 2 vvm and 50 to 300 rpm, respectively. Samples were taken every 24 h for the analyses of mycelial biomass, EPS, and residual sugar concentration.

4. Estimation of Cell Mass and EPS Concentration

Samples taken at various time intervals were centrifuged at 12,000 ×*g* for 20 min. The resulting supernatant was mixed with four volumes of absolute ethanol, stirred vigorously and left overnight at 4 °C. The precipitated EPS was centrifuged at 10,000×*g* for 20 min, and the supernatant was discarded. The EPS precipitate was lyophilized and the weight of the EPS was estimated. Cell dry weight was measured after repeated washing with distilled water and drying at 70 °C overnight to a constant weight. The filtrate from membrane filtration was analyzed quantitatively for residual sugar concentration by HPLC (Shimadzu Co., Kyoto, Japan) using an Aminex HPX-42C column (0.78×30 cm, Bio-Rad Laboratories, Hercules, CA, USA), equipped with a refractive index detector (Shimadzu Co., Kyoto, Japan) [21].

5. Preparation of IPS

Mycelia, obtained by filtration of culture broth for isolation of water-soluble IPS, were washed three times with distilled water by repeated centrifugation, then resuspended in distilled water. The resulting suspension was heated for 6 h in an autoclave at 121 °C to extract IPS from mycelia, followed by filtration. The filtrates were then put through a process of ethanol precipitation, dialysis, and lyophilization. The lyophilized samples were dissolved in distilled water, and then water-soluble IPS was separated by centrifugation for 60 min at 10,000×*g* [22].

6. Fermentation Kinetics

Specific growth rate (μ/d) of the fungus was calculated from the equation: $\mu=(1/X)(dX/dt)$; where X is the cell concentration (g/*l*) at a time t (d). The specific consumption rate of substrate, $Q_{S/X}$ (g/g/d) was estimated according to the following equation: (dS/dt) (1/X); where S is the substrate concentration (g/*l*) at a time t (d). The specific production rate of EPS, $P_{P/X}$ (g/g/d) was estimated by the equation: (dP/dt)(1/X); where P is the concentration of EPS (g/*l*) at a time t (d). The yield of EPS on substrate, $Y_{P/S}$ (g/g) was estimated by the equation: (dP/dt)/(dS/dt) [23].

7. Compositional Analysis

The total sugar content in each sample was determined by the phenol-sulfuric acid method [24] using glucose as the standard. Sugar composition was analyzed by gas chromatography (Varian Co., Model: Star 3600CX, Lexington, MA) with a fused silica capillary column (Na form, 300 mm×0.25 mm, Supelco Inc., Bellefonte, PA) and a flame ionization detector. Total protein was determined by the Lowry method [25] with bovine serum albumin as the standard. The composition of amino acid was analyzed by amino acid analyzer (Amersham Pharmacia Biotech Ltd., Cambridge, UK) with a high performance ion exchange column (No. 3906, 200 mm×4.6 mm).

8. Neuroblastoma Cell Culture and Treatment

Human neuroblastoma cells (SK-N-SH) were obtained from the Korean Cell Line Bank of Seoul National University (Seoul, Korea). The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Hyclone Inc., Logan, UT, USA), 100 units/ml of penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2, and after 7 days growth were seeded in the petri dishes (2×10^5 cells/ml). After 24 h, the cells were treated in the petri dishes by incubation for 3 h at 37 °C in the presence of IPS or EPS.

9. Cell Viability Assay

Cell viability was assessed by the MTT {3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)} assay based on the reduction of a MTT into formazan dye by active mitochondria [26]. Briefly, The cells were placed in 96-well culture dishes (Nunclon™, Roskilde, Denmark) at a density of 5×10^4 cells/ml in MEM culture medium containing 10% FBS at 37 °C, 5% CO_2. After 24 h, the cells were washed and placed in culture medium with different concentrations of either IPS or EPS (0.5, 1.0, and 2.0 mg/ml) for 24, 48, and 72 h, respectively. Thereafter, 20 μl of MTT solution (5 mg/mL in PBS) was added to each well of microtiter plate and incubated for 4 h. After washing, the formazan dye precipitates, the amount of which was proportional to the number of live cells, were dissolved in 100 μl of DMSO. The absorbance was read at 540 nm with a microtiter plate reader (Thermo Electron Co., Vanta, Finland). The inhibition rate of cell growth was calculated by the following formula: mean value of {(control group-treated group)/control group}×100%. Triplicate wells were analyzed per each concentration.

10. Single Cell Gel Electrophoresis (Comet Assay)

For standard comet assay, three slides were prepared from cells obtained from each group treated with different concentrations [27]. About 4×10^6 cells were mixed with 80 μl of 0.7% low-melting agarose in PBS at 37 °C in microtube, than spread over a window microscopic slide specially designed for this assay locally, precoated with 150 μl of 0.5% normal-melting agarose in PBS. Slides were placed immediately in cold lysis buffer (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, and 1% Triton X-100, 4 °C) for a minimum of 1 h. After lysis, the slides were drained and placed in a horizontal gel electrophoresis tank surrounded by ice and filled with fresh cold electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) to a level of approximately 0.25 cm above the slides. Slides were kept in the high pH buffer for 20 min to allow DNA unwinding. Electrophoresis was then carried out for 20 min at 25 V and 300 mA. The slides were then drained and flooded slowly with three changes of neutralization buffer (0.4 M Tris, pH 7.5) for 5 min each, and then stained with 30 ml of ethidium bromide (20 mg/*l*) and covered with cover slips. To prevent additional DNA damage from visible light, all the steps described above were conducted under a dimmed light. A total of 50 randomly selected cells per slide were an-

alyzed. Image analysis was performed with a fluorescence microscope (Zeiss Axiovert L410 Inc., Jena Germany) attached to a digital camera (Olympus Inc., Tokyo, Japan) equipped with an excitation filter of 549 nm, a barrier filter of 590 nm, and a 100-W mercury lamp. The images were analyzed by the Kinetic Imaging Komet 5.0. The percentage of DNA in the comet tail (designated as "DNA damages") was automatically calculated. At least three slides per one experimental condition with 50 randomly selected cells per slide were analyzed.

