Enhancement of β-D-Glucans Production by *Agaricus blazei* Murill by Nitrogen Supplementation

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Abstract Temporal changes of cell growth pattern and intracellular content of β-D-glucans were investigated with off-gas data in *Agaricus blazei* culture where glucose was intermittently fed. It was observed that the time point of carbon source depletion coincided with the point of sudden drop in the carbon dioxide evolution rate (CER), and that the sole supplementation of glucose was not enough to maintain active cell growth and glucan content. On the other hand, when yeast extract, a typical nitrogen source, was supplemented together with glucose when the CER suddenly dropped because of carbon source depletion, an active cell growth could be maintained until the end of the culture and the glucan content did not decrease with culture time, significantly enhancing glucan productivity.

Key words: β-D-Glucans production, *Agaricus blazei*, nitrogen supplementation

Many mushroom (basidiomycetes) species are considered to be a remarkable natural source, because of their outstanding efficacy in preventing and curing a variety of diseases. Therefore, mushrooms have been used as a traditional remedy in Asia for a long time. Polysaccharides with an antitumor activity have been isolated from various species of mushrooms [4]. These active polysaccharides, β-D-glucans, have relatively high molecular weight and are considered to exert their antitumor activity through raising the host immunity rather than direct toxicity to tumor cells [7]. Having been reported to have an over 90% inhibition rate against the growth of Sarcoma 180/mice, β-

D-glucans from *Agaricus blazei* Murill have attracted much attention as new bioactive molecules [1, 3, 6].

There is a limitation, however, in producing glucans at an economically feasible productivity by the traditional extraction method from the fruit body of *A. blazei*, mainly because solid state culture to produce fruit bodies in quantity requires a large space, very long culture time, and improvement of many difficult engineering problems associated with solid materials handling. A promising alternative to overcome these difficulties is submerged culture of *A. blazei* mycelia in a fermenter [5]. Submerged culture has the potential advantages of higher mycelial production in a compact space and a shorter incubation time with lesser chance of contamination [2]. In addition, exopolysaccharides (EPS), which are also known to have antitumor activity, can concurrently be synthesized and secreted. However, there are several problems to be solved. It is often difficult to have reproducible results, since basidiomycetes, including *A. blazei*, do not make spores in a liquid medium, thus making it very difficult to start the culture with a precise amount of inoculum. Often, their metabolism of growth and polysaccharides synthesis are very complicated, and therefore, not clearly known.

In this study, temporal changes in cell growth pattern and intracellular glucan content were investigated with off-gas data in *A. blazei* culture. The information obtained was applied to enhancing glucans production by supplementing a nitrogen source.

Agaricus blazei Murill was kindly provided by the Rural Development Administration, Korea. Ten-ml aliquots of its mycelium suspension in 20% glycerol were stored at −80°C. The seed culture medium contained (per liter): glucose 20 g, soybean oil 30 ml, yeast extract 4 g, soytone peptone 2 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, $FeCl_3 \cdot 6H_2O$

