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Effect of Cultural Conditions on Polysaccharide Production and its Monosaccharide Composition in *Phellinus linteus* L13202

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배양 조건에 따른 상황 버섯의 다당류 생산 및 단당류 구성 변화

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ABSTRACT: The effect of cultural conditions on mycelial growth, polysaccharide production in *Phellinus linteus* and its monosaccharide composition was studied. *P. linteus* showed the highest growth (0.9 g/100 ml) on glucose but the polysaccharide production was the highest (13.7%) on mannose. The fungus grew very well at neutral pH (0.9 g/100 ml) but the growth was reduced to 0.47 g per 100 ml at alkaline pH. For the different pH, the yield of polysaccharide was in the range of 5~8%. The highest yield of 7.94% was obtained at pH 5. Also a variation in monosaccharide composition was observed for different carbon sources and pH. The composition ranges of glucose, mannose, and galactose of polysaccharide were 80~95%, 3~12%, and 2~10% depending on carbon sources, respectively. In contrast, the variation of composition range of three monosaccharides was narrower for different pH than that for carbon sources. These results suggested the possibility of the improvement of production and the physiological modification of the polysaccharide.

KEYWORDS: *Phellinus linteus*, mycelial growth, polysaccharide production

In view of developing new anti-tumor substances, numerous polysaccharides from bacteria, algae, higher plants and fungi have been investigated for anti-tumor and immunomodulating activities (Franz, 1989). Most of polysaccharides with anti-tumor and immunostimulating activities were originated from fungi, especially mushrooms. Among anti-tumor polysaccharides, β-(1-3)-glucan with glucose branched at C-6 has been shown to be the most efficient. The relationship between structure and activity was thus established as for the polymer(Lee, 1994).

Many studies on maximal mycelial growth have been carried out with useful mushrooms such as *Flammulina velutipes* (Song *et al*, 1995a), *Lentinus edodes* (Park and Lee, 1991), and *Poria cocos* (Hong *et al*, 1991). Not many studies, however, were done for modification and production improvement (Park *et al*, 1989; Park *et al*, 1994) of polysaccharide with immunostimulating activity. Polysaccharide modifications and improved production have been made by chemical method, enzymatic method, physiological method, and the application of genetic engineering technology. In case of anti-

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Table. 1. Composition of media used in this study

Medium	Composition (per liter)
Potato dextrose agar (PDA)	potato 200 g, dextrose 20 g, agar 20 g
Yeast extract-malt extract-glucose (YMG)	yeast extract 4 g, malt extdract 10 g glucose 4 g
Yeast extract-malt extract (YM)	yeast extract 4 g, malt extract 10 g
Peptone-Yeast extract-glucose (PYG)	peptone 1.25 g, yeast extract 1.25 g glucose 14 g
GP medium	glucose 30 g, peptone 3 g, K_2HPO_4 10 g $MgSO_4$ 0.7 g
GPY medium	glucose 20 g, polypeptone 2 g yeast extract 2 g, $NaNO_3$ 3 g, NaH_2PO_4 10 g, KCl 0.5 g, $MgSO_4$ 0.5 g

tumor polysaccharides, many chemical modifications have thus been made to improve the activity (Demleitner *et al*, 1992; Kishida *et al*, 1992). The biocatalytic techniques using polysaccharide-modifying enzymes can be restricted by factors such as the absence of abundant enzyme sources and slow or inefficient polymer production (Yalpani, 1988). But it was known that production yield of polysaccharides can be improved through the manipulation of a number of environmental parameters including the use of excess substrate concentration, low incubation temperature, and nitrogen, phosphorus, or sulphur limitation.

The new polysaccharide from mycellium of *Phellinus linteus* was reported to have a strong immunostimulating activity (Song *et al*, 1995b). The polysaccharide was characterized as protein-bound hetero-polysaccharide. The aim of this study was to improve the production yield and modify the polysaccharide from *Phellinus linteus* by changes of environmental factors.

Materials and Methods

Strain

The *Phellinus linteus* L13202, which was obtained from the american type culture collection, was used and preserved on the PDA plate.

Preparation of inoculum

Ten pieces of 4×4 mm from solid media of PDA were put into 100 m*l* YMG broth in 250 m*l* Erlenmeyer flask. The broth was cultured at 30°C on a rotary shaker for 10 days and homogenized at 10,000 rpm for 3 min. The 10 m*l* of homogenate was used as an inoculum.

Culture conditions

The effect of various media on mycelial growth was studied with different media (Table 1). Three flasks were used for each different media. Among the tested media, GP medium was selected because it gave a good mycelial growth. In study of carbon source effect on growth and polysaccharide production, 100 m*l* of GP medium containing 3% of carbon source was used. For pH experiment, the pH of GP medium was adjusted from 5 to 9 with HCl or NaOH after autoclaving. The fungus was grown for 12 days on a shaker at 30°C.