RESULTS

1. Effect of Temperature and Initial pH in Flask Cultures

To find optimal temperature for the production of mycelial biomass and EPS, the fungal cells were cultivated in shake flasks under various temperatures (22-31 ºC), where the optimum temperature was found to be 28 ºC (Fig. 1(a)). This temperature optimum of the fungus is quite similar to the results obtained from other liquid culture of macrofungi [23,28]. To investigate the effect of initial pH on the production of mycelial biomass and EPS, the fungus was cultivated under the different initial pHs (3.0-9.0) in shake flask cultures. As shown in Fig. 1(b), fungal growth was the highest at an initial pH 5, whereas EPS production was the maximum at an initial pH 8. This pH optimum is comparable, in that many other macrofungi have identical neutral pH optima for both cell growth and EPS production during submerged culture [22,29,30].

2. The Optimal Combinations of the Media Constituents in Flask Cultures

To examine the effect of carbon sources on the production of mycelial biomass and EPS, various carbon sources, which are frequently used for macrofungal cultivation, were provided at a concentration of 20 g/*l*. As shown in Table 1, high levels of mycelial biomass and EPS were obtained when glucose, maltose, sucrose and sorbitol were used as the sole carbon source. In the present study, glucose was chosen for the remainder of this study due to its high EPS productivity. The optimal concentration of glucose for both mycelial growth and EPS production was determined to be 30 g/*l* (Fig. 2(a)). Meanwhile, among the nine nitrogen sources examined, trypton, soy peptone, meat peptone, poly peptone and yeast extract were relatively favorable to the mycelial growth of *C. takaomontana* (Table 1). The maximum production of EPS was achieved at the medium of 3 g/*l* of yeast, whereas the concentration of mycelial biomass was increased in parallel with the concentration of yeast extract employed (Fig. 2(b)). In comparison with organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower my-

Table 1. Effect of carbon and nitrogen sources on mycelial growth and exopolysaccharide (EPS) production in *Cordyceps takaomontana* DG-07[a]

	Cell dry weight (g/*l*)[b]	EPS (g/*l*)[b]	Final pH[b]
Carbon source			
Maltose	8.12±1.60	0.86±0.12	5.74±0.21
Sucrose	9.00±0.14	0.57±0.28	6.05±0.01
Glucose	8.80±1.59	0.95±0.11	6.65±0.69
Fructose	7.50±0.99	0.57±0.19	5.92±0.10
Xylitol	7.18±0.81	0.66±0.03	6.77±0.19
Galactose	5.04±1.18	0.26±0.08	6.44±0.21
Xylose	0.64±0.33	0.38±0.19	4.79±0.19
Lactose	2.59±0.82	0.28±0.14	6.43±0.13
Sorbitol	9.32±0.42	0.64±0.21	5.74±0.03
Nitrogen source			
Yeast extract	8.78±0.10	1.74±0.15	5.55±0.08
Polypeptone	10.50±0.05	0.85±0.83	5.30±0.14
Meat peptone	9.60±0.06	1.34±0.49	5.68±0.14
Soy peptone	11.58±0.01	1.63±0.03	5.75±0.02
Tryptone	11.26±0.07	1.27±0.72	5.52±0.05
Ammonium sulfate	5.62±0.07	0.77±0.68	2.53±0.56
Ammonium nitrate	4.14±0.07	0.04±0.02	4.87±0.03
$(NH_4)_2HPO4$	7.18±0.02	0.02±0.13	3.85±0.21
NH_4Cl	4.96±0.07	0.66±0.94	2.86±0.55

[a]Fermentations were carried out in shake flasks for 5 days at 28 ºC with initial pH 8

[b]Values are mean±S.D. of triple determinations

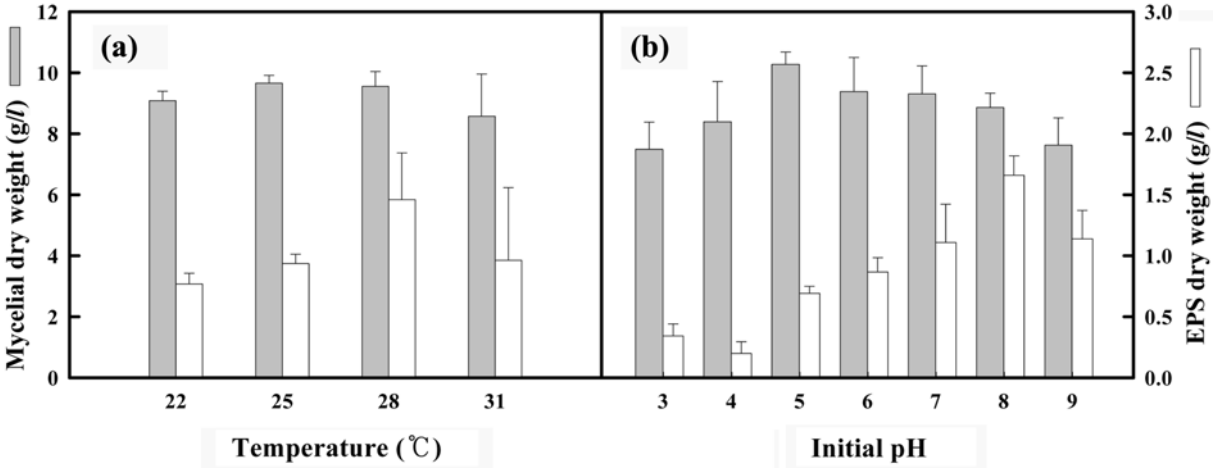

Fig. 1. Effect of temperature (a) and initial pH (b) on mycelial growth and exopolysaccharide (EPS) production in shake flask culture of *Cordyceps takaomontana* DG-07. The results are expressed as the average of triple determinations with ±S.D.