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0.2 mg. A 250-ml Erlenmeyer flask containing 40 ml of the seed medium was inoculated with 10 ml of mycelium suspension from the stock culture, and the culture was incubated at 28°C and 150 rpm in a rotary shaker for 5 days. Ten ml of seed culture was transferred to 90 ml of the seed culture medium in a 500-ml Erlenmeyer flask for further activation. After 2 days of incubation, the second seed culture was transferred to a 500-ml Erlenmeyer flask with a working volume of 100 ml or a 2.5-l jar fermentor (KoBiotech., Korea). In either case, the inoculum size was 10% (v/v). The shake flask culture medium contained (per liter): carbon source(s) 50 g, yeast extract 4 g, soytone peptone 2 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, $FeCl_3 \cdot 6H_2O$ 0.2 mg. The medium for bioreactor culture contained (per liter): glucose 4 g, dextrin 16 g, yeast extract 4 g, soytone peptone 2 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, $FeCl_3 \cdot 6H_2O$ 0.2 mg. Whenever glucose was exhausted, a concentrated feed medium containing only 400 g/l of glucose, or 400 g/l of glucose with 40 g/l of yeast extract, and 20 g/l of soytone peptone was fed to the fermenter to raise the glucose concentration to about 10 g/l. The temperature was maintained at 28°C and pH was controlled at 5.0 with 2 N HCl or 2 N NaOH. The cell concentration was determined by measuring dry cell weight (DCW). The concentration of glucose was determined by a glucose analyzer (YSI 2700, Yellow Spring Instrument, Yellow Springs, OH, U.S.A.). To extract β-D-glucans from the mycelium, 20 ml of distilled water was added to 1 g of wet mycelium, and the mixture was homogenized with a Waring blender. Extraction was carried out at 121°C for 30 min at 1.2 bar. The resulting suspension was filtered through a syringe filter (0.45 μm, Sartorius, Germany). The filtrate was analyzed by high performance liquid chromatography (HPLC) (YoungLin, Korea) with a GPC column (Ultrahydrogel™ 1000, Waters Co., MA, U.S.A.) and an evaporated light scattering detector (ELSD) (SEDEX 75, Sedere, France) for quantitative analysis of β-D-glucans. Oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) were measured on-line by a gas analyzer (GMATE3000, Lokas, Korea) connected to an IBM-compatible PC on which data acquisition and control programs (AutoLab, Lokas, Korea) were installed.

A. blazei Murill was cultivated in the 2.5-l jar fermenter. When glucose was depleted, the glucose feed medium was fed into the fermenter to raise its concentration to about 10 g/l. The time profiles of cell and glucose concentrations are shown in Fig. 1a. The glucose concentration increased until 24 h and, during this period, the cells grew exponentially with a specific growth rate of about 0.080/h. Thereafter, the glucose concentration began to rapidly decrease, probably due to dextrin depletion, which will be discussed below. The cell mass concentration was about 9.5 g/l at 36 h, when the carbon sources were almost completely depleted with a cellular yield of 0.47 g cells/g carbon sources. After 36 h, glucose was fed intermittently and its concentration
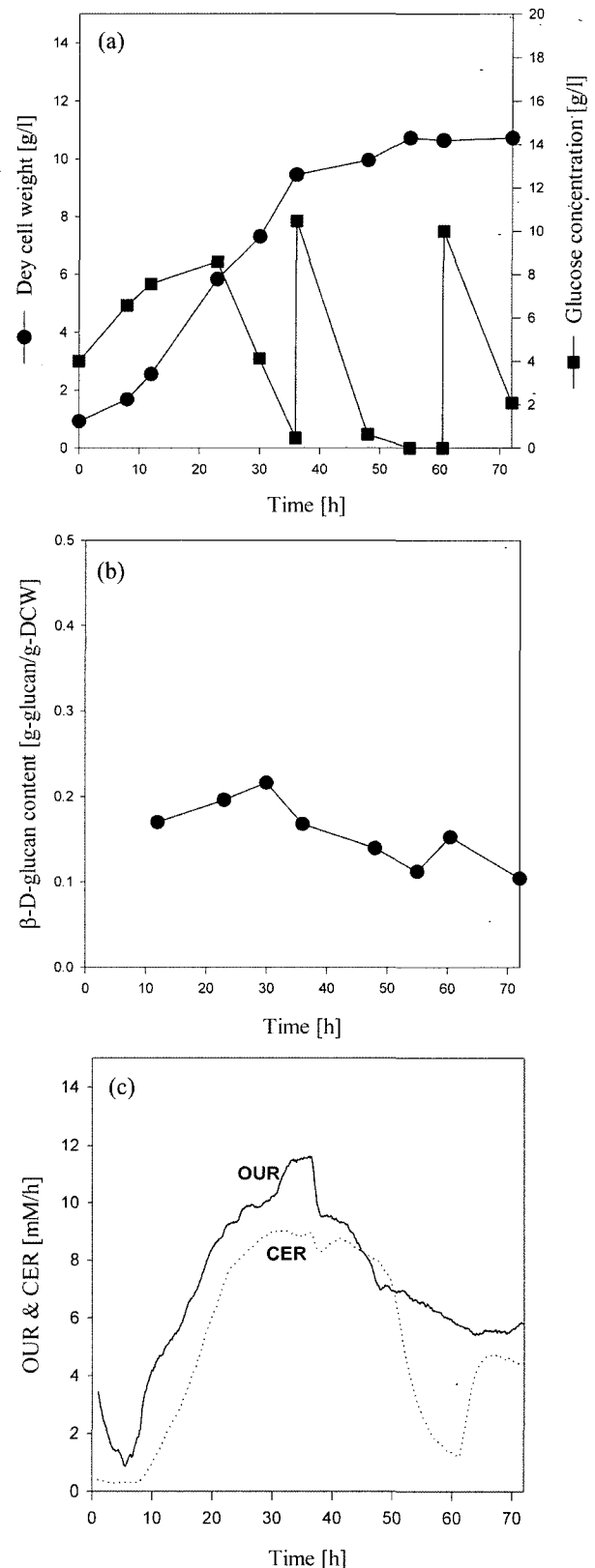

