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Study of mycelial growth and bioactive polysaccharide production in batch and fed-batch culture of *Grifola frondosa*

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Abstract

The fermentation of *Grifola frondosa* was investigated in the shake flasks and a 5-L jar fermenter in batch and fed-batch modes. In the shake-flask experiments, the preferable mycelial growth and exopolysaccharide (EPS) production was observed at relatively low pH; maltose and glucose were preferred carbon sources for high mycelial production. The EPS was doubled after 13 d of cultivation when glucose was increased from 2% to 4%. Yeast extract (YE) (0.4%) in combination with corn steep powder (CSP) (0.6%) and YE (0.8%) in combination with CSP (1.2%) were preferred nitrogen sources for high mycelial production and EPS production, respectively. All plant oils tested significantly stimulate cell growth of *G. frondosa* but they failed to enhance EPS production. The EPS products usually consisted of two fractions of different molecular sizes varied by the plant oils used. The fed-batch fermentation by glucose feeding was performed when the glucose concentration in the medium was lower than 0.5% (5 g/L), which greatly enhanced the accumulation of mycelial biomass and EPS; the mycelial biomass and EPS were 3.97 g/L and 1.04 g/L before glucose feeding, which reached 8.23 g/L and 3.88 g/L at 13 d of cultivation. In contrast, the mycelial biomass and EPS in the batch fermentation were 6.7 g/L and 3.3 g/L at 13 d of cultivation.

Keywords: *Grifola frondosa*; Exopolysaccharides; Submerged culture; Fed-batch fermentation; Batch culture

1. Introduction

Grifola frondosa, also known as hen-of-the-woods or maitake, is a *Basidiomycete* fungus belonging to the order *Aphyllopherales*, family *Polyporaceae* (Lincoff, 1981); it is an edible mushroom and has been marketed in Asia for medicinal use. The commercial production of maitake began in 1981 in Japan (Takama et al., 1981); since then, production and consumption of this mushroom has been increased rapidly in the world. By 1997, world production of maitake reached 331,000 ton (an increase of 40.6-fold compared with 1991) (Shen, 2001).

G. frondosa (maitake) has gained in popularity among consumers, not only because of its excellent flavor, but also because of its claimed medicinal properties; it was shown to have both anti-tumor and anti-viral properties (Hobbs, 1996; Jong and Birmingham, 1990; Lindequist et al., 2005; Mizuno and Zhuang, 1995). Other medicinal uses of this mushroom include blood pressure regulation (Adachi et al., 1988; Kabir et al., 1987), control of diabetes (Horio and Ohtsuru, 2001; Kubo et al., 1994), reduction of cholesterol (Fukushima et al., 2001; Kubo and Nanba, 1996, 1997), treatment of chronic fatigue syndrome (CFS) (Jong and Birmingham, 1990; Nanba, 1993), protection of liver function (Kubo and Nanba, 1996; Ooi, 1996) and anti-HIV activity (Nanba et al., 1999, 2000). The major anti-tumor substances, which have been obtained from extracts of the fruit body and liquid-cultured mycelium, were attributed to polysaccharides (Mizuno et al., 1995). These polysaccharides have

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been identified as many types of glucans in that more than 20 anti-tumor polysaccharides have been isolated and purified from *G. frondosa*; each active polysaccharide has a basic structure of a (1–6)-β-branched (1–3)-β-D-glucan and heteroglycan or heteroglycan-protein complex as the major component (Mizuno et al., 1986; Ohno et al., 1986; Nanba et al., 1987). Clinical studies have shown that these polysaccharides stimulate the mammalian immune system by activating macrophage and subsequently increasing the T-cell cascade in that they enhance the secretion of cytokines like TNF-α, IFN-γ, IL-1β, etc. and potentiate the delayed-type hypersensitivity response associated with tumor growth suppression (Adachi et al., 2002; Kodama et al., 2003; Reshetnikov et al., 2001; Wasser and Weis, 1999). In this regard, β-glucan has been used as an immunotherapeutic agent for cancer treatment in Japan since 1986 (Kodama et al., 2002; Konno et al., 2002; Lindequist et al., 2005).

