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Process development for mycelial growth and polysaccharide production in *Tricholoma matsutake* liquid culture

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In this study, the effects of agitation and aeration on mycelial growth and exo-polysaccharide production were examined in batch cultures of *Tricholoma matsutake*. Agitation was varied from 100 to 300 rpm and aeration was varied from 0.5 to 1.5 vvm. Mycelial growth was 21.87 g/l at 150 rpm, and exo-polysaccharide production was 8.79 g/l at 1.5 vvm. When we analyzed the polysaccharide extractions from the cultured mycelium and the culture broth of *T. matsutake*, 1.4 g of crude polysaccharide was found per 100 g of dried weight in the cultured mycelium, and 1.47 g/l of polysaccharides was found in the culture broth. In addition, the amounts of β-Glucan in the soluble polysaccharide fractions of the cultured mycelium and culture broth were 75.4% and 83.6%, respectively. The cultured mycelium and the culture broth contained a higher amount of β-Glucan than that of the fruiting body.

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[**Key words:** *Tricholoma matsutake*; Exo-polysaccharide; Mycelial growth; Culture broth; β-Glucan]

Historically, mushrooms have been an important edible resource for medicinal purposes. Recently, a number of bioactive molecules, including antitumor substances, have been found in various higher Basidiomycetes mushrooms. Unlike existing anti-cancer chemical medications, the polysaccharides from mushrooms are known to have no toxic side effects, and polysaccharide therapy has shown very promising results in prolonging patient life span (1). Polysaccharides derived from mushroom are known to have potent immunomodulating properties. These polysaccharides have unique chemical compositions, consisting primarily of β-Glucan (2, 3). For decades, a diverse range of different mushrooms has been extensively studied for their potential beneficial clinical impacts in both *in vivo* and *in vitro* model systems. From this work, a number of new antitumor and immunomodulating polysaccharides have been identified (4–8). *Tricholoma matsutake*, which is an ectomycorrhizal parasite mushroom, belongs to the Basidiomycotina, Agaricales and Tricholomataceae families (9). Liquid culture has the potential to increase mycelial production in a compact space and shorter time with less chance of contamination. The production of mycelia by liquid culture has been shown to be a promising alternative for producing the fruiting body. It has been reported that when the mycelia of *Lentinus edodes* were grown in a liquid culture, the time for fruiting body formation was shortened (10). *T. matsutake* is very popular and expensive in East Asia due to its unique flavor and taste. Production of the fruiting body is limited, and cultivating the fruiting bodies from mycelia in artificial culture medium has proven difficult. Although demand for this mushroom has increased, only a few studies have examined the liquid cultural characteristics of this fungus in Korea. Thus, if it is possible to overcome the drawbacks associated with cultivating *T. matsutake* in liquid culture, the cultured mycelia and the beneficial substances that it produces may be used in processed foods on a commercial scale. Therefore, in this study, we investigated the effects of aeration rate and agitation speed on mycelial growth and exo-polysaccharide (EPS) production of *T. matsutake* in a 5-l fermentor. In addition, the β-Glucan content in the cultured mycelium and culture broth was analyzed and compared with that of the fruiting body.

MATERIALS AND METHODS

Strain and media The mycelia were isolated from the fruiting body of *T. matsutake*, the higher basidiomycetes mushroom, which was collected from Uljin Gyeong-Buk, Korea. They were cultured on an agar plate containing *T. matsutake* medium (TMM) that was supplemented with antibiotics (50 μg/ml of streptomycin and 60 μg/ml of ampicillin). The TMM agar contained 20 g/l glucose, 1.5 g/l soytone and 1.5 g/l yeast extract. The basal medium for mycelial growth was DMK, which contained 40.0 g/l dextrin, 6.0 g/l yeast extract, 1.0 g/l KH_2PO_4 and 0.5 g/l $MgSO_4 \cdot 7H_2O$. The composition of the medium for mycelial growth in the 5-l bioreactor was modified and contained 40.0 g/l glucose, 30.0 g/l yeast extract, 1.5 g/l KH_2PO_4 and 1.0 g/l $MgSO_4 \cdot 7H_2O$ (11).