Fig. 1. Time profiles of (a) cell mass and residual glucose concentrations, (b) intracellular content of glucans, and (c) OUR and CER in an *A. blazei* culture with pulse addition of glucose.

changed in the range of 0-10 g/l. The cells, however, did not actively grow any longer, probably due to the depletion of some nutrients essential for cell growth.

The increase of glucose concentration at the beginning of the culture is of interest and a peculiar feature. It seems that the cells metabolized dextrin to produce glucose and simultaneously consumed glucose for growth until 24 h when the rate of glucose formation from dextrin hydrolysis was greater than the rate of its consumption by the cells. Generally, it is known that the utilization of polysaccharides such as dextrin is repressed in the presence of monosaccharides, which are preferentially used by cells. In order to examine the dextrin-hydrolyzing activity of *A. blazei* Murill, a separate culture was run: when 10 g/l of dextrin was supplemented to the culture broth at the mid stage of fermentation, dextrin was completely hydrolyzed only in 30 min (data not shown).

The intracellular content of glucans gradually decreased after 30 h (Fig. 1b). There are two possible explanations for this. Firstly, nutrients other than the carbon source might have been depleted, and consequent deterioration of growth conditions might be responsible for the decrease in glucan content for unidentified reasons. Secondly, the extended exposure to shear stress by agitation might have caused the cells to acquire a tighter and more shear-resistant cell wall structure. In such a condition, the yield of glucan from such cell wall would be incomplete, resulting in underestimation of the glucan content. However, HPLC chromatograms in Fig. 2 clearly show that the content of lower molecular compounds (retention time 16–20 min)
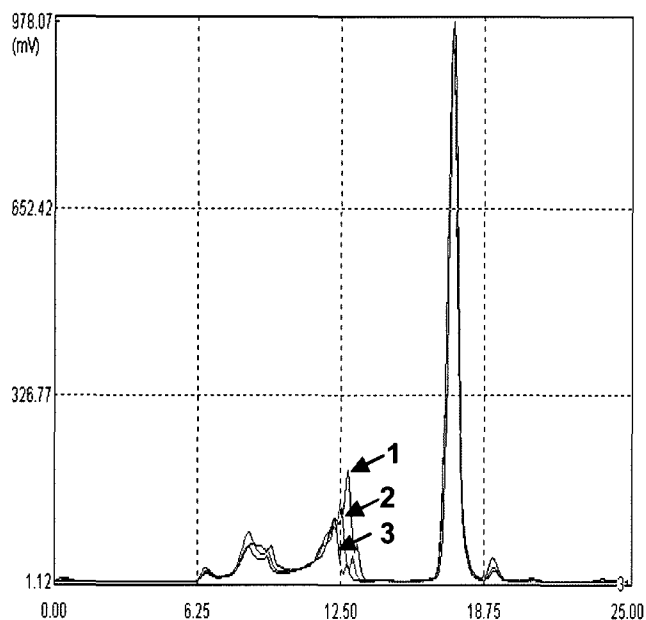
co-extracted with glucans (retention time 6–14 min) did not change, while the glucan content decreased with time. Therefore, it was concluded that the main reason for the decrease in the glucan content was deterioration of growth conditions.