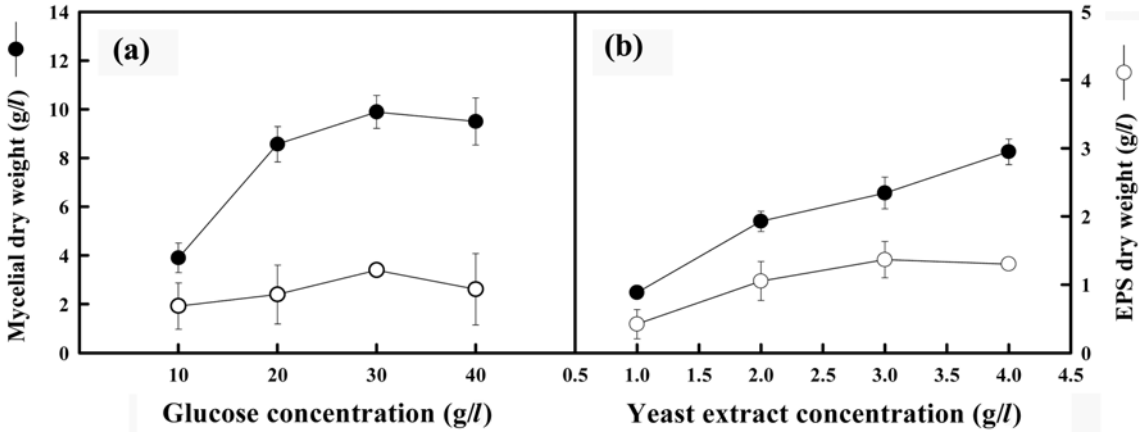

Fig. 2. Effect of carbon (a) and nitrogen concentration (b) on mycelial growth and exopolysaccharide (EPS) production in shake flask culture of *Cordyceps takaomontana* DG-07. The results are expressed as the average of triple determinations with ±S.D.

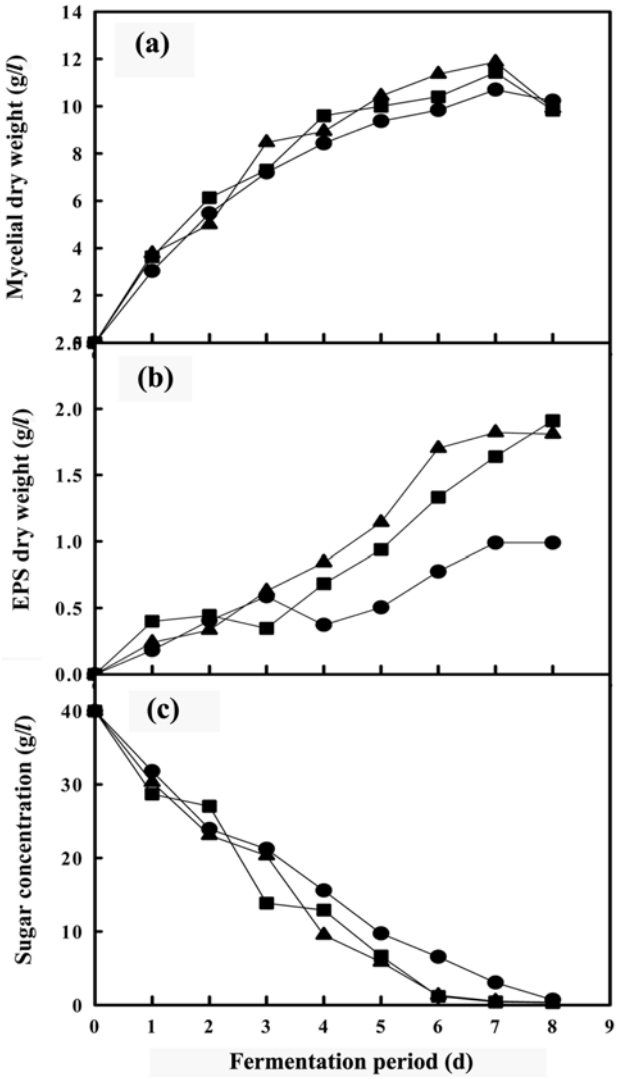

Fig. 3. Time profiles of mycelial dry weight (a), exopolysaccharide (EPS) production (b), residual sugar (c) during submerged culture of *Cordyceps takaomontana* DG-07 in a 5-*l* stirred-tank reactor at different aeration rates. 0.5 vvm (●), 1 vvm (■), 2 vvm (▲).

Table 2. Fermentation kinetics of *Cordyceps takaomontana* DG-07 under the different aeration conditions

Kinetic parameters	Aeration rate (vvm)		
	0.5	1	2
Specific growth rate, μ (d^{-1})	0.151	0.155	0.163
Maximum cell mass, X_{max} (g/*l*)[a]	10.701	11.433	11.866
Maximum EPS, P_{max} (g/*l*)[a]	0.988	1.640	1.821
Specific consumption rate of substrate, $Q_{S/X}$ (g/g/d)	0.472	0.481	0.488
Specific production rate of EPS, $P_{P/X}$ (g/g/d)	0.011	0.016	0.025
Yield of EPS on substrate, $Y_{P/S}$ (g/g)	0.023	0.033	0.051

[a]At day 7

celial biomass and EPS production (Table 1). As for mineral sources, the mixed elements originally contained in MCM (g/*l*) 0.5 $MgSO_4 \cdot 7H_2O$, 1 K_2HPO_4, and 0.46 KH_2PO_4) led to the best results for both mycelial growth and EPS formation, compared to other combinations of mineral sources tested (data not shown).