Inoculum preparation The seed culture was cultivated for 15 days in 100 ml of the DMK media in a 250-ml flask that was inoculated with 10 ml of an activated stock solution frozen at −70 °C. For mycelial growth, the mycelia were homogenized with a Heidolph DIAX 600 homogenizer (VWR International, West Chester PA, USA), and 10% of the seed culture broth was inoculated into 100 ml of the DMK media in a 250-ml flask. They were then cultivated for 15 days at 26 °C with 120 rpm in a shaking incubator (Vision Scientific Co., Ltd., Buchun, Korea). Fermentation was carried out in a 5-l bioreactor (Korea Fermentor Co., Seoul, Korea). The culture medium was inoculated

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with 10% (v/v) of the seed culture. The aeration rate and agitation speed were varied from 0.5 to 1.5 vvm and from 100 to 300 rpm, respectively, to assess the effects of these parameters.

Analytical methods After 15 days of cultivation, the culture broth was centrifuged at 5000 rpm for 20 min. Precipitated mycelia were washed three times with distilled water and then dried for 24 h at 60 °C. The EPS derived from the liquid culture broth was prepared by ethanol precipitation with three times volume followed by standing at 4 °C overnight, filtered with 0.45 μm Whatman filter paper and then dried in a drying oven to a constant weight. The dry weight of mycelia and EPS was quantified by subtracting the dry weight of the filter paper from the total weight. The residual glucose in the cultured broth was determined by using a glucose assay kit (Sigma Diagnostics St. Louis, MO, USA) and glucose analyzer (YSI Inc., Yellow Springs, Ohio, USA) according to the manufacturer's instructions.

Extraction and quantitative analysis of β-Glucan The exo-polysaccharide fraction derived from the liquid culture broth of *T. matsutake* was prepared by ethanol precipitation, dialyzed for 7 days and then lyophilized. Polysaccharide fractions that were derived from the cultured mycelia and fruiting body of *T. matsutake* were prepared by ethanol precipitation and lyophilized following hot water extraction. Each fraction of the crude polysaccharide extract was solubilized with distilled water and centrifuged, and insoluble fractions were eliminated. The concentration of β-Glucan in each soluble fraction was determined using a BGSTAR kit (Wako Pure Chemical Industries, Ltd., Doshomachi, Chuo-Ku, Osaka, Japan) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Effect of culture pH In order to investigate the effect of culture pH on mycelial growth and EPS production, *T. matsutake* was cultivated in solutions of different acidity (uncontrolled pH and pH of 6) in a 5-l bioreactor. In the case of cultivation without pH control, pH did not change significantly during cultivation. Residual glucose was slowly consumed and remained 17.89 g/l at the 14th day of culture for the *T. matsutake* liquid culture at uncontrolled pH. Mycelial growth of *T. matsutake* increased continuously, and the highest mycelial biomass (19.11 g/l) was obtained at the 11th day of culture. After the 4th day of culture, EPS production of *T. matsutake* increased continuously, and the highest EPS production (8.42 g/l) was obtained at the 11th day of culture (Fig. 1A). Residual glucose was slowly consumed and remained 16.67 g/l at the 14th day of culture for the *T. matsutake* liquid culture at a pH of 6.0 using 1 N HCl and 1 N NaOH. Mycelial growth of *T. matsutake* increased continuously after the 5th day of culture, and the highest mycelial biomass (19.68 g/l) was obtained at the 11th day of culture. After the 4th day of culture, EPS production of *T. matsutake* increased continuously, and the highest EPS production (7.80 g/l) was obtained at the 11th day of culture. These results suggest that pH control does not significantly affect mycelial growth and EPS production (Fig. 1B).

