Mycobiology 36(3) : 178-182 (2008)

Submerged Culture of *Phellinus linteus* for Mass Production of Polysaccharides

June Woo Lee[1], Seong Jin Baek[2] and Yong Seok Kim*

[1]*Department of Food Processing and Cooking, Kyungbuk College, Yeongjusi 750-712, Korea*
[2]*Biotoxtech Co., Cheongwongun, Chungbuk, 363-883, Korea, Central Research Institute, Ilyang Pharm. Co. Ltd., Yonginsi 449-900, Korea*

(Received September 5, 2008. Accepted September 19, 2008)

In order to increase the mycelial production of *Phellinus linteus*, which exhibits potent anticancer activity, some ingredients of the medium used to culture *P. linteus* were investigated. The optimal medium composition for the production of *Phellinus linteus* was determined to be as follows: fructose, 40 g/*l*; yeast extract, 20 g/*l*; K_2HPO_4, 0.46 g/*l*; KH_2PO_4, 1.00 g/*l*; $MgSO_4 \cdot 7H_2O$, 0.50 g/*l*; $FeCl_2 \cdot 6_2O$, 0.01 g/*l*; $MnCl_2 \cdot 4H_2O$, 0.036 g/*l*; $ZnCl_2$, 0.03 g/*l*; and $CuSO_4 \cdot 7H_2O$, 0.005 g/*l*. The optimal culture conditions were determined to be as follows: temperature, 28°C; initial pH, 5.5; aeration, 0.6 vvm; and agitation, 100 rpm, respectively. Under optimal composition and conditions, the maximum mycelial biomass achieved in a 5 *l* jar fermentor was 29.9 g/*l*.

KEYWORDS : Cultural condition, Optimal media, *Phellinus linteus*, Polysaccharide

Various mushrooms have been used in traditional medicine for a long time. The higher Basidiomycetes have become a subject of great interest due to their diverse nutritional, medicinal, and pharmaceutical properties. Over the past three decades, many polysaccharides and polysaccharide-protein complexes have been isolated from the fruiting bodies and mycelia of mushrooms (Wasser, 2002).

Phellinus linteus, a yellow-orange colored mushroom that grows well on mulberry trees, is a well-known fungus in the genus *Phellinus* in the family Hymenochaetaceae, and it has been used as an herb in traditional medicine for many years in Asian countries (Kim *et al*., 2004). In traditional medicine, it has been known to have curative effects on stomachaches, inflammation, tumors and so on. It is also used to improve overall health and prevent various diseases, such as gastroenteric disorders, lymphatic disease and cancers (Cho *et al*., 2002).

In 1968 (Ikekawa *et al*., 1968), it was first reported that the hot water extract from the fruiting body of *Phellinus linteus* inhibited the growth of sarcoma 180 to about 96.7%. Since then, many investigators have documented a wide variety of further reports (Chi *et al*., 1996; Chung *et al*., 1993; Kang *et al*., 1997; Han *et al*., 1999). The active polysaccharide from *Phellinus linteus* stimulates humoral and cell-mediated immunity (Kim *et al*., 1996; Song *et al*., 1995). Acidic polysaccharides and proteoglycans from *P. linteus* activate protein tyrosine kinase and protein kinase C (Kim *et al*., 2003a, b). The ethanol extract showed strong antiangiogenic and antioxidant activities (Song *et al*., 2003).

As *P. linteus* is very rare in nature, the amount of wild mushrooms available is not sufficient for commercial exploitation. In order to obtain polysaccharides from mushrooms, most investigators have focused their efforts on cultivating mushrooms on solid artificial media (for fruiting body production) rather than submerged culture (Brochers *et al*., 1999; Kues and Liu, 2000). Obviously submerged cultures give rise to the potential advantages of higher mycelial production in a compact space and shorter period of time without significant contamination (Zhong and Tang, 2004; Hwang *et al*., 2003; Berovic *et al*., 2003). Therefore, in the present study, the submerged cultivation conditions were optimized for the production of polysaccharides from *P. linteus*.