3. Effect of Aeration Rate in the Stirred-tank Reactor

To investigate the effect of oxygen supply on both mycelial growth and EPS production, a set of cultures were performed under the three different aeration conditions. Although mycelial growth was not significantly affected by altering aeration rate (Fig. 3(a)), a critical influence of aeration rate on EPS production was observed (Fig. 3(b)). The highest levels of both cell mass (10.7 g/*l*) and EPS production (1.9 g/*l*) were observed at day 7 under the aeration rate of 2 vvm (Fig. 3(a) and 3(b)). However, a significantly lower EPS production was obtained at 0.5 vvm than that achieved at 1 and 2 vvm. Several investigators have also reported that relatively high aeration rates are not always required for the production of mycelial biomass and metabolites [31-34]. The sugar concentrations were decreased sharply from the early period of culture, and then almost depleted at the end of culture period (Fig. 3(c)). As illustrated in Table 2, all kinetic parameters obtained at 2 vvm were the highest among tested aeration conditions. Taken together, the results indicated that the production of biomass was not significantly influenced

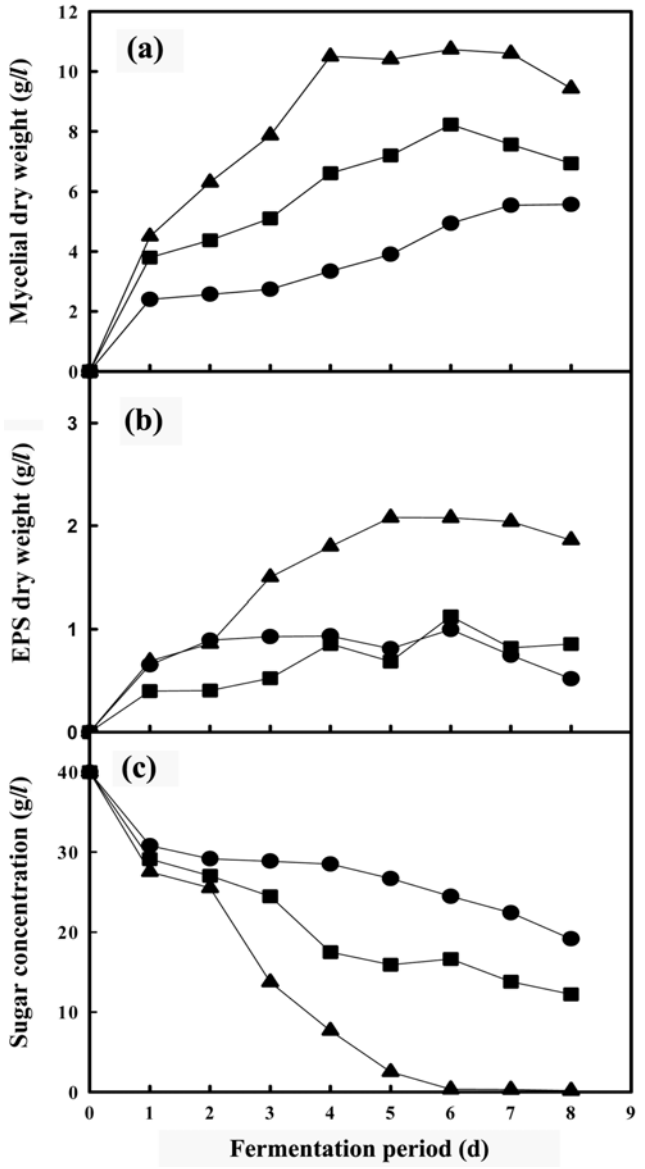

Fig. 4. Time profiles of mycelial dry weight (a), exopolysaccharides production (b), residual sugar (c) during submerged culture of *Cordyceps takaomontana* DG-07 in a 5-*l* stirred-tank reactor at different agitation speeds. 50 rpm (●), 150 rpm (■), 300 rpm (▲).

by the aeration rates, whereas EPS production was very sensitive to oxygen supply.

4. Effect of Agitation Speed in the Stirred-tank Reactor

A set of batch cultures were performed in a 5-*l* stirred-tank bioreactor at various agitation intensities of 50, 150, and 300 rpm at a fixed aeration rate of 1 vvm. The mycelial biomass and EPS products were increased by shifting the agitation speed from 50 to 300 rpm because of enhanced mixing of the fermentation broth, thereby increasing uptake of oxygen and nutrients. The maximum cell mass (10.1 g/*l*) and EPS production (2.1 g/*l*) were achieved at day 5 under 300 rpm (Fig. 4(a) and 4(b)). The total sugar concentrations as a function of agitation speed are shown in Fig. 4(c). The utilization of sugars also increased with agitation speed when shifted from 50 to 300 rpm. The growth kinetic data of *C. takaomontana* under different agitation speed are shown in Table 2. As observed in the results from different aeration rate, the highest kinetic performances were achieved at 300 rpm (Table 3). This result suggests that high agitation in combination with high aeration is strongly required for the submerged culture of *C. takaomontana* for the preparation of both mycelial biomass and EPS production. There are similar results that the increased agitation speed enhanced the yields of biomass and EPS in other submerged cultures of macrofungi [30,35,36].

Table 3. Fermentation kinetics of *Cordyceps takaomontana* DG-07 under the different agitation conditions

Kinetic parameters	Agitation rate (rpm)		
	50	150	300
Specific growth rate, μ (d^{-1})	0.088	0.132	0.155
Maximum cell mass, X_{max} (g/*l*)[a]	4.933	8.233	10.733
Maximum EPS, P_{max} (g/*l*)[a]	0.996	1.124	2.083
Specific consumption rate of substrate, $Q_{S/X}$ (g/g/d)	0.255	0.303	0.506
Specific production rate of EPS, $P_{P/X}$ (g/g/d)	0.012	0.019	0.034
Yield of EPS on substrate, $Y_{P/S}$ (g /g)	0.047	0.062	0.067

[a]At day 6

Table 4. Chemical composition of carbohydrate and amino acid in intracellular polysaccharide (IPS) and exopolysaccharide (EPS) obtained from submerged mycelial culture of *Cordyceps takaomontana* DG-07

	Composition (%)	
	IPS	EPS
Carbohydrate		
Mannose	49.07	36.68
Galactose	26.37	19.28
Glucose	21.42	43.00
Others	3.11	1.02
Amino acid		
Aspartic acid	9.96	12.03
Threonine	10.72	21.20
Serine	8.68	14.82
Glutamic acid	9.32	7.71
Proline	0.35	11.20
Glycine	10.30	14.87
Alanine	9.73	nd[a]
Cysteine	0.63	13.35
Valine	6.44	7.75
Methionine	0.70	0.80
Isoleucine	3.35	4.32
Leucine	3.38	5.70
Histidine	1.31	3.04
Tyrosine	1.76	3.88
Phenyalanine	2.32	1.53
Lysine	5.46	6.75
Arginine	2.42	3.75