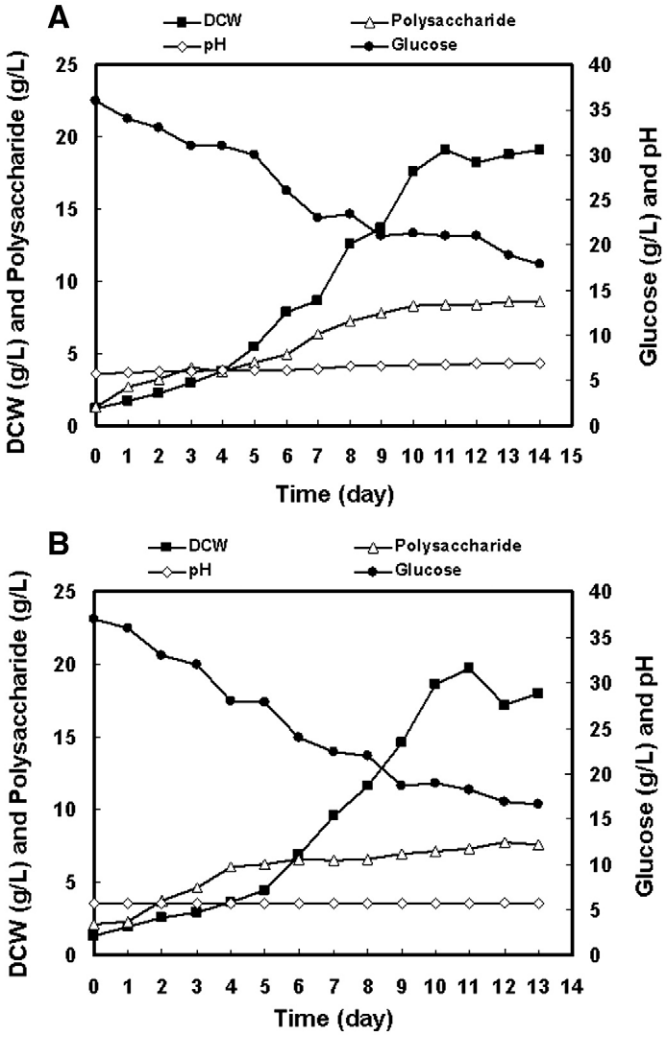

FIG. 1. The effect of culture pH on mycelial growth, polysaccharide production and residual glucose in a 5-l jar fermentor. The mycelial were cultivated for 14 days at 26 °C at 250 rpm, 1.0 vvm and a 10% inoculum size in modified medium. The working volume in the 5-l jar fermentor was 3.0 *l*. (A) The profiles of cell growth, glucose, pH, DO and polysaccharide production without controlling the initial pH. (B) The profiles of cell growth, glucose, pH, DO and polysaccharide production with an initial pH of 6.0.

Effects of agitation on mycelial growth and EPS production When the agitation level was varied, we observed a higher level of mycelial growth at lower agitation speeds, as expected. The maximum mycelial biomass (21.87 g/l) was obtained at 150 rpm. In contrast, the opposite effect was observed in EPS production, where a higher level of EPS production was achieved at the highest agitation speed. The maximum EPS production (8.86 g/l) was obtained at 300 rpm. Residual glucose was slowly consumed at higher agitation speed. EPS production did not increase for residual glucose concentrations lower than 20 g/l, and the residual glucose concentration increased when EPS production decreased (8th day of culture) (Fig. 2). Agitation is an important parameter for substrate, heat and dissolved oxygen (DO) transfer, and it creates a shear stress that causes morphological changes. DO and shear stress are important factors that affect β-Glucan production. Different optimal DO levels have been reported for maximal mycelial growth and EPS production in fungi (12–14). The β-(1-3)-glucan of *Alcaligenes faecalis* ATCC 31479 is optimally produced at higher DO levels and under lower shear stress conditions (15). The β-(1-3)-glucan accumulation in the filamentous mycelia of *Schizophyllum commune* is higher under DO limited conditions (16). In contrast, β-Glucan production for *Fusarium oxysporum* in a stirred-jar fermentor requires higher DO levels for bioactive polymer production, and DO requirements vary when different medium sources are used (17). In the present study, when the DO was depleted after the 7th day of culture, mycelial growth of *T. matsutake* increased continuously, but EPS production was not enhanced (Fig. 2). Therefore, these results indicate that higher levels of mycelial growth in *T. matsutake* can be achieved at lower DO levels and that increased EPS production can occur at higher DO levels.

Effects of aeration on mycelial growth and EPS production The maximum mycelia biomass (20.85 g/l) and EPS production (8.79 g/l) were observed at aeration rates of 0.5 and 1.5 vvm, respectively. The mycelial growth and EPS production profiles at different aeration conditions were quite similar to those observed at the different agitation conditions (Fig. 3). Previous studies have reported similar results for EPS production in batch culture (18). Aeration aids produce secondary metabolites by promoting the mass transfer of substrates, products and oxygen. Therefore, aeration intensity is an important factor in EPS production (13). Although residual glucose was present long enough to enhance mycelial growth and EPS production, polysaccharides were gradually degraded, and EPS production slowly increased after the 8th day of culture (Fig. 3). It

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