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Optimization of *in Vitro* Cultivation of *Inonotus obliquus*[*1]

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ABSTRACT

This study was performed to get the basic information concerned to the optimum culture condition of *Inonotus obliquus*. Several solid media, PDA, MEA and Czapek-Dox, and three liquid media were adopted for the *in vitro* cultivation. Some main features of the fungal morphological characteristics under cultivation conditions were observed and described. Preliminary results showed that appearance of the mycelial mat, hyphal size and substrate pigmentation differed according to the media. The PDA medium was the most favorable substrate for the growth on solid culture, followed by MEA and Czapek-Dox media. Concerned to the addition of amino acids, 5 amino acids, such as alanine, alginine, isoleucine, leucine and threonine, enhanced to the mycelial growth. Isoleucine was shown the best fungal growth. An important morphological hyphal structure for the fungus, the setae, was found in abundance and diverse its shape and size. In liquid culture, fresh potato broth was the best growth stimulant of the fungus, followed by Malt extract and potato broth. Addition of yeast extract to the liquid media had improved the biomass, but not laccase production.

Keywords : *Inonotus obliquus*, solid culture, liquid culture, optimum culture condition, PDA, MEA, potato broth, amino acids

1. INTRODUCTION

Far East Asian countries have been used many mushrooms (Kong *et al.*, 1991; Lee *et al.*, 2000; Shin *et al.*, 2000a; Jonathan and Fasidi, 2001; Kim *et al.*, 2001; Fang and Zhong, 2002; Hwang *et al.*, 2003; Shin *et al.*, 2004) for centuries in folk medicine for the common gastrointestinal diseases, such as gastritis and stomach ulcer etc. The fact that there were few cancer patients in certain villages of Asia, where people had a habit of drinking Chaga as tea was supported by the experimental results showing the positive anti-tumor effect of its extractives. The sterile sclerotium of *I. obliquus* is called Chaga in Russian. Chaga has also been used as a cure for skin, heart, liver diseases and tuberculosis (Shivrina, 1967; Kier, 1961; Loviagina and Shivrina, 1962). Nowadays, the medicinal value of *I. obliquus* is commonly recognized in many countries of Asia, Europe and America (Kahlos *et al.*, 1987; Kahlos *et al.*, 1996; Ichimura *et al.*,

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1998; Mizuno *et al.*, 1999; Shin *et al.*, 2004) and the interest to this fungus as a health-improving preparation has been increased.

The tree species found this fungus, are mostly genus *Betula* (*B. lutea* Michaux, *B. populifolia* Marsh, *B. papyrifera* Marshall, *B. lenta*, *B. ermanii genuine*, *B. grossa* Sieb, *B. maximowicziana*, *B. verrucosa*, *B. pubescens*, *B. alleghaniensis*, and *B. platyphylla* var. *japonica*). This fungus was rarely found on alder (*Alnus* genus), rowan (*Sorbus* genus), hop hornbeam (*Ostrya* genus) and oak (*Quercus* genus) (Shin, 2001a). In Eastern European countries, the sclerotium of this fungus has been used as one of folk medicines since the 16th or 17th century (Kahlos and Hiltunen, 1983). Also, the Khanty of West-Siberia used this fungus to prevent and treat heart disease, liver disease, stomach disease and even tuberculosis (Saar, 1991).