[a]nd, not detected

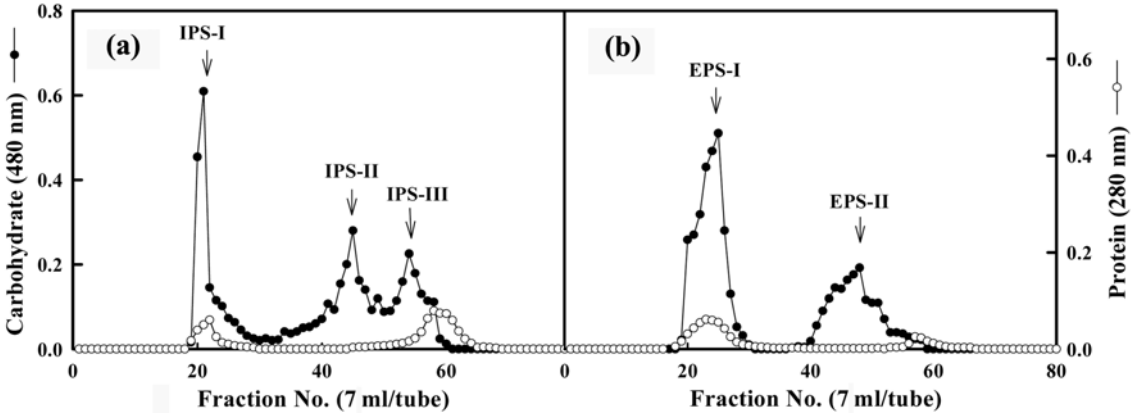

Fig. 5. Elution profiles of crude intracellular polysaccharides (IPS) and exopolysaccharides (EPS) in Sepharose CL-6B chromatography. Elutes were analyzed by measuring at 480 nm for carbohydrates (●) and at 280 nm for proteins (○): (a) IPS: (b) EPS.

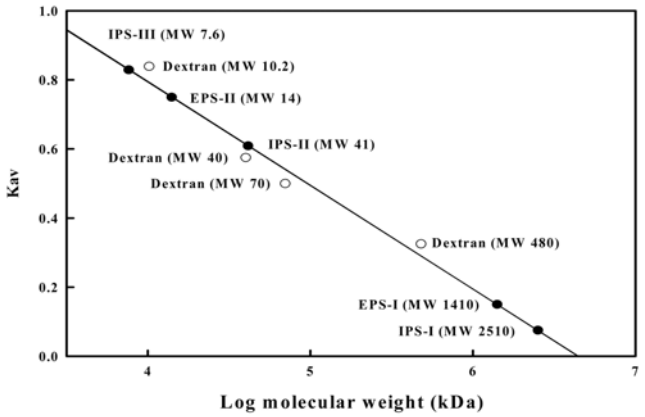

Fig. 6. Determination of molecular weight of the intracellular polysaccharide (IPS) and exopolysaccharide (EPS) obtained from *Cordyceps takaomontana* DG-07 in Sepharose CL-6B chromatography. Elutes were analyzed by measuring the absorbance at 480 nm for carbohydrate. Kav=(Ve−Vo)/(Vt−Vo) (Vo: void volume; Vt: total volume; Ve: elution volume). Three groups of IPS and two groups of EPS are denoted as closed circles and four mark dextrans are denoted as open circles.

5. Characterization of IPS and EPS

A compositional analysis revealed that IPS and EPS obtained from a stirred-tank bioreactor consisted of carbohydrates (93.5%, 87%) and proteins (6.5%, 13%), respectively. The detailed compositions of carbohydrate and amino acid in IPS and EPS are illustrated in Table 4. Both IPS and EPS commonly consisted of mannose, galactose, and glucose but their ratios were different. By a Sepharose CL-6B gel permeation chromatography, three fractions of IPS and two fractions of EPS were obtained (Fig. 5(a) and 5(b), respectively). The molecular weights of IPS-I, IPS-II, and IPS-III were estimated to be about 2510, 41, 7.6 kDa, while those of EPS-I and EPS-II were about 1410, 14 kDa, respectively (Fig. 6). Among five different fractions, IPS-II and EPS-II were polysaccharides without containing any peptide moieties, whereas the other three fractions were peptide-polysaccharide complex.

6. Inhibitory Effects of IPS and EPS on the Growth of Neuroblastoma Cells

We investigated some of the biological activities of IPS and EPS, and found that they had inhibitory effects on the growth of human neuroblastoma (SK-N-SH) cell lines. Cultures of cells were treated with crude IPS and EPS at various concentrations (e.g., 0.5, 1, and 2 mg/ml) for 24, 48, 72 h. As shown in Fig. 7, the results demon-

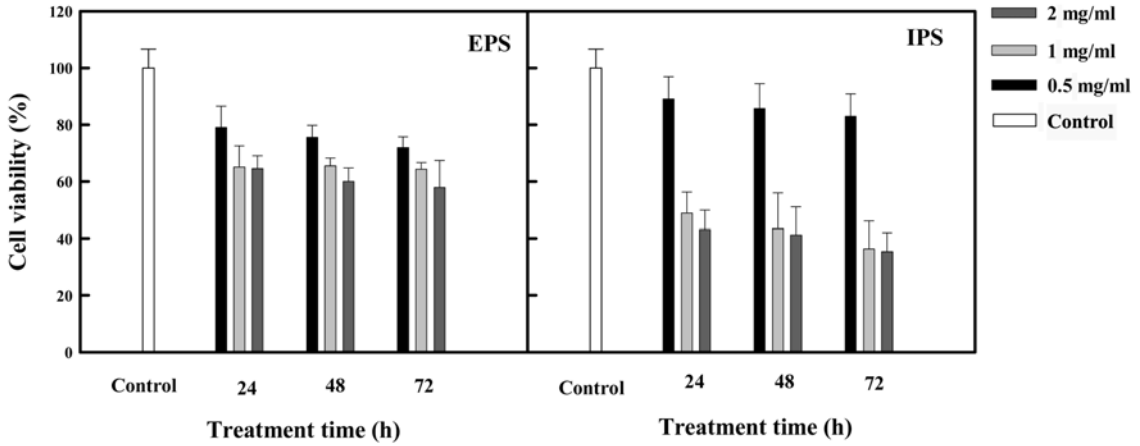

Fig. 7. Inhibitory effect of intracellular polysaccharide (IPS) and exopolysaccharide (EPS) on SK-N-SH cells. The results are expressed as the average of triple determinations with ±S.D.

