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Effects of Dissolved Oxygen Control on Cell Growth and Exopolysaccharides Production in Batch Culture of *Agaricus blazei*

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Abstract−To investigate the effects of DOC on cell growth and EPS production, DOC was controlled at three different levels of 10, 20, and 40% of air-saturation by manipulating agitation speed in a series of batch cultures of *A. blazei*. The cellular and EPS productivities increased with the DOC level up to 20%. However, DOC had no significant effects over 20%. When DOC was controlled at 20%, the cellular and EPS productivities were observed to increase 1.6-fold and 2.2-fold, respectively, as compared to the control case with no DOC control in which DOC dropped to and thereafter remained at almost zero. Another batch culture was carried out with the DOC controlled at 20% by manipulating the amount of oxygen supply at a rather low agitation speed of 100 rpm. In this case, the cellular productivity was comparable to that of the former case in which DOC was controlled at the same level of 20% by manipulating agitation speed in the range of 100-450 rpm. However, the EPS productivity was about 15% lower than the former case, implying that a sufficient level of agitation is also important for EPS production.

Key words: *Agaricus blazei*, Cell Growth, Exopolysaccharides Production, Dissolved Oxygen Control

INTRODUCTION

Agaricus blazei, a basidiomycete fungus, has been traditionally used as a health food source for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis. Recent studies on this fungus have demonstrated many interesting biological activities, including antitumor, anticarcinogenic, and antimutagenic effects. These studies also have suggested that the bioactive substance in *A. blazei* is polysaccharides, 1, 3-β-D-glucans [Menoli et al., 2001; Takaku et al., 2001]. They are closely related to one another in their structure, but vary in their degree of branching, water solubility, and the nature of their side chains. Having been reported to have over 90% inhibition rate against Sarcoma 180 in mice, β-D-glucans from *A. blazei* have attracted a great attention as new bioactive molecules [Dong et al., 2002; Kawagishi et al., 1989; Mizuno et al., 1998].

A. blazei has normally been produced in solid cultures using substrates such as grain, sawdust or wood. It usually takes several months to cultivate the fruiting body of *A. blazei*, and product quality control is very difficult. There is a great need to supply the market with a large amount of high-quality *A. blazei* products.

For these reasons, in recent years, the submerged culture of *A. blazei* mycelia has received great interest as a promising alternative for efficient production of polysaccharide. Submerged culture has potential advantages of higher cell production in a compact space and a shorter incubation time with lesser chance of contamination [Friel and McLoughlin, 2000]. In addition, exopolysaccharides (EPS), which are also known to have biological effects with the polysaccharide extracted from mycelia, can be concurrently produced and secreted. The recovery of EPS from the culture broth requires relatively simple steps and, thus, is less costly than the recovery of polysaccharide from mycelia [Bae et al., 2000; Cavazzoni and Adami, 1992].

Although many investigators have attempted to obtain optimal submerged culture conditions for cell growth and EPS production with several fungi including *A. blazei*, the nutritional requirements and environmental conditions for submerged cultures have not been clearly demonstrated [Choi, 1999; Ha et al., 1997; Park et al., 2001].

In our previous work, the optimal culture conditions such as pH, inoculum size, and medium composition were identified through batch culture experiments, and the importance of dissolved oxygen concentration (DOC) control was realized [Kim et al., 2004; Na et al., 2004]. However, no detailed research results have been reported regarding the effects of DOC on cell growth and EPS production by *A. blazei*.

In this study, the effects of DOC and its control method on cell growth and EPS production in batch culture of *A. blazei* were investigated in a bioreactor.