Inonotus obliquus (Pers. Ex. Fr.) Pilat is a white-rot fungus belonging to Hymenochaetaceae. The external appearance of the sclerotium of *I. obliquus* is a crusty and black surface, split up into large scales, with a brittle charcoal-like consistency. The internal appearance of the sclerotium is dark brown, because of the pigments produced by hyphae dye in wood tissues (Shin *et al.*, 2000a; 2000b; 2001a; 2001b; 2001c; 2002). In nature, this fungus invades mainly living birch trees through wounds, which cause decay and eventually lead to death of the tree. So far, many researches (Kahlos and Hiltunen, 1983; Kahlos *et al.*, 1987; Kahlos *et al.*, 1996; Ichimura *et al.*, 1998; Mizuno *et al.*, 1999; Shin *et al.*, 2000a; 2000b; 2001a; 2001b; 2001c; 2002; 2004) have been devoted to analyzing the biologically active compounds in *I. obliquus*, so that render this fungus medicinal value. The results revealed that mycelia (sclerotium) of *I. obliquus* contain a wide variety of active triterpenes. Other researchers have found active polysaccharides, which are common components in most medicinal mushrooms. Recent publications on laboratory-scale cultivations of different medicinal mushrooms have been focused mainly on genera *Phellinus*, *Ganoderma*, *Cordyceps* and *Paecilomyces*, while reference on *in vitro* cultivation of *I. obliquus* appears to be rather rare. Although *I. obliquus* is a valuable medicinal fungus, its growth in nature as a phytoparasite causes damage to the forest. Therefore, *in vitro* cultivation of this fungus would present scientific and practical interest.

In this study, the optimization of culture conditions of *I. obliquus* under the laboratory condition was performed. Hopefully, this study would be of some useful starting data for the future research on mass production of this precious fungus.

2. MATERIALS and METHODS

2.1. Strain

Inonotus obliquus CBNU-1 was obtained from the culture collections of the School of Forest Resources, Chungbuk National University. The stock culture was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured every second month.

2.2. Culture Media

2.2.1. Solid Culture

Three media for agar culture were tested for *I. obliquus* CBNU-1 as follows; PDA, Malt extract agar (MEA), and Czapek-Dox. For each medium, the growth of *I. obliquus* was observed and measured based on five agar plates. In order to know the effect of aminoacids addition on the cultures, six amino acids, alanine, alginine, isoleucine, leucine, serine and threonine, were added. Concentration of amino acid was 0.1% (wt/v).

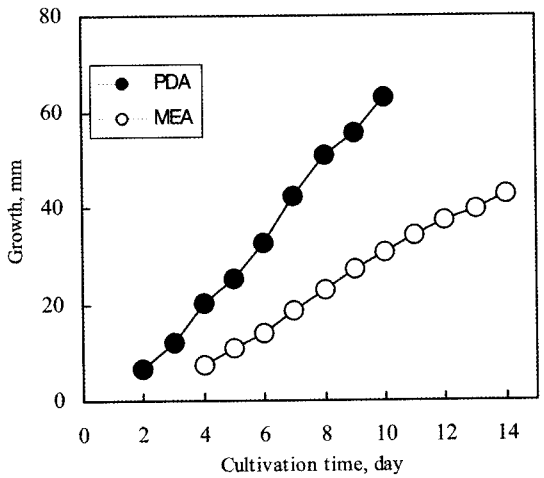

Fig. 1. Growth of *I. obliquus* on PDA and MEA media.

2.2.2. Liquid Culture

Media for flask cultures were used Medium No. 1 (potato dextrose broth, 2.4%) and Medium No. 2 (fresh potato broth, 2% dextrose) and Medium No. 3 (Malt extract, 2%). All media were prepared at initial pH 6.0 and autoclaved at 121°C for 20 minutes. For each liquid medium, five 25-㎖ aliquots were delivered into 100-㎖ flasks.

2.3. Cultivation Conditions

The culture was grown statically at 26°C. The mycelial growth on solid culture was recorded every day by measuring the diameter of the mycelial mat until it covered about 90% of the plate surface. The biomass production on liquid culture was estimated by weights after harvesting the mycelial mats. The procedure of drying and weighing was repeated to ensure constant weights of the samples. Laccase activity of the cultural broth was quantitatively estimated via color reaction with syringaldazine (Cho *et al.*, 2004; Jarosz-Wilkolazka *et al.*, 2004).