Table 5. The results of comet assay for detecting the levels of DNA damage in SK-N-SH cancer cells induced by the intracellular polysaccharide for 3 h

Treatment conc. (mg/ml)	DNA damage[a] (%)
0	4.13±3.28
0.5	10.40±2.73
1.0	20.83±7.49
2.0	24.40±11.87

[a]DNA damage was noted as the ratio of comet tail of DNA to sum of tail and head of DNA taken average from 100 comets. Values are means±S.E.

strated that crude IPS showed higher inhibitory effect on growth of SK-N-SH cells in a dose-dependent manner. After 72 h treatment with crude IPS at a concentration of 2 mg/ml, the inhibitory effect of SK-N-SH cells was 64.6±0.05%. To confirm whether IPS induced apoptosis of SK-N-SH cells, we detected DNA damage (50-300 kb fragments) using the comet assay because this assay can detect various forms of DNA strand breakage dependent on the pH of electrophoresis, and is generally accepted as specific for cell apoptosis [37-39]. Cells were exposed to four different concentrations of crude IPS together with 0, 0.5, 1, and 2 mg/ml for 3 h. The levels of DNA strand breaks were expressed as the percentage of DNA in the comet tails. As shown in Table 5, the levels of DNA strand breaks in cells exposed to crude IPS were dose-dependently higher than those in control cells, which is in parallel with the results of MTT assay (Fig. 7).

DISCUSSION

In the present study, a critical conclusion was derived that both aeration and agitation were critical factors in the submerged culture of *C. takaomontana*, particularly for the production EPS. The yields of EPS at the high aeration rates (e.g., 1 and 2 vvm) were approximately more than two times achieved at a low aeration rate (0.5 vvm). However, cumulated studies revealed that degree of oxygen requirement in submerged culture of *Cordyceps* or other macrofungi differs by species. For example, we previously reported that a high aeration rate of 4 vvm significantly increased mycelial growth, whereas the highest EPS was achieved at a moderate aeration rate (2 vvm) in submerged mycelial culture of *Cordyceps militaris* [8]. Other investigators also stressed the importance of aeration and agitation in fungal submerged culture, but their results were controversial. Hsieh and co-workers have demonstrated that high oxygen levels >40% were found to inhibit both cell growth and polysaccharide production with a higher rate of glucose consumption in submerged culture of a mushroom *Grifola frondosa* in a 5-*l* stirred-tank fermenter. [34]. Similar results were found in mycelial submerged culture of a macrofungus *Ganoderma lucidum* [40]. Although higher speeds enhanced mixing efficiency and polysaccharide release, higher shear stress had a detrimental effect on mycelial growth and polysaccharide formation. Indeed, high aeration rate and high agitation intensity are important but not always required for the enhanced production of fungal growth and their metabolites [31,32,41].

Nowadays, macrofungi are distinguished as important natural resources of immunomodulating and anticancer agents. A wide variety of bioactive compounds with anticancer activity from macrofungi have been addressed, including polysaccharides, protein bound polysaccharides, terpenoids, and steroids [14,42]. Among those compounds, different types of antitumor polysaccharides have been isolated such as β-glucan, galactoglucomannan, galactomannoglucan, xylogalactoglucan [42,43]. Among them, β-glucans are most important polysaccharides with immunomodulating and antitumor activity. β-Glucans are glucose polymers that can exist as a non-branched (1→3)-β-linked backbone or as a (1→3)-β-linked backbone with (1→6)-β-branches, and they occur as a primary component in the cell walls of higher fungi in the great deals. Heteroglucan side chains contain glucuronic acid, xylose, galactose, mannose, arabinose and ribose that may combined with other components. Glycans are other polysaccharides that have frequently been found in macrofungi. These polysaccharides, in general, contain units other than glucose in their backbone. They are classified as galactans, fucans, xylans, and mannans by the individual sugar components in the backbone.

It has been demonstrated that the structural features of those polysaccharides are important for anticancer activity [14]. For example, water soluble polysaccharides from fruit body of *Ganoderma lucidum* exhibiting antitumor activity contained the backbone with either β-(1→3)-linked D-glucose or α-(1→4)-linked D-mannan [44]. Moreover, biological activities are closely related with molecular size, solubility in water, branching frequency and forms.

Polysaccharides derived from macrofungi have high capacity for carrying biological information because they have great potential for structural variability. Despite the structural and functional similarities, they differ in their effectiveness against specific tumors and in their ability to elicit various cellular responses, particularly cytokine expression and production [45]. For example, the structure of β-D-glucans has a relationship with the binding characteristics with receptors that the triple helical solution conformation, molecular weight, branching ratio, solubility in water and charge of the glucan polymer are important determinations in receptor ligand interaction and antitumor activity [46].

To data, there are a large number of studies on anticancer activities of macrofungal (protein-bound) polysaccharides and their molecular mechanisms have been documented. Shi et al. [47] obtained water-insoluble polysaccharides from mycelia of *Grifola frondosa* and provided evidence that they inhibited human gastric cancer cells through apoptotic induction. Lavi et al. [48] reported that a hot-water-soluble fraction of the mycelium of the liquid cultured mushroom *Pleurotus ostreatus* had pro-apoptotic activities against HT-29 colon cancer cells *in vitro*, upregulating the pro-apoptotic molecules Bax and cytosolic cytochrome-c. Potential biological activities of a chemically sulfated polysaccharide (S-GAP-P), which was derived from water-insoluble polysaccharide of *Grifola frondosa* mycelia, have been reported [49]. S-GAP-P inhibited the proliferation of human gastric carcinoma SGC-7901 cells and induced apoptosis. The results from *in vivo* experiments demonstrated that S-GAP-P significantly inhibited the tumor growth and enhanced the peritoneal macrophages phagocytosis in S180-bearing mice. Wang et al. [50] prepared four different fractions of ethanolic extract of *Cordyceps sinensis* using supercritical carbon dioxide, demonstrating that the fraction having the highest total polysaccharide content was the most active fraction to scavenge free radicals and inhibit

the proliferation of human colorectal and hepatocellular carcinoma cells. Bae et al. [51] reported that polysaccharides extracted from mushroom *Phellinus gilvus* were capable of inhibiting melanoma growth *in vivo* and the mechanism of polysaccharides with anti-tumor effects involved the promotion of cellular apoptosis.