MATERIALS AND METHODS

1. Microorganism

The microorganism used in this study was *A. blazei*, which had been kindly provided by The Rural Development Administration, South Korea. Its freeze dried culture was resuscitated in M1 liquid medium. The composition of M1 medium was: 10 g l^{-1} glucose, 40 g l^{-1} dextrin, 4 g l^{-1} yeast extract, 2 g l^{-1} soytone peptone, 2 g l^{-1} KH_2PO_4, 0.6 g l^{-1} $MgSO_4 \cdot 7H_2O$, and 0.2 mg l^{-1} $FeCl_3 \cdot 6H_2O$. The first stock culture was grown in a 250 ml flask containing 50 ml of

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M1 medium at 28 °C on a rotary shaker incubator at 150 rpm for 5 days. The second stock culture was inoculated with 10% (v/v) of the first stock culture broth and then cultivated in a 500 ml flask containing 100 ml of M1 medium at 28 °C on a rotary shaker incubator at 150 rpm. Glycerol (20%, v/v) stock samples were made from the culture broth after 2 days of culture and stored at −70 °C.

2. Inoculum Preparation

For the first seed culture, 50 ml of M1 medium in a 250 ml flask was inoculated with 5 ml of 20% (v/v) glycerol stock and incubated on a rotary shaker incubator at 150 rpm and 28 °C for 5 days. The second seed culture was inoculated with 10 ml of the first seed culture broth and then cultivated in a 500 ml flask containing 100 ml of M1 medium at 28 °C on a rotary shaker incubator at 150 rpm for 2 days. After the second seed cultivation, the pellet-containing culture broth was homogenized aseptically by using a Waring blender (31BL92, Coleparmer Co., NH) for 10 seconds. The homogenized culture broth was used as inoculum for submerged cultures in a bioreactor.

3. Batch Culture in a Bioreactor

Batch cultures were conducted in an automated 7 L bioreactor (LK230, KoBioTech Co., South Korea). The control of DOC was done by employing a software (Autolab LK 930, Lokas Automation Co., South Korea). It provided data logging, interactive parameter processing and supervisory control of the process. The composition of the batch culture medium was: 6 g l^{-1} glucose, 24 g l^{-1} dextrin, 4 g l^{-1} yeast extract, 2 g l^{-1} soytone peptone, 2 g l^{-1} KH_2PO_4, 0.6 g l^{-1} $MgSO_4 \cdot 7H_2O$, and 0.2 mg l^{-1} $FeCl_3 \cdot 6H_2O$. Three liters of culture medium was inoculated with 10% (v/v) of the seed culture and then cultivated for 5 days at 28 °C. The pH was controlled at 5.00±0.05 by automatic addition of 2 N HCl and 2 N NH_4OH. To control DOC at a desired saturated level, two methods were applied: One by manipulating agitation speed with a fixed aeration rate of 1 vvm; and the other by increasing pure oxygen supply as cell mass increased while keeping the aeration rate and the agitation speed at 1 vvm and 100 rpm, respectively. As the control case, a culture was also carried out with no DOC control. The agitation speed and aeration rate were 100 rpm and 1 vvm, respectively, in the control.

4. Analytical Methods

Samples taken from the bioreactor were filtered with a filter paper (Whatman #1, Whatman Inc., NJ). The filtrate was further filtered by using a membrane filter (0.2 μm, Millipore). The resulting filtrate was analyzed by high performance liquid chromatography (HPLC) (L6200, Hitachi Co., Japan) with an Ultrahydrogel™ 1,000 column (0.78×30 cm, Waters Co., MA) and an evaporated light scattering detector (ELSD) (SEDEX 75, Sedere Co., France) for quantitative analysis of EPS concentration. The residual glucose concentration was measured by a glucose analyzer (YSI 2700, Yellow Springs Instruments Co., OH). Dry weight of mycelium was measured after repeated washing of the mycelial pellets with distilled water and drying overnight at 80 °C to a constant weight.

RESULTS AND DISCUSSION

1. Effects of DOC in Batch Culture

In our previous work in which batch cultures of *A. blazei* were carried out at a fixed agitation speed and an aeration rate of 100 rpm and 1 vvm, respectively, with no DOC control, it was observed that DOC dropped rapidly with active cell growth from the early stage of the culture, becoming almost nil at about 24 hours of cultivation, and thereafter cell growth became slow because of the oxygen limitation [Kim et al., 2004].