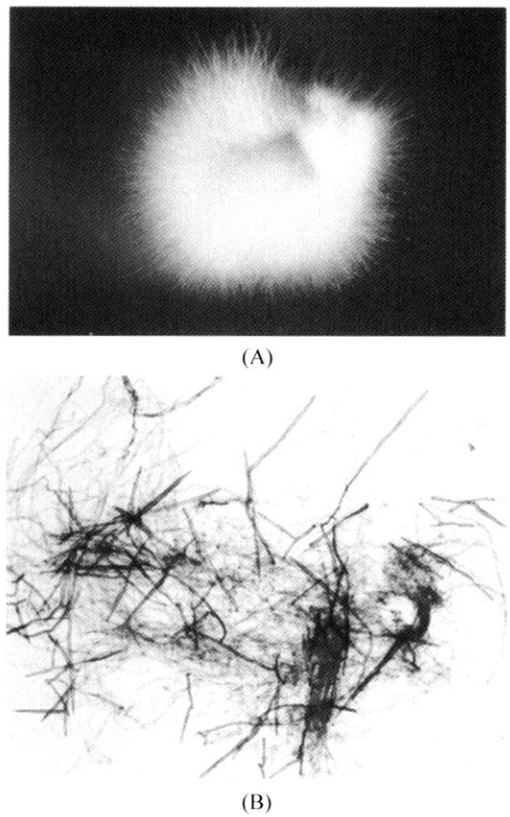

Fig. 2. White aerial hyphae of *I. obliquus* on PDA and MEA media.

3. RESULTS and DISCUSSIONS

3.1. Growth of *I. Obliquus* on Solid Culture

The growth rates and appearances of *I. obliquus* were distinctively different among the three tested media. The growth curves on PDA and MEA are shown on Fig. 1. On PDA medium, the fungus grew faster than that on MEA, and formed abundant white aerial hyphae. On the 3rd day after inoculation, the agar piece looked like cotton bud, from which a thick white mat began to expand (Fig. 2A). An important morphological hyphal structure for the fungus, the setae, was found in abundance

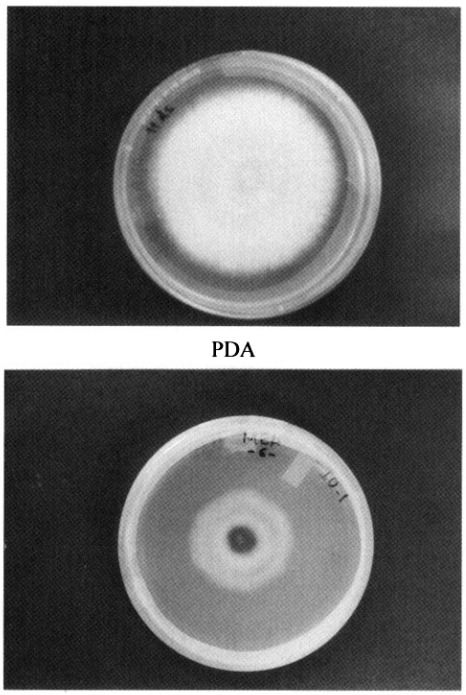

Fig. 3. Effect of growth media on growth of *I. obliquus*.

and diverse of shape and size (Fig. 2B). Meanwhile, it took four days for the culture on MEA to show a measurable growth zone, which formed a rather pale mat slowly expanding on the agar (Fig. 3). The fungus spread on Czapek-Dox agar with an average rate compared with PDA and MEA. However, the mycelial mat was so thin that it was not much distinctive from the medium itself.

Chang (2001) reported that the mycelial growth and density of *I. obliquus* were the highest in the medium of BDA (birch dextrose agar) followed by the order of PDA, yeast meium (YM) and MEA (pH 4.7). Shin (2001) reported birch wood sap did not significantly enhanced the growth of *I. obliquus*. In this experiment PDA resulted in the best mycelial growth. According to Chang (2001), optimal temperature for the mycelial growth and density of *I. obliquus* were

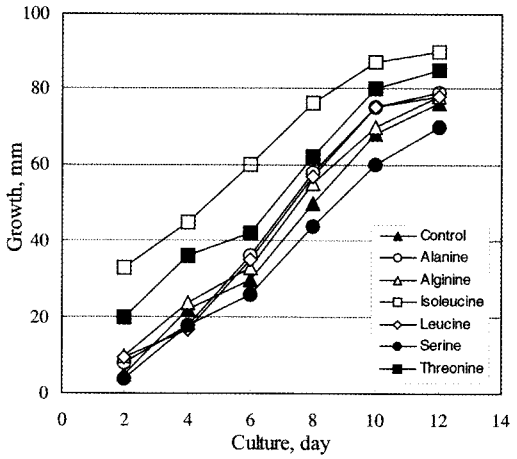

Fig. 4. Effect of amino acid addition on the mycelial growth of *I. obliquus*.