Isolation of polysaccharide from mycelia

The polysaccharide was extracted from the mycelia by boiling. After cultivation for 12 days, the grown mycellium was harvested by filtration and dried to a constant weight on

oven at 60°C. The dried mycellium was suspended in an appropriate amount of water and homogenized at 15,000 rpm for 3 min. The homogenate was heated at 100°C for 1 h with refluxing then centrifuged (5,000 rpm×5 min). The supernatant was saved. The pellet was resuspended in water and the suspension was subjected to boiling. The procedure was repeated two more times. All three supernatants were combined and treated with ethanol up to 80%. The mixture was stood at 4°C overnight and centrifuged to precipitate the polysaccharide. The polysaccharide was dissolved in water and dialyzed for 2 days. Non-dialyzable fraction was freeze-dried to afford the polysaccharide.

Monosaccharide analysis of polysaccharide

Monosaccharide analysis was carried out according to the previous method (Song *et al*, 1995b). Briefly, a sample of polysaccharide (2 mg) was hydrolyzed with 2N trifluoroacetic acid (TFA) at 121°C for 1 h. After removing TFA by repeated evaporation, sugars in the hydrolyzate were converted to their corresponding alditol acetate. The alditol acetate was identified by GC analysis. Values are an average of two times analyses.

Results and Discussion

Mycelial growth of *P. linteus on various* media

Many different media, which has been used for cultivation of fungi and mushroom, were tested for selecting medium which gave good growth of *P. linteus*. Table 2 showed that *P. linteus* grew well on YMG, PYG, and GP media but did not on YM and GPY media. Thus GP medium was selected for further study because it gave a good mycelial growth and this medium was used for cultivation of *Ganoderma tsugae* (Zhang *et al*, 1994), previously. The slow growth on GPY medium may reflect relatively large amount of nitrogen source.

Table 2. Mycelial growth of *P. linteus* on different media

Medium	Mycelial dry weight (g/100 ml)
YMG	0.93±0.07
YM	0.37±0.02
PYG	0.92±0.07
GP	0.90±0.03
GPY	0.26±0.03

Table 3. Carbon source effect on growth and polysaccharide production of *P. linteus*

C source	Dried mycelium (g/100 ml)	Polysaccharide yield (%)
Glucose	0.90±0.04	4.76±0.41
Galactose	0.25±0.02	8.66±1.53
Mannose	0.46±0.03	13.70±1.02
Arabinose	0.23±0.03	11.90±1.14
Xylose	0±0	N.D.*
Rhamnose	0.02±0.002	N.D.
Sorbitol	0.04±0.003	N.D.
Mannitol	0.04±0.005	N.D.
Sucrose	0.02±0.004	N.D.
Starch	0.24±0.05	5.49±0.57

* Not determined.

Carbon source effect on mycelial growth

When *P. linteus* was cultivated on liquid medium containing different carbon sources for 12 days at 30°C with shaking, the mycelial growth and yield of polysaccharide production were investigated and found to be different for the used carbon sources (Table 3). The amount of the dried mycelia ranged from 0 g to 0.9 g. The fungus showed the best growth on glucose as carbon source. The growth on galactose, mannose, arabinose, and starch was relatively good to give about 0.2~0.5 g of the mycelium. But the growth on xylose, rhamnose, sorbitol, mannitol, and sucorse was little observed and the mycelium less than 0.04 g was obtained. In contrast, a white mutant of *Ganoderma lucidum* showed

a good growth on xylose, sorbitol, and mannitol as on glucose (Cho *et al*, 1993). *Flammulina velutips*, an edible mushroom, showed maximum mycelial growth on mannitol (Song *et al*, 1995a). Thus each mushroom seems to require different carbon source for maximum mycelial growth. The variation in growth on different carbon source could be explained by the lack of suitable hydrolase or transport system.

Carbon source effect on polysaccharide production

The polysaccharides were extracted from the dried mycelia as above mentioned method. The production yield of the polysaccharide was in range of 5~14% (Table 3). The highest yield was obtained on the mycelia grown on mannose and the lowest one was on glucose. Although the growth was relatively good on mannose and arabinose, the production yield was the highest. For the glucose, the result was reverse. The pattern of polysaccharide production was not thus consistent with that of growth in *P. linteus*. These results indicated that carbon source can be used to improve the production yield of polysaccharide and that the good mycelial growth was not a determining factor on high production yield of polysaccharide in *P. linteus*. Similar result was observed in *Ganoderma lucidum* (Sone *et al*, 1985). However, Park *et al* (1994) reported that good growth was closely related to the protein-bound polysaccharide production in *Coriolus versicolor*.

Carbon source effect on monosaccharide composition of polysaccharide

Monosaccharide of the extracted polysaccharide was determined as their alditol acetate. While *P. linteus* was cultured on different carbon sources, all extracted polysaccharides from the mycelia had a similar monosaccharide composition (Table 4). Thus the polysaccharide was found to be glucan with a small amount of galactose and mannose. But ratios between main sugars of glucose, galactose, and mannose were different for all polysaccharides. Polysaccharides from mycelia grown on glucose and mannose was composed of 90% of glucose, about 5% of mannose galactose but the one from mycelia on galactose contained about 80% of glucose and about 10% of mannose and galactose. In some polysaccharides a trace amount of fucose and xylose were also detected. These differences in monosaccharide composition of polysaccharide, microheterogeneity, might have an important effect on their biological activity.