In this work, a series of batch cultures were carried out at three different DOC of 10, 20, and 40% of air-saturation (Figs. 2-4), to investigate its effects on cell growth and EPS production by *A. blazei*, DOC was regulated at the set point by manipulating agitation speed at a fixed aeration rate of 1 vvm. As the control experiment, a batch culture with no DOC control was also carried out (Fig. 1). The agitation speed and aeration rate were 100 rpm and 1 vvm, respectively,

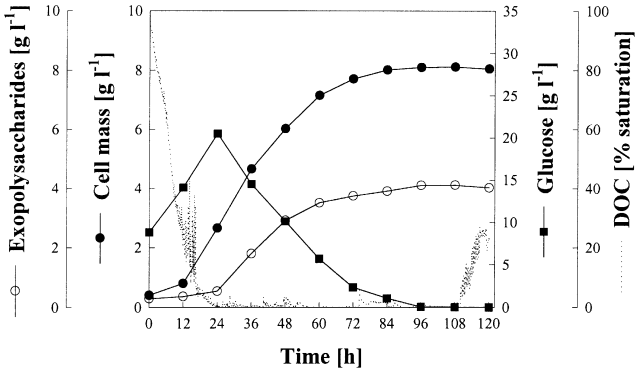

Fig. 1. Time profiles of cell growth, EPS production, glucose consumption, and DOC in batch culture of *A. blazei* with no DOC control.

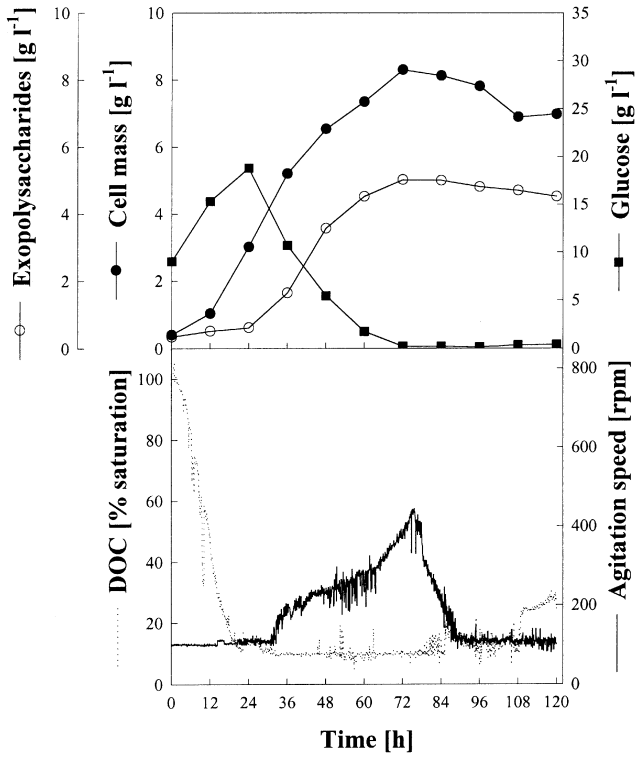

Fig. 2. Time profiles of cell growth, EPS production, glucose consumption, DOC, and agitation speed in batch culture of *A. blazei* with DOC controlled at 10% by manipulating agitation speed.