Likewise, polysaccharide-peptide complex (PPC) obtained from macrofungi also has anticancer activity. Cui et al. [52] isolated a novel PPC from cultured mycelia of *Grifola frondosa* and found that it significantly inhibited the proliferation of gastric carcinoma tumor cells. They showed evidence that PPC could suppress cancer cell growth and reduce cell survival via arresting cell cycle and inducing apoptosis of tumor cells. Cao et al. [53] prepared other types of PPC from fruiting body of *Ganoderma lucidum* and showed effects as anti-tumors in mice, indicating that the proliferation of human umbilical cord vascular endothelial cells was inhibited by PPC in a dose-dependent fashion, but not because of cytotoxicity. Moreover, addition of PPC also led to a reduction of Bcl-2 anti-apoptotic protein expression and an increase of Bax pro-apoptotic protein expression of the cells. PPC derived from another medicinal mushroom *Coriolus versicolor* has been also considered as a biological response modifier with potential pharmaceutical applications [54]. PPC obtained at different days from the *C. versicolor* culture was tested *in vitro* for its immune function on human normal peripheral blood mononuclear cells and cytotoxicity on the human leukemia Molt 4 cells. They derived an important finding that those biological activities were dependent on culture duration. Thus, they demonstrated a correlation between culture duration of mushroom and biological potency of PPC, suggesting that monitoring the harvest duration is critical for the quality control of polysaccharopeptide in the biotechnological industry. The medicinal mushroom *Phellinus linteus* also has inhibitory effect against proliferation and colony formation of SW480 human colon cancer cells [55].

The reports about the use of macrofungal polysaccharides for treating neural cancer cells are limited. More recently, several compounds derived from macrofungi were employed for treatment of human neuroblastoma, and molecular mechanism was suggested to be linked to caspase-3 activation through an up-regulation of Bax rather than a down-regulation of Bcl-2 [56,57].

In conclusion, we found for the first time the optimal submerged culture condition for the preparation of bioactive IPS and EPS in *C. takaomontana*. Indeed, a critical conclusion was derived that both agitation and aeration were important factors for the production of mycelial biomass and EPS. Moreover, IPS and EPS obtained from mycelia of *C. takaomontana* has inhibitory effect on neuroblastoma (SK-N-SH) cells, suggesting a possible use of mycelial extract as chemotherapy for neuroblastoma cancer cells. Although enough data are not shown here, the inhibitory effect of fungal polysaccharides on neuroblastoma cells was mediated by apoptosis.

ACKNOWLEDGMENTS

This work was supported by Daegu University Research Grant 2008.

REFERENCES

1. N. Nikoh and T. Fukatsu, *Mol. Biol. Evol*., **17**, 629 (2000).
2. Y. Kobayasi, Botanical Institute, Tokyo (1941).
3. R. A. Samson, Centralbureau voor Schimmelcultures, Baarn (1974).
4. J. H. Koh, J. M. Kim, U. J. Chang and H. J. Suh, *Biol. Pharm. Bull*., **26**, 84 (2003).
5. W. Chen, Z. Zhao, S. F. Chen and Y. Q. Li, *Bioresour. Technol*., **99**, 3187 (2008).
6. X. B. Mao, T. Eksriwong, S. Chauvatcharin and J. J. Zhong, *Process Biochem*., **40**, 1667 (2005).
7. S. W. Kim, H. J. Hwang, C. P. Xu, J. M. Sung, J. W. Choi and J. W. Yun, *J. Appl. Microbiol*., **94**, 120 (2003).
8. J. P. Park, Y. M. Kim, S. W. Kim, H. J. Hwang, Y. J. Cho, Y. S. Lee, C. H. Song and J. W. Yun, *Process Biochem*., **37**, 1257 (2002).
9. J. H. Xiao, D. X. Chen, J. W. Liu, Z. L. Liu, W. H. Wan, N. Fang, Y. Xiao, Y. Qi and Z. Q. Liang, *J. Appl. Microbiol*., **96**, 1105 (2004).
10. J. H. Xiao, D. X. Chen, Y. Xiao, Z. L. Liu, W. H. Wan, N. Fang, B. B. Tan, Z. Q. Liang and A. Y. Liu, *Process Biochem*., **39**, 2241 (2004).
11. A. S. Daba and O. U. Ezeronye, *Afr. J. Biotechnol*., **2**, 672 (2003).
12. F. Levander and P. Rådström, *Appl. Environ. Microbiol*., **67**, 2734 (2001).
13. P. J. Looijestenijn, I. C. Boels, M. Kleerebezem and J. Hugenholtz, *Appl. Environ. Microbiol*., **65**, 5003 (1999).
14. S. P. Wasser, *Appl. Microbiol. Biotechnol*., **60**, 258 (2002).
15. J. W. Bok, L. Lermer, J. Chilton, H. G. Klingeman and G. H. Towers, *Phytochemistry*, **51**, 891 (1999).
16. P. H. Leung, Q. X. Zhang and J. Y. Wu, *J. Appl. Microbiol*., **101**, 275 (2006).
17. J. O. Toth, B. Luu and G. Ourisson, *Tetrahedron Lett*., **24**, 1081 (1983).
18. W. C. Wu, J. R. Hsiao, Y. Y. Lian, C. Y. Lin and B. M. Huang, *Cancer. Chemother. Pharmacol*., **60**, 103 (2007).
19. A. T. Yap and M. L. Ng, *Int. J. Med. Mushrooms*., **5**, 339 (2003).
20. G. Y. Kim, S. I. Roh, S. K. Park, S. C. Ahn, Y. H. Oh, J. D. Lee and Y. M. Park, *Biol. Pharm. Bull*., **26**, 1418 (2003).
21. J. T. Bae, J. Sinha, J. P. Park, C. H. Song and J. W. Yun, *J. Microbiol. Biotechnol*., **10**, 482 (2000).
22. Y. O. Kim, H. W. Park, J. H. Kim, J. Y. Lee, S. H. Moon and C. S. Shin, *Life Sci*., **79**, 72 (2006).
23. E. J. Cho, J. Y. Oh, H. Y. Chang and J. W. Yun, *J. Biotechnol*., **127**, 129 (2006).
24. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem*., **28**, 350 (1956).
25. O. H. Lowry, N. J. Rosebrough, L. Farr and R. J. Randall, *J. Biol. Chem*., **193**, 265 (1951).
26. T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).
27. R. R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J. C. Ryu and Y. F. Sasaki, *Environ. Mol. Mutagen*., **35**, 206 (2000).
28. L. Selbmann, S. Onofri, M. Fenice, F. Federici and M. Petruccioli, *Res. Microbiol*., **153**, 585 (2002).
29. H. J. Hwang, S. W. Kim, C. P. Xu, J. W. Choi and J. W. Yun, *J. Appl. Microbiol*., **94**, 708 (2003).
30. C. P. Xu, S. W. Kim, H. J. Hwang, J. W. Choi and J. W. Yun, *Process Biochem*., **38**, 1025 (2002).
31. Y. J. Tang and J. J. Zhong, *Enzyme. Microb. Technol*., **32,** 478 (2003).
32. A. W. Hayes, N. D. Davis and U. L. Diener, *Appl. Microbiol*., **14**, 1019 (1966).
33. J. Y. Oh, E. J. Cho, S. H. Nam, J. W. Choi and J. W. Yun, *Process*