Although bioactive polysaccharides were obtained form both the fruit body and liquid-cultured mycelium of *G. frondosa* (Mizuno et al., 1986; Ohno et al., 1986; Suzuki et al., 1989; Zhung et al., 1994), most previous efforts have been focused on cultivating this mushroom on solid artificial media (for fruit body production) rather than in submerged cultures (for mycelial extract and/or exopolysaccharide (EPS) production). However, because the solid culture of *G. frondosa* takes long time to complete a fruiting body, investigators have recently exerted their efforts to prepare this mushroom from submerged culture in the form of mycelium for use in the formulation of nutraceuticals and functional foods (Lee et al., 2003, 2004). Submerged culture gives rise to potential advantages of higher mycelial production in a compact space and shorter time with fewer chances of contamination (Friel and McLoughlin, 2000; Yang and Liau, 1998; Yang et al., 2003). In addition, it ensures standardized quality and year around production (Chang, 2001).

A few attempts have been made to obtain optimal submerged culture conditions for polysaccharide production from *G. frondosa* (Lee et al., 2003, 2004); however, currently available reports on nutritional requirements and environmental conditions in submerged cultures are still very limited in that optimal submerged culture conditions have not been demonstrated extensively by far. In the present study, optimization of mycelial growth and bioactive polysaccharide production of *G. frondosa* was evaluated in shake flasks and a jar fermenter in batch and fed-batch modes, particularly the control of glucose feeding for enhancing mycelial biomass and polysaccharide production were demonstrated.

2. Methods

2.1. Microorganism and inoculum

G. frondosa used in this study was obtained from Taiwan Agricultural Research Institute, Wufeng, Taiwan. The strain *G. frondosa* was maintained on potato dextrose agar (PDA) slants. Unless otherwise mentioned, the slant was incubated at 25 °C for 14 days, and then stored at 4 °C. The mycelium was activated by culturing at 25 °C for 7 days on a modified agar plate which consisted of the following: 4 g/L yeast extract, 10 g/L malt extract, 2 g/L glucose, 1.0 g/L molasses, 10 mL/L mineral salt solution (120 g/L $MgSO_4 \cdot 7H_2O$, 6 g/L NaCl, 20 g/L KH_2PO_4, 20 g/L $CaCl_2$, 10 g/L $FeSO_4 \cdot 7H_2O$, and 1.8 g/L $ZnCl_2$) and 15 g/L agar. The experimental inoculums were prepared in 250 mL Erlenmeyer flasks containing 100 mL of the medium with four units of a cutter square of activated mycelia. Mycelia agar squares (5 mm × 5 mm) were obtained by punching out the agar plate culture with a sterilized self-designed cutter. The composition of inoculum medium was the same as that of activating medium except that agar was not included. After the culture was incubated on a New Brunswick rotary shaker (Model G24) at 25 °C, 150 rpm for 7 days, the whole medium including mycelium was poured into a sterilized blender (Osterizer, Taiwan) and homogenized for 30 s to be used as the inoculums in the following experiments (Hsieh et al., 2006).

2.2. Flask culture conditions

The flask culture experiments were performed in 250-mL flasks containing 100 mL of fermentation medium after inoculating with 10% (v/v) of the seed culture. The fermentation medium consisted of the following components: 4 g/L yeast extract (YE), 6 g/L corn steep powder (CSP), 20 g/L glucose, 1 g/L $(NH_4)_2SO_4$, and 10 mL mineral salt solution. The culture was incubated at 25 °C on a rotary shaker incubator at 150 rpm, and 100 mL samples were collected for analyzing biomass dry weight, EPS, intracellular polysaccharide (IPS) productions.

The effects of factors affecting cell growth and the production of components such as EPS, IPS were studied using shake-flask culture on rotary incubator shaker (Wisdom 721 SR-Incubator-Shaker) as described above. Effects of carbon sources on *G. frondosa* culture were studied by substituting various carbon sources such as molasses (ML), sucrose (S), maltose (M) and fructose (F) for glucose in fermentation medium; effects of nitrogen sources on *G. frondosa* culture were studied by varying the concentrations of YE and CSP in fermentation medium. Effects of plant oils on *G. frondosa* culture were also studied using shake flask culture. Plant oils such as soy, peanut and olive oils were supplemented, all at volume fractions of 0.1% and 1%.

2.3. Batch and fed-batch fermentation in jar fermentor

Fermentation was carried out in a fully instrumented and computer controlled 5-L jar fermentor (Biotop, BTF-A-5, Taiwan), equipped with a pH probe (Mettler Toledo 2200) and a dissolved oxygen probe. Agitation was provided by a standard six-blade impeller and aeration was provided by a ring sparger. The fermentation medium, which consisted of the same components as indicated in the