Materials and Methods

Mushroom. The fruiting body from *P. linteus* IY003 was previously reported to possess antitumor and antioxidant activities (Lee *et al*., 2006). To obtain a pure culture of *P. linteus* IY003, a single spore was isolated from the fruiting bodies of *P. linteus* IY003, placed in 2% water agar and incubated for 2 days at 28°C. The mycelia germinated from the single spore were transferred to potato dextrose agar (PDA) and incubated for 10 days at 28°C.

Inoculum preparation. *P. linteus* IY003 was initially grown on PDA in a petri dish and then transferred to seed culture medium. The seed culture was grown in a 500 *ml* flask containing 100 *ml* of medium at 28°C on a rotary shaker incubator at 120 rpm for 7 days.

Culture condition. The flask culture experiments were performed in a 500 *ml* flask containing 100 *ml* of media after inoculation with 10% (v/v) of the seed culture. The fermentation medium was inoculated with 10% (v/v) of

*Corresponding author <E-mail : kys0908@chol.com>

the seed culture and then cultivated at 28°C in a 5 *l* Jar fermentor (KoBiotech Co., Korea). Unless otherwise specified, fermentations were performed under the following conditions: temperature, 28°C; aeration rate, 0.6 vvm; agitation speed, 100 rpm; initial, pH 5.5; and working volume, 3.5 *l*.

Analytical methods. The weight of the mycelium in the culture medium was determined by filtration through preweighted dried Whatman filter paper No. 2 (Whatman International. UK) under suction, after washing the filter 3 times with water, and drying to constant weight at 105°C for 20 hrs. Residual fructose in the medium was assayed by the DNS method. Viscosity measurements were performed on samples collected from the fermentor at regular intervals using a Brookfield RVTDV digital viscometer (Brookfield Engineering Laboratories, USA) fitted with a small sample adapter. Antioxidant activity and nitric oxide (NO) production were assayed as previously reported (Lee *et al*., 2006).

Preparation of partially purified polysaccharides. Culture broth of *P. linteus* IY003 was extracted 4 times with 2 volumes of distilled water at 121°C for 30 min. After centrifugation (3,000 ×g), the supernatant was concentrated by evaporation at 75°C. Three volumes of ethanol were added to the supernatant, which was then stored at 4°C for 24 hours. After centrifugation, the precipitate was ultrafiltrated (10 kDa cut-off) and freeze-dried.

Results and Discussion

Effects of carbon and nitrogen sources. To find a suitable carbon source, *P. linteus* IY003 was cultured in media containing various carbon sources at a concentration of 4% (w/v) for 7 days. Of the 9 carbon sources examined, fructose, maltose, glucose and mannose were favorable to mycelial growth (Table 1). Fructose proved to be the best carbon source for mycelial production. Contrary to our findings, other researchers have reported that mannose was the most suitable carbon source, and they emphasized that different carbon sources slightly altered the compositions of polysaccharides (Lee *et al*., 1995).

Table 1. Effects of various carbon sources on the mycelial growth of *Phellinus linteus* IY003

Carbon sources	Dry mycelial weight (g/*l*)
Glucose	26.8
Galactose	9.6
Fructose	29.9
Mannose	25.8
Maltose	29.2
Sucrose	16.3
Lactose	15.4
Cellulose	18.3
Corn starch	19.1

P. linteus IY003 was cultured for 7 days at 28°C in medium containing 4% of the carbon source, 2% yeast extract, 0.036% K_2HPO_4, 0.1% KH_2PO_4, 0.05%, $MgSO_4 \cdot 7H_2O$, Mineral salt 1 *ml*, pH 5.5.