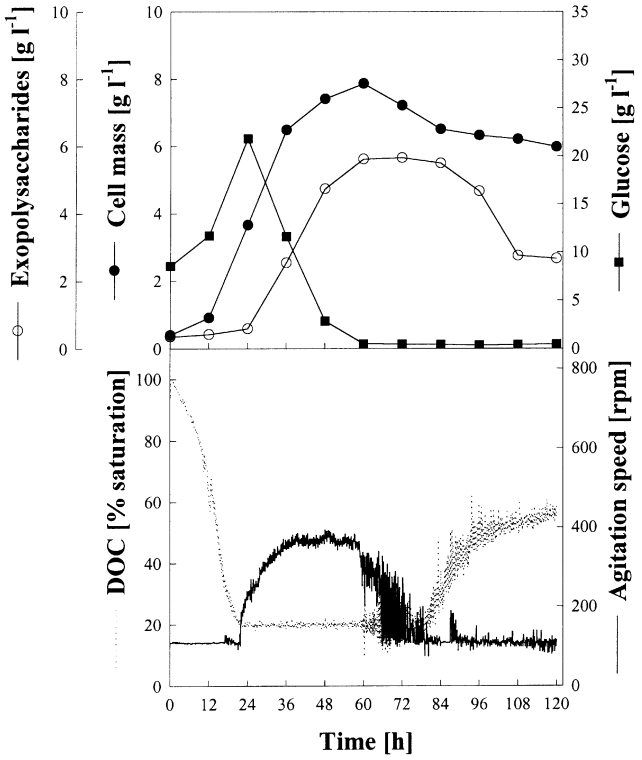

Fig. 3. Time profiles of cell growth, EPS production, glucose consumption, DOC, and agitation speed in batch culture of *A. blazei* with DOC controlled at 20% by manipulating agitation speed.

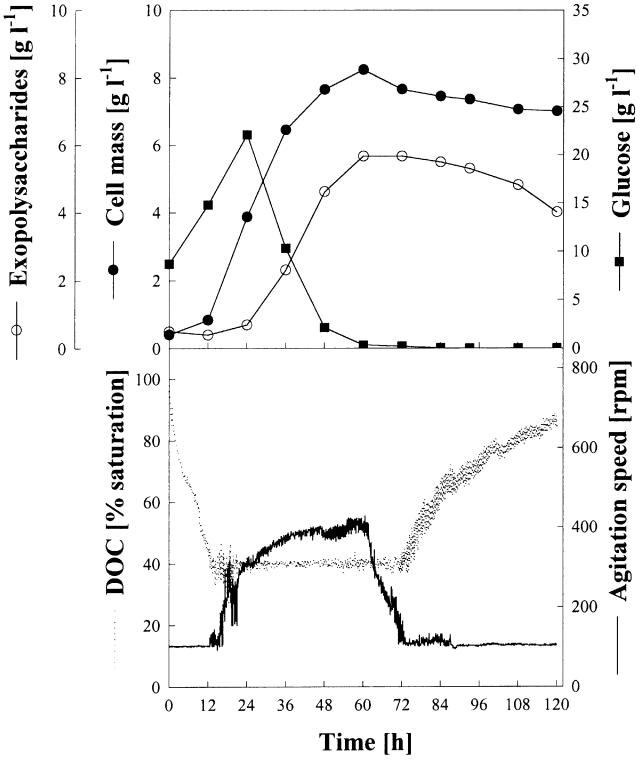

Fig. 4. Time profiles of cell growth, EPS production, glucose consumption, DOC, and agitation speed in batch culture of *A. blazei* with DOC controlled at 40% by manipulating agitation speed.

in the control.

Typical time courses of cell growth, EPS production, glucose consumption, DOC and agitation speed are well represented by their profiles given in Fig. 3, for the case when DOC was controlled at 20%. The glucose concentration increased till about 24 hours of cultivation, and due to the glucose concentration in this period, the cell mass increased exponentially with a maximum specific growth rate of 0.092 h^{-1}. Thereafter, the glucose concentration began to rapidly decrease due to dextrin depletion, and thus the growth was retarded as already discussed in the previous report [Kim et al., 2004]. EPS started to be produced at about 12 hours of cultivation and accumulated. The maximum cell mass was attained after 60 hours of cultivation when glucose was completely exhausted, coinciding with the time-point of maximum EPS concentration. This clearly demonstrated that EPS production was growth associated. The agitation speed was gradually increased with the increase of oxygen demand due to active cell growth until 60 hours of cultivation. Thereafter, however, it was immediately decreased with an abrupt decrease in oxygen demand due to glucose depletion. The DOC was controlled closely in overall.