Biochem., **42**, 3352 (2007).
34. C. Hsieh, C. J. Liu, M. H. Tseng, C. T. Lo and Y. C. Yang, *Enzyme. Microb. Technol*., **39**, 434 (2006).
35. S. D. Baets, S. D. Laing, C. Francois and E. Vandamme, *J. Ind. Microbiol. Biotechnol*., **29**, 181 (2002).
36. T. M. Sobczuk, F. G. Camacho, E. M. Grima and Y. Chirti, *Bioprocess Biosyst. Eng*., **28**, 243 (2005).
37. T. Godard, E. Deslandes, P. Lebailly, C. Vigreux, F. Sichel, J. M. Poul and P. Gauduchon, *Histochem. Cell. Biol*., **112**, 155 (1999).
38. P. L. Olive and J. P. Banath, *Exp. Cell. Res*., **221**, 19 (1995).
39. S. Yasuhara, Y. Zhu, T. Matsui, N. Tipirneni, Y. Yasuhara, M. Kaneki, A. Rosenzweig and J. A. Martyn, *J. Histochem. Cytochem*., **51**, 873 (2003).
40. F. C. Yang and C. B. Liau, *Process Biochem*., **33**, 547 (1998).
41. J. L. Casas López, J. A. Sánchez Pérez, J. M. Fernández Sevilla, E. M. Rodríguez Porcel and Y. Chisti, *J. Biotechnol*., **116**, 61 (2005).
42. M. F. Moradali, H. Mostafavi, S. Ghods and G. A. Hedjaroude, *Int. Immunopharmacol*., **7**, 701 (2007).
43. V. E. C. Ooi and F. Liu, *Int. J. Med. Mushrooms*., **11**, 195 (1999).
44. G. Y. Wang, J. Zhang, H. Li, C. Zhang, T. Mizuno, H. Ito, H. Mayuzumi, H. Okamoto and J. Li, *Biosci. Biotechnol. Biochem*., **57**, 894 (1993).
45. A. T. Borchers, J. S. Stern, R. M. Hackman, C. L. Keen and M. E. Gershwin, *Proc. Soc. Exp. Biol. Med*., **221**, 281 (1999).
46. M. Okazaki, Y. Adachi, N. Ohno and T. Yadomae, *Biol. Pharm. Bull*., **18**, 1320 (1995).
47. B. J. Shi, X. H. Nie, L. Z. Chen, Y. L. Liu and W. Y. Tao, *Carbohydr. Polymer*., **68**, 687 (2007).
48. I. Lavi, D. Friesem, S. Geresh, Y. Hadar and B. Schwartz, *Cancer. Lett*., **244**, 61 (2006).
49. X. Nie, B. Shi, Y. Ding and W. Tao, *Int. J. Biol. Macromol*., **39**, 228 (2006).
50. B. J. Wang, S. J. Won, Z. R. Yu and C. L. Su, *Food Chem. Toxicol*., **43**, 543 (2005).
51. J. S. Bae, K. H. Jang, H. Yim and H. K. Jin, *Cancer. Lett*., **218**, 43 (2005).
52. F. J. Cui, Y. Li, Y. Y. Xu, Z. Q. Liu, D. M. Huang, Z. C. Zhang and W. Y. Tao, *Toxicol. In Vitro*., **21**, 417 (2007).
53. O. Z. Cao and Z. B. Lin, *Life Sci*., **78**, 1457 (2006).
54. C. L. Lee, X. Yang and J. M. F. Wan, *Enzyme. Microb. Technol*., **38**, 14 (2006).
55. G. Li, D. H. Kim, T. D. Kim, B. J. Park, H. D. Park, J. I. Park, M. K. Na, H. C. Kim, N. D. Hong, K. Lim, B. D. Hwang and W. H. Yoon, *Cancer. Lett*., **216**, 175 (2004).
56. Y. H. Choi, M. K. Huh, C. H. Ryu, B. T. Choi and Y. K. Jeong, *Int. J. Mol. Med*., **14**, 227 (2004).
57. R. Z. Thomas, R. G. Helen and D. C. Michael, *Toxicology*, **231**, 210 (2007).