Table 2. Effects of various nitrogen sources on the mycelial growth of *Phellinus linteus* IY003

Nitrogen sources	Dry mycelial weight (g/*l*)
Peptone	25.9
Yeast extract	29.9
Malt extract	5.7
Tryptone	18.1
Soytone	10.2
Casein	9.2
Beef extract	15.1
Corn steep liquor	12.3
$NaNO_3$	2.7
NH_4Cl	1.0

P. linteus IY003 was cultured for 7 days at 28°C in medium containing 2% of the nitrogen source, 4% fructose, 0.036% K_2HPO_4, 0.1% KH_2PO_4, 0.05%, $MgSO_4 \cdot 7H_2O$, Mineral salt 1 *ml*, pH 5.5.

Of the nitrogen sources tested, the best mycelial growth was observed in the medium containing yeast extract (Table 2). However, inorganic nitrogen sources, such as sodium nitrate ($NaNO_3$) and ammonium chloride (NH_4Cl), were hardly utilized. In comparison with the results reported by other investigators, the nitrogen source required for a liquid culture of *P. linteus* IY003 was different from that of *P. linteus* LI3202, i.e. mycelial growth was most favorable in medium containing peptone in most *P. linteus* fermentation processes (Lee *et al*., 1995). The optimum medium composition for *P. linteus* IY003 was determined from the results, as shown in Table 7.

Effects of pH and temperature. In order to investigate the effects of initial pH on mycelial growth, *P. linteus* IY003 was cultivated in the optimized medium under different initial pHs (3.0~7.0) in a shake flask culture. Table 3 shows the effects of initial pH on the mycelial growth

Table 3. Effects of initial pH on the mycelial growth of *Phellinus linteus* IY003

Initial pH	Dry mycelial weight (g/*l*)
3.0	4.1
4.0	13.9
4.5	22.3
5.0	25.9
5.5	29.9
6.0	28.0
7.0	18.6

P. linteus IY003 was cultured for 7 days at 28°C in the medium containing 4% fructose, 1.5% yeast extract, 0.036% K_2HPO_4, 0.1% KH_2PO_4, 0.05%, $MgSO_4 \cdot 7H_2O$, Mineral salt 1 *ml*.

Table 4. Effects of temperature on the mycelial growth of *Phellinus linteus* IY003

Temperature (°C)	Dry mycelial weight (g/*l*)
20	14.1
25	16.1
28	29.9
30	29.4
35	27.3

P. linteus IY003 was cultured for 7 days in medium containing 4% fructose, 1.5% yeast extract, 0.036% K_2HPO_4, 0.1% KH_2PO_4, 0.05%, $MgSO_4 \cdot 7H_2O$, Mineral salt 1 *ml*, pH 6.0.

of *P. linteus* IY003. The highest mycelial concentration was obtained at a pH of 5.5. The optimum environmental conditions for the mycelial growth of *Phellinus* mushrooms in liquid cultures depend on the strain. Contrary to our findings, the optimum pH for mycelial growth of *P. linteus* L13202 was 7.0 (Lee *et al*., 1995). It has been reported that the optimal pH for mycelial growth of a wide variety of mushrooms is also acidic (Kim *et al*., 2002; Park *et al*., 2001), and several *P. linteus* strains have an optimal pH of 5~7 (Choi and Lee, 2000; Kim *et al*., 2000).

To determine the optimal temperature for mycelial growth, *P. linteus* IY003 was cultivated in shake flask culture at various temperatures (20~35°C), where the optimum temperature was found to be 28°C (Table 4). It is comparable that many kinds of mushrooms have relatively low optimal growth temperatures, ranging from 20 to 25°C in their submerged cultures.

Effects of aeration and agitation. Mycelium production by *P. linteus* IY003 was investigated with aeration rates of 0.2, 0.4, 0.6, 0.8 and 1.0 vvm. The production of mycelia increased as the aeration rate increased. However, the optimum aeration rate was found to be 0.6 vvm, and mycelia production tended to decrease at an aeration rate of 1.0 vvm (Table 5).

To examine the effects of agitation speed on mycelial production, *P. linteus* IY003 was cultivated at 50, 100, 150, and 300 rpm, under the standard conditions (28°C, pH 5.5, 0.6 vvm). The optimum agitation speed was found to be 100 rpm (Table 6).