Table 1. *A. blazei* cultures at different DOC levels

	DOC level [%]				
	No DOC control	10	20	40	20[a]
Process time [h][b]	96	72	60	60	60
Maximum cell mass [g l^{-1}]	8.10	8.30	7.87	8.24	7.86
Maximum EPS concentration [g l^{-1}]	4.10	5.02	5.61	5.67	4.82
Cellular productivity [g l^{-1} h^{-1}]	0.084	0.115	0.131	0.137	0.131
EPS productivity [g l^{-1} h^{-1}]	0.042	0.070	0.094	0.095	0.080
Specific growth rate [h^{-1}]	0.079	0.084	0.092	0.095	0.088
Specific substrate consumption rate [g g^{-1} h^{-1}]	0.039	0.050	0.064	0.061	0.064
Specific EPS production rate [g g^{-1} h^{-1}]	0.005	0.008	0.012	0.011	0.010
Cellular yield on substrate [g g^{-1}]	0.270	0.277	0.262	0.275	0.262
EPS yield on substrate [g g^{-1}]	0.137	0.167	0.187	0.189	0.161

[a]: DOC control by supplying pure oxygen, [b]: Time of glucose depletion.

Results of the experiments for the effects of DOC level are shown in Fig. 1-4, and summarized and compared in Table 1 and Fig. 6. With no DOC control as in the control case, DOC immediately dropped to and remained nil. Cell growth significantly, and thus cellular productivity, increased with a faster substrate depletion as the DOC level was increased up to 20%. But, no significant influence of DOC on cell growth was observed over 20%. The maximum cell mass concentration, being observed in the range of 7.87-8.30 g l^{-1}, was not practically influenced by DOC level, implying that DOC had no influence on the cellular yield. The time courses of EPS concentration followed the similar trend to those of cell growth. However, on the contrary to the maximum cell mass concentrations attained, the maximum EPS concentration and thus EPS yield increased with the DOC level up to 20%, showing no further increase at 40% of DOC.

With the DOC controlled at a high enough DOC level of 20% or 40%, the cellular and EPS productivities increased about 1.6-fold and 2.2-fold, respectively, compared to the control experiment with no DOC control.

It was still in question whether the increased cellular and EPS productivities with increased DOC level were solely due to an increased driving force the transfer of oxygen molecules to the cells. Another possibility speculated was that an increased agitation to maintain the DOC at the increased set point might have caused an increased shear force exerted on the cell surface, and that this increased shear force could reduce the thickness of boundary layer on the cell surface and thus could have more oxygen molecules be

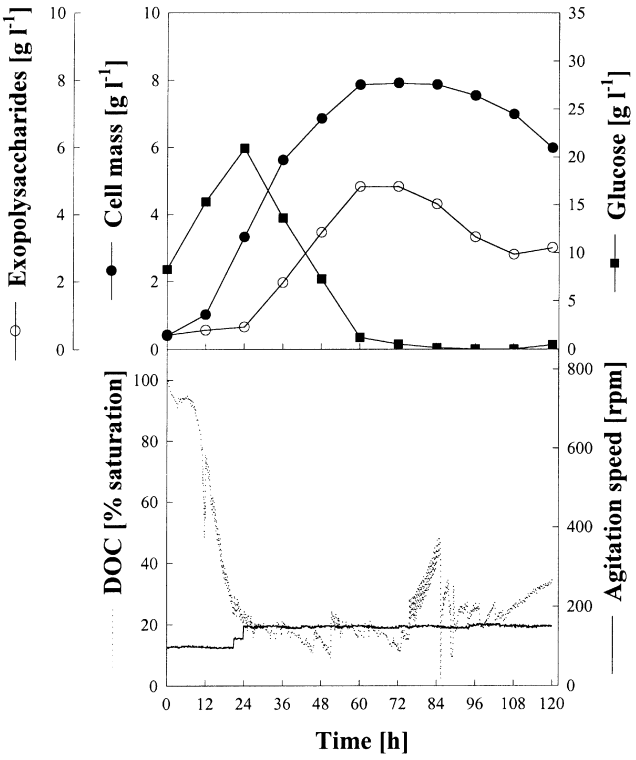

Fig. 5. Time profiles of cell growth, EPS production, glucose consumption, DOC, and agitation speed in batch culture of *A. blazei* with DOC controlled at 20% by manipulating pure oxygen supply.

