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On-Line Estimation of Cell Growth from Agitation Speed in DO-Stat Culture of a Filamentous Microorganism, *Agaricus blazei*

Na, Jeong-Geol[1], Hyun Han Kim[2], and Yong Keun Chang[2]*

[1] Department of Fossil Energy and Environment Research, Korea Institute of Energy Research, Daejeon 305-343, Korea
[2] Department of Chemical and Biomolecular Engineering, KAIST, Daejeon 305-701, Korea

Abstract A simple, but effective on-line method for estimating the mycelial cell mass concentration from agitation speed data, a most readily-available process variable, has been developed for DO-stat cultures of *Agaricus blazei*. The dynamic change of dissolved oxygen concentration (DOC) in the initial transient period and the change in yield were considered in the development of the estimation algorithm or estimator. Parameters in the estimation algorithm were calculated from the agitation speed data at 20% of DOC. The proposed estimator could accurately predict the cell mass concentration regardless of DOC levels in the tested range of 10~40%, showing a good extrapolation capability.

Keywords: on-line estimation, agitation speed, DO-stat culture, *Agaricus blazei*

INTRODUCTION

Because of its outstanding efficacy in preventing and curing a variety of diseases, *Agaricus blazei*, a basidiomycete fungus, is considered to be a remarkable natural resource [1,2]. However, its high production cost is the major constraint which prevents its extensive use, and thus, the development of an optimal production process is needed.

In dealing with a number of engineering problems, including culture condition optimization and control, one needs an accurate kinetic model and an efficient measurement method which can be used in a real-time situation [3]. In our previous work [4], a kinetic model with mechanistic features was developed for batch cultures of *A. blazei*. However, the measurement of its cell mass concentration still acted as a major obstacle. *A. blazei* forms mycelia during cultivation and therefore rendering it impossible to determine the cell mass concentration from optical density data, which is most commonly used to measure bacterial cell mass concentrations. Its concentration can be determined only using the dry-cell weight method, which is quite time-consuming. For this reason, a more time-efficient method for the determining the *A. blazei* concentration from the data of readily-measurable quantities is necessitated.

There have been several efforts to make on-line estimations of the specific growth rate, and thus, the cell mass concentration, or to analyze metabolic states from the time changes of off-gas data, such as the oxygen uptake rate (OUR) or carbon dioxide evolution rate (CER) in batch and/or fed-batch cultures [5,6]. OUR and CER are easy to measure on-line. However, these measurements require an expensive analytical device, such as a mass spectrometer, or require frequent on-line calibration. Therefore, it would be useful to develop a simple and cost-effective method for the on-line estimation of the cell mass concentration from other process variables which are easier to measure than gas data. We have developed a method to produce on-line estimates of the specific growth rate, and thus, cell mass concentration of *Brevibacterium ketoglutamicum* from agitation speed in a DO-stat culture [7]. However, the method was too simple to be applied for the culture of filamentous microorganisms [8,9], including *A. blazei*, which required some modification or augmentation.

In this study, the behavior of agitation speed in relation to cell growth and oxygen uptake rate in DO-stat batch cultures of *A. blazei* was investigated. A relatively simple, but accurate method for the estimation of the specific growth rate from the relationships between the agitation speed and cell growth was subsequently developed.

MATERIALS AND METHODS

Microbial Strain and Stock Culture

The microorganism used in this study was *A. blazei*, which was kindly provided by the Rural Development Administration, Korea. 10 mL aliquots of its mycelium suspension in 20% glycerol were stored at -80°C.

***Corresponding author**
Tel: +82-42-869-3927 Fax: +82-42-869-3910
e-mail: ychang@kaist.ac.kr

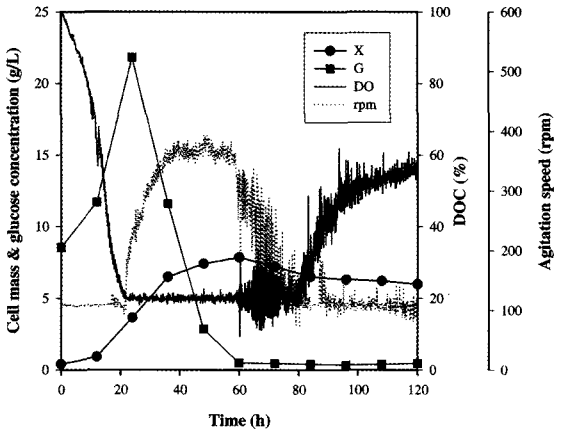

Fig. 1. Profiles of cell mass (●), residual glucose concentration (■), DOC, and agitation speed in batch culture of *A. blazei* with DOC controlled at 20% of air saturation.

Culture Media and Conditions

The composition of the medium for the seed culture was (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; and $FeCl_3 \cdot 6H_2O$, 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. For further activation, 10 mL of seed culture was transferred to 90 mL of seed culture medium in a 500 mL Erlenmeyer flask. Two days after inoculation, the second seed culture was transferred to a 7 L jar fermentor (Kobiotech., Incheon, Korea) with a working volume of 3 L. The inoculum size was 10% (v/v). The composition of the medium for the bioreactor culture was (per liter): glucose, 6 g; dextrin, 24 g; yeast extract, 4 g; soytone peptone, 2 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; and $FeCl_3 \cdot 6H_2O$, 0.2 mg. Fermentation was carried out in a computerized fermentor system (Autolab LK 930, Lokas, Daejeon, Korea). The temperature was maintained at 28°C and pH was controlled at 5.0 with 2 N HCl or 2 N NaOH.

Analytical Methods

The 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.

DO-Stat Operation

The dissolved oxygen concentration (DOC) was controlled at a desired saturation level using a PI control algorithm. The agitation speed was manipulated, with a fixed aeration rate of 1 vvm.

RESULTS AND DISCUSSION

Typical time courses of *A. blazei* cell growth, glucose concentration, DOC, and agitation speed are well represented by their profiles (shown in Fig. 1) for a batch culture in which DOC was controlled at 20%. The rapid increase in glucose concentration in the beginning of culture was a result of the hydrolysis of dextrin in the medium, as previously reported [10-12]. There is a clear correlation between the cell growth and the dynamics of agitation speed. The manipulation variable, agitation speed, differed over time as the oxygen demand of *A. blazei* cells changed. The time course of the culture was classified into four distinct phases. In the initial phase (<24 h, Phase I), DOC decreased to reach a set point, and the cell mass concentration increased exponentially. At 24 h, the agitation speed began to rapidly increase in order to balance the oxygen transfer rate (*OTR*) with the rapidly increasing oxygen uptake rate (*OUR*) due to active cell growth (<36 h, Phase II). Thereafter, the agitation speed was maintained at a high level of about 400 rpm, and sluggish cell growth was observed (<60 h, Phase III). DOC was closely maintained at the set point in Phase II and Phase III. Finally, due to nutrient depletion, the agitation speed immediately decreased with an