The metabolic activity profile of *A. blazei* Murill was also examined in a real-time situation through off-gas analysis. Figure 1c shows the profiles of oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) during the culture discussed above. OUR after an initial dip increased until 36 h with the cells actively growing, although its increasing rate became lower after 24 h when glucose concentration began to decrease. CER started to increase only after 12 h and, thereafter, remained almost constant until 36 h. As glucose was depleted at 36 h, both OUR and CER decreased rapidly. At this point, a dosage of glucose roughly equivalent to 10 g/l was supplemented to the fermenter. Despite the glucose supplementation, OUR
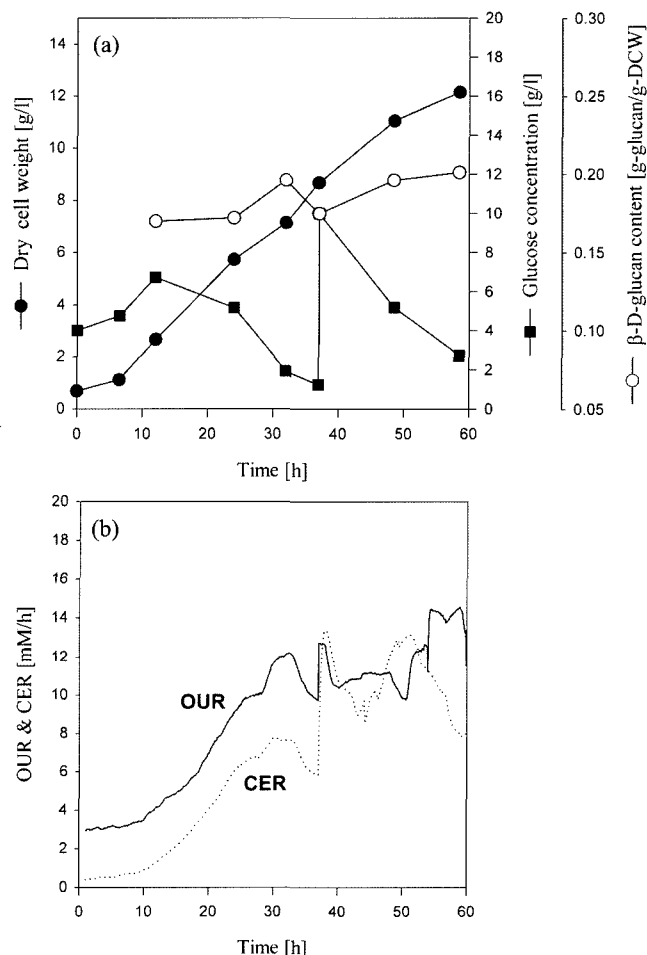

Fig. 2. HPLC chromatograms of the extracts of *A. blazei* mycelia sampled at different culture times (1 at 30 h; 2 at 55 h; and 3 at 72 h).

Fig. 3. Time profiles of (a) cell mass, residual glucose concentrations, and intracellular content of glucans, and (b) OUR and CER in an *A. blazei* culture with pulse addition of glucose and nitrogen source.

kept decreasing. The glucan content also monotonically decreased like OUR after 36 h as mentioned above. On the contrary to OUR, CER increased rapidly soon after glucose supplementation and then decreased as the glucose concentration decreased. Based on these results, it was concluded that the point of carbon source depletion could be detected in real time by observing changes in CER, and that changes in growth activity and glucan content could qualitatively be monitored by observing the OUR profile.

For efficient production of glucans, it is important to prevent the decrease of glucan content as well as to maintain active growth for an extended period of time. However, as can be seen in Fig. 1a, cell growth was much retarded and the glucan content was significantly reduced in the later part of the culture in which only glucose was intermittently fed. This implies that some nutrients other than the carbon sources are needed to be supplemented also. For this reason, yeast extract, a typical nitrogen source, was supplemented together with glucose when CER began to decrease because of depletion of glucose at 37 h in the next run. The concentration of yeast extract supplemented was about 1.5 g/l. As shown in Fig. 3a, with the supplementation of nitrogen source, active cell growth continued up to the end of the culture and the glucan content did not decrease, apposed to the previous culture with only glucose supplementation. Both OUR and CER were higher than those in the previous culture (Fig. 3b). The supplementation of yeast extract enhanced the glucan productivity by about 50%.

Acknowledgment

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