30°C, but at higher temperatures, *e.g.* 40°C, the mycelia were dead. The mycelial growth and its density of KNAC (Korean National Agricultural College) 3005 strain was the highest at 30°C followed by the order of 25, 20, 15, 35, 10, and 5°C. Optimal pH for the mycelial growth and density of 40°C was revealed to be 6.0. Above or below pH 6.0, the mycelial growth and density were shown to be retarded. Shin (2001) also reported the optimal conditions of the mycelial growth of *I. obliquus* were as follows; temperatures are from 25 to 29°C, pH from 5.9 to 6.4, and the best wood meal was beech (*Fagus crenata*). That's why we used the optimum culture condition of 26°C and pH 6.0.

3.2. Effect of Amino Acids Addition on the Mycelial Growth During PDA Culture

Mycelial growth of *I. obliquus* on PDA medium was compared based on the addition of six amino acids. Except serine, five amino acids enhanced to the mycelial growth. Isoleucine was given the best growth rates, and resulted in almost two times higher growth than that of the control. Threonine was also shown higher growth

Table 1. Biomass production of *I. obliquus* on flask cultures

Media	Dry biomass (g/L)	Final pH	Laccase
No. 1	1	5.8	+
No. 1 + YE	1.22	5.82	±
No. 2	2.36	5.49	++
No. 2 + YE	2.66	5.84	+
No. 3	1.65	5.2	+
No. 3 + YE	2.00	5.29	±

Note: YE: yeast extract (0.1%); Dry biomass (12 days).
Laccase activity was quantitatively estimated (++: strong; +: medium; ±: weak).

than the other amino acids.

3.3. Growth of *I. Obliquus* on Liquid Cultures

Three media were used for static flask cultivation of *I. obliquus*. The cultivation was carried out for 12 days, and stopped as the fungus occupied the whole surface of the medium in flask. The yeast extracts (0.1%) were supplemented to three different media as an additional source of vitamin B groups which is essential for fungal growth.

As shown in Table 1, No. 2 medium, fresh potato broth, gave the best results in both mycelial production and laccase synthesis. In the relation to biomass production, the media could be ranked in the following order: No. 2 medium, fresh potato broth>No. 3 medium, Malt extract>No. 1 medium, Difco potato broth. Fresh potato broth was certainly more nutritious than that of canned Difco product. In this experiment, yeast extract enhanced to the biomass production for approximately 12% in medium No. 2 and 17% in two media No. 1 and No. 3, but those laccase activities were not so high. Aerial hyphae were white and abundant in those flasks. As the fungus was growing, all media became more acidic. In addition, light brown pigmentation of the mycelia was observed in Malt extract media.

4. CONCLUSIONS

In this study, *I. obliquus* was cultivated *in vitro* on several agar and liquid media commonly used for fungi cultivation. Some main features of the fungal morphological characteristics under cultivation conditions were observed and described. Preliminary results showed that appearance of the mycelial mat, hyphal size and substrate pigmentation differed according to the media. The PDA medium was the most favorable substrate for the growth on solid culture, followed by MEA and Czapek-Dox media. Concerned to the addition of amino acids, 5 amino acids, alanine, alginine, isoleucine, leucine and threonine, enhanced to the mycelial growth. Isoleucine was given the best growth rates, and resulted in almost two times higher growth than that of the control. An important morphological hyphal structure for the fungus, the setae, was found in abundance and diverse its shape and size. In liquid culture, fresh potato broth was the best growth stimulant of the fungus, followed by Malt extract and Difco potato broth. Addition of yeast extract to the liquid media had improved the biomass, but not laccase production.

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