Table 4. Carbon source effect on monosaccharide composition in polysaccharide from *P. linteus*

C source	Monosaccharide component (%)*				
	Man	Gal	Glc	Xyl	Fuc
Glucose	5.3	4.9	89.9	+**	—
Galactose	11.8	9.3	78.7	—	—
Mannose	5.1	5.9	88.9	—	+
Arabinose	7.3	7.3	85.4	+	+
Starch	2.9	2.1	95.0	—	—

*Aberage values of two times analyses.
**Less than 1%.

On exopolysaccharide production, there are many reports that the culture medium can drastically affect the nature, composition, as well as the biosynthetic reaction rate and yield. For examples, in *Xanthomonas juglandis*, potassium-and magnesium-limited conditions afforded xanthan with a higher glucose, mannose, and glucuronic acid and reduced branch content (Kennedy and Bradshaw, 1984). Therefore, the formulation of the more refined and chemically-defined medium might be a possible and good approach to modify the polysaccharide structure in *P. linteus*.

Table 5. The effect of pH on growth and polysaccharide production of *P. linteus*

pH	Dried mycelium (g/100 ml)	Polysaccharide yield (%)
5	0.70±0.06	7.94±1.02
6	0.74±0.05	5.16±0.63
7	0.90±0.04	6.0 ±0.71
8	0.60±0.01	5.51±0.62
9	0.47±0.14	6.22±0.60

Table 6. The effect of pH on monosaccharide composition in polysaccharide from *P. linteus*

pH	Monosaccharide component (%)*			
	Man	Gal	Glc	Fuc
5	4.2	5.4	90.4	+**
6	4.3	3.8	91.9	+
7	7.7	5.6	86.7	+
8	6.7	7.9	85.4	+
9	6.0	5.2	82.8	+

*Average vakyes of two times analyses.
**Less than 1%.

The pH effect on mycelial growth

The result of the pH experiments showed that *P. lintues* grew well in acidic and neutral pH but did not in alkaline pH (Table 5). Thus the fungus gave 0.9 g of the mycelium at pH 7.0 but only 0.5 g of mycelia was produced at pH 9.0. A similar phenomenon was also observed in *Coriolus versicolor, Lentinus edodes* (Park and Lee, 1991), *Ganoderma lucidum* (Cho *et al*, 1993), and *Naematoloma sublateritium*(Kang *et al*, 1994). Thus mycelial growth appears to favor the acidic and neutral pH.

The pH effect on polysaccharide production

The pH effect was not much drastic in polysaccharide production as carbon source and the range in production yield was 5~8%. No close relation between pH and the yield of polysaccharide in *P. linteus* was observed (Table 5). But in *Coriolus versicolor*, the optimum pH for production of protein-bound polysaccharide was at the range of 5-6 and at above pH 6, the production yield was reduced rapidly (Park *et al*, 1994).

The pH effect on monosaccharide composition in polysaccharide

All polysaccharides extracted from mycelia grown on different pH were also found to be glucan containing mannose and galactose (Table 6). The microheterogeneity was also observed. With increasing pH, the glucose amount in the polysaccharide generally decreased from 92% to 83% but the variation in the amounts of mannose and galactose was only 4%.

In summary, above results suggested that both growth and production yield should be considered in improvement of polysaccharide production. In order to modify the polysaccharide, the change of carbon source is more effective than that of pH. Although relatively few of existing monosaccharides are commonly found in the composition of polysaccharides, the various permutation available for linkage between the monomer units via glycosidic linkages is large. When the further variations in sugar ring conformation are considered, it is clear that the variety of possible chemical structures for biopolymer produced by microorganisms is enormous. Therefore the microheterogeneity of polysaccharide may be significant in the modification of the polysaccharide.

적 요

상황(*Phellinus linteus*)의 균사체 생장과 다당류 생산 및 다당류에서의 단당류 구성비에 대한 배양 조건의 영향을 조사하였다. 여러 가지 배지를 사용하여 실험한 결과 GP(glucose-peptone)배지에서 균사 생장이 가장 양호하여, 이 배지를 사용하여

탄소원 및 pH 영향를 조사하였다. 탄소원중 glucose에서 최대 균사체 생장 (0.9 g/100 ml)을 보였고, 다당류의 수율은 5~14%로서 mannose에서 최대이었다. 최적 pH는 7이었으며, 산성 pH에서는 비교적 좋은 생장을 보였으나, 알칼리 pH에서는 생장이 급격히 감소하였다. 한편 pH에 따른 다당류 수율은 5~8%로 큰 변화를 보이지 않았다. 또한 탄소원 및 pH에 따른 단당류 구성비의 변화가 관찰되었다. 즉 탄소원에 따라서 다당류에서 glucose, mannose, 및 galactose 함량은 각각 80~95%, 3~12%, 그리고 2~10%이었다. 한편 pH 변화에 따라서, glucose는 83~92%, mannose는 4~8%, 그리고 galactose는 5~8%의 구성비를 보였다. 이 결과는 배양 조건에 의한 항암면역활성 다당류의 구조의 변환 및 생산 증강이 가능함을 시사한다.

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