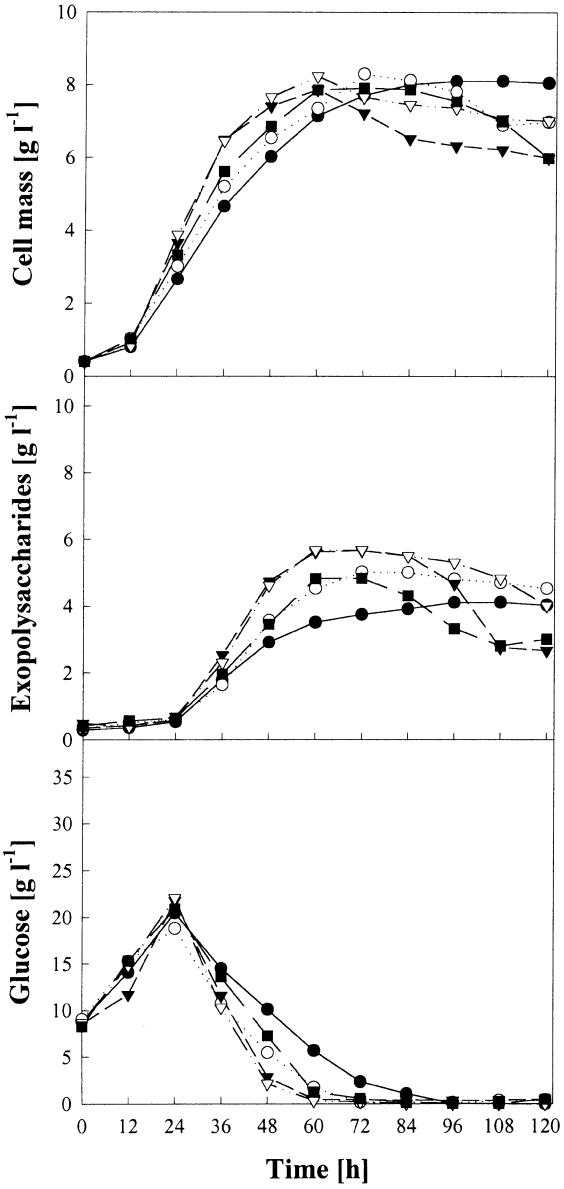

Fig. 6. Effects of DOC and its control method on the cell growth and EPS production in batch culture of *A. blazei* (●: No DOC control, ○: 10%, ▼: 20%, ▽: 40%, ■: 20% by manipulating pure oxygen supply).

transferred to the cells even at the same DOC level. To check for this possibility, another run of batch culture was performed, in which the DOC was controlled at 20% by supplying pure oxygen with a total aeration rate of 1 vvm, while keeping the agitation speed at a rather low level of 100 rpm to minimize shear force. The results are presented and compared with other experimental results in Fig. 5, Fig. 6, and Table 1. In this case of pure oxygen supply with a less intensive agitation at 100 rpm, the maximum cell mass and the cellular productivity were 7.86 g l^{-1} and 0.131 g l^{-1} h^{-1}, which was comparable to those of the former case in which DOC was controlled at the same level of 20% by manipulating the agitation speed in the range of 100-450 rpm. However, the EPS concentration and its productivity of 4.82 g l^{-1} and 0.080 g l^{-1} h^{-1}, respectively, were lower by about 15% than those of the former case, while being still

significantly higher than those of the control case with no DOC control. Such observation results suggest that DOC is the major parameter influencing both cell growth and EPS production as already reported for other microorganisms [Flores et al., 1994; Park et al., 1993; Peters et al., 1989; Wecker and Onken, 1991]. The results also suggest that rather intensive agitation is important for EPS production for unknown reasons. It is quite probable that good EPS removal from the cell surface due to a rigorous agitation in the former case might have promoted EPS secretion, enhancing its productivity.

In general, fungi are physically weak; therefore, the agitation speed has to be controlled within a certain range to avoid cell damage by excessive shear force. In our work, no damage of cells was observed although the agitation speed was in the range of up to 450 rpm, which is much higher than the agitation speed normally employed in higher fungi cultures [Kim et al., 2003]. At such a high level of agitation, the cells were observed to form pellets during the entire culture period irrespective of DOC level. Smaller pellets were observed at a higher agitation level.

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