Table 5. Effects of aeration on the mycelial growth of *Phellinus linteus* IY003

Aeration (vvm)	Dry mycelial weight (g/*l*)
0.2	16.0
0.4	17.7
0.6	29.9
0.8	27.2
1.0	22.8

P. linteus IY003 was cultured for 7 days at 28°C at 200 rpm in the medium containing 4% fructose, 1.5% yeast extract, 0.036% K_2HPO_4, 0.1% KH_2PO_4, 0.05%, $MgSO_4 \cdot 7H_2O$, Mineral salt 1 *ml*, pH 6.0.

Table 6. Effects of agitation on the mycelial growth of *Phellinus linteus* IY003

Agitation (rpm)	Dry mycelial weight (g/*l*)
50	22.0
100	29.9
150	24.2
300	19.5

P. linteus IY003 was cultured for 7 days at 28°C by 0.6 vvm in medium containing 4% fructose, 1.5% yeast extract, 0.036% K_2HPO_4, 0.1% KH_2PO_4, 0.05%, $MgSO_4 \cdot 7H_2O$, Mineral salt 1 *ml*, pH 6.0.

Comparison to other media. Media typically used to cultivate higher fungi (i.e., potato dextrose broth (PDB), mushroom complete medium (MCM) and Czapeck medium) were compared to the optimized medium (Table 8). Mycelial growth was best on the optimized medium, followed by MCM, PDB, and Czapeck (Table 8), with maximum mycelium production rates of 29.9, 24.2, 18.6, and 11.9 g/*l*, respectively. The initial pH of the fermentation broth of *P. linteus* IY003 slowly increased according to growth until it reached a maximum of 6.0 at the end of fermentation. However, the pH of the Czapeck media decreased.

Results of fermentation in a Jar fermentor. Fig. 1 shows the typical time courses of mycelial growth and fructose consumption in a 5 *l* jar fermentor. Under opti-

Table 7. Optimized conditions for culture of *Phellinus linteus* IY003

Factors	Components	Composition
Media	Fructose	40.0 (g/*l*)
	Yeast extract	20.0 (g/*l*)
	K_2HPO_4	0.46 (g/*l*)
	KH_2PO_4	1.00 (g/*l*)
	$MgSO_4 \cdot 7H_2O$	0.50 (g/*l*)
	$FeCl_2 \cdot 6_2O$	0.01 (g/*l*)
	$MnCl_2 \cdot 4H_2O$	0.036 (g/*l*)
	$ZnCl_2$	0.03 (g/*l*)
	$CuSO_4 \cdot 7H_2O$	0.005 (g/*l*)
Conditions	Temperature	28°C
	pH	5.5
	Time	7days
	Aeration	0.6 vvm
	Agitation	100 rpm

Table 8. Comparison of the mycelial growth of *Phellinus linteus* IY003 in various media

Media	Initial pH	Final pH	Dry mycelial weight (g/*l*)
Optimized media	5.5	6.01	29.9
Potato dextrose broth	5.5	5.84	18.6
MCM	5.5	5.99	24.2
Czapeck	5.5	5.21	11.9

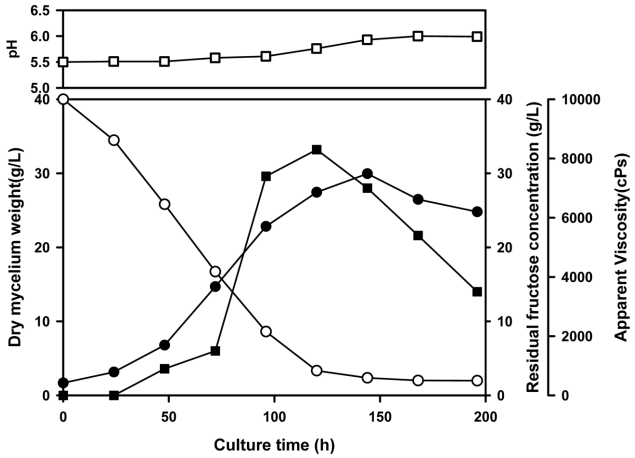

Fig. 1. Mycelial growth and fructose consumption from *Phellinus linteus* in a 5 *l* fermentor. Mycelial weight (●), Residual fructose (○), Viscosity (■), pH (□).

mum culture conditions, the maximum mycelial production rate was 29.9 g/*l* at 144 h of fermentation. To the best of our knowledge, the mycelial production obtained in this study is the highest yield amongst liquid cultures of *P. linteus* reported in the literature. The apparent viscosity of the fermentation broth of *P. linteus* IY003 increased rapidly during the exponential phase, but a rapid decrease was detected in a later stage. This drop in viscosity may be attributable to the occurrence of severe fragmentation in mycelial clumps of *P. linteus* IY003. Based on the results reported by another investigator, the apparent viscosity was influenced by mycelial fragmentation (Hwang *et al*., 2004).

Yield and characterization of polysaccharides. The dry weight of polysaccharides extracted by hot water from the culture broth of *P. linteus* IY003 was 5.4 g. This extract showed high antioxidant activities (72.86%) by inhibiting lipid peroxidation in rat liver homogenate treated with 1mg/ml of the extract. Moreover, the production of nitric oxide (NO) in RAW264.7 (RAW) cells was 28.1 ± 1.8 nM (10 mg/*ml* treatment). This result indicated that hot water-extracted polysaccharides from the culture broth of *P. linteus* IY003 may have immunomodulating and antioxidant activities.

References

Berovic, M., Habijanic, J., Zore, I., Wraber, B., Hodzar, D., Boh, B. and Pohleven, F. 2003. Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. *Journal of Biotechnology* 103:77-86.

Brochers, A. T., Stem, J. S., Hackman, R. M., Keen, C. L. and Gershwin, M. E. 1999. Mushrooms, tumors, and immunity. *Proc. Soc. Exp. Biol. Med.* 221:281-293.

Chi, J. H., Ha, T. M., Kim, Y. H. and Ryo Y. D. 1996. Studies on the main factors affecting the myceliall growth of *Phellinus linteus*. *Kor. J. Mycol.* 24:214-222.

Cho, J. H., Cho, S. D., Hu, H., Kim, S. H., Lee, S. K., Lee Y. S. and Kang K. S. 2002. The role of ERK1/2 and p38 MAP kinases in the prevention mechanism of mushroom *Phellinus linteus* against the inhibition of gap junction intercellular communication by hydrogen peroxide. *Carcinogen* 23:1164-1169.

Choi, K. H. and Lee, C. W. 2000. Submerged culture of *Phellinus linteus* in a stirred tank and an airlift fermenter. *J. Kor. Ins. Chem. Engin.* 38:310-315.

Chung, K. S., Kim, H. S., Kim, K. Y., Han, M. W. and Kim, K. H. 1993. Effects of Kp, an antitumor protein polysaccharide from myceliall culture of *Phellinus linteus* on the humoral immune response of tumor bearing ICR mice to sheep red blood cells. *Arch. Pharm. Res.* 16:336-338.

Han, S. B., Lee, C. W., Jeon, Y. J., Hong, N. D., Yoo, I. D. and Yang, K. H. 1999. The Inhibitory effect of polysaccharides isolated from *Phellinus* linteus on tumor growth and metastasis. *Immunopharmacology* 41:157-164.

Hwang, H. J., Kim S. W., Choi, J. W. and Yun, J. W. 2003. Production and characterization of exopolysaccharides from submerged culture of *Phellinus linteus* KCTC 6190. *Enzyme Microbiol. Technol.* 33:309-319.

Hwang, H. J., Kim, S. W., Xu, C. P., Choi, J. W. and Yun, J. W. 2004. Morpholoical and rheological properties of the three different species of basidomycetes *Phellinus* in submerged cultures. *J. Appl. Microbiol.* 96:1296-1305.

Ikekawa, T., Nakanishi, M., Uehara, N. and Chihara G. 1968. Antitumor action of some basidomycetes, especially *Phellinus linteus*. *Gann*. 59:155-157.

Kang, T. S., Lee, D. G. and Lee, S. Y. 1997. Isolation and mycelial submerged cultivation of *Phellinus* sp. *Kor. J. Mycol.* 25:257-267.

Kim, G. Y., Oh, Y. H. and Park, Y. M. 2003a. Acidic polysaccharide isolate from *Phellinus linteus* induces nitric oxide-mediated tumoricidal activity of macrophages through protein tyrosine kinase and protein kinase C. *Biochem. Biophys. Res. Comm.* 309:399-407.

Kim, G. Y., Park, S. K., Lee, M. K., Lee, S. H., Oh, Y. H., Kwak, J. Y., Yoon, S., Lee, J. D. and Park, Y. M. 2003b. Proteoglycan isolated from *Phellinus linteus* activates murine B lymphocytes via protein kinase C and protein tyrosine kinase. *Int. Immunopharmacol.* 3:1281-1292.

Kim, H. M., Han, S. B., Oh, G. T., Kim, Y. H., Hong, D. H., Hong, N. D. and Yoo, I. D. 1996. Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom *Phellinus linteus*. *Int. J. Immunopharmacol.* 18:295-303.

Kim, J. L., Kwon, H. K., Chun, G. T., Kim, J. W. and Lee, K. K. 2000. Studies on cultural characteristics for high density fermentation of *Phellinus linteus* WI-001. *Kor. J. Appl. Microbiol. Biotech.* 28:105-110.

Kim, S. H., Song, Y. S., Kim, S. K., Kim, B. C., Lim, C. J. and Park, E. H. 2004. Anti-inflammatory and related pharmacological activities of the n-BuOH subfraction of mushroom *Phellinus linteus*. *J. Enthnopharmacol.* 93:141-146.

Kim, S. W., Hwang, H. J., Park, J. P., Cho, Y. J., Song, C. H. and Yun, J. W. 2002. Mycelial growth and exo-polymer production by submerged culture of various edible mushrooms under different media. *Letters Appl. Microbiol.* 34:56-61.

Kues, U. and Liu, Y. 2000. Fruiting body production in basidomycetes. *Appl. Microbiol. Biotechnol.* 53:141-152.

Lee, J. H., Cho, S. M., Ko, K. S. and Yoo, I. D. 1995. Effect of cultural conditions on polysaccharide production and its mononsaccharide composition in *Phellinus linteus* L13202. *Kor. J. Mycol.* 23:325-331.

Lee, J. W., Baek, S. J., Bae, W. C., Park, J. M. and Kim, Y. S. 2006. Antitumor and antioxidant activities of the extracts from fruiting body of *Phellinus linteus*. *Mycobiology* 34:230-235.

Park, J. B., Kim, S. W., Hwang, H. J. and Yun, J. W. 2001. Optimization of submerged culture conditions for the myceliall growth and exo-polymer production by *Cordyceps militaris*. *Letters Appl. Microbiol.* 33:76-81.

Song, K. S., Cho, S. M., Lee, J. H., Kim, H. M., Han, S. B., Ko, K. S. and Yoo, I. D. 1995. B-lymphocyte-stimulating polysaccharide from mushroom *Phellinus linteus*. *Chem. Pharm. Bull.* 43:2105-2108.

Song, Y. S., Kim, S. H., Sa, J. H., Jin, C. B., Lim, C. J. and Park, E. H. 2003 Anti-angiogenic, antioxidant and xanthine oxidase inhibition activities of the mushoroom *Phellinus linteus*. *J. Enthnopharmacol.* 88:113-116.

Wasser, S. P. 2002. Medicinal mushrooms as a source of antitumor and immunomodulationg polysaccharides. *Appl. Microbiol. Biotech*. 60:258-274.

Zhong, J. J. and Tang, Y. J. 2004. Submerged cultivation of medicinal mushrooms for production of valuable bioactive metabolites. *Adv. Biochem. Engin. Biotech*. 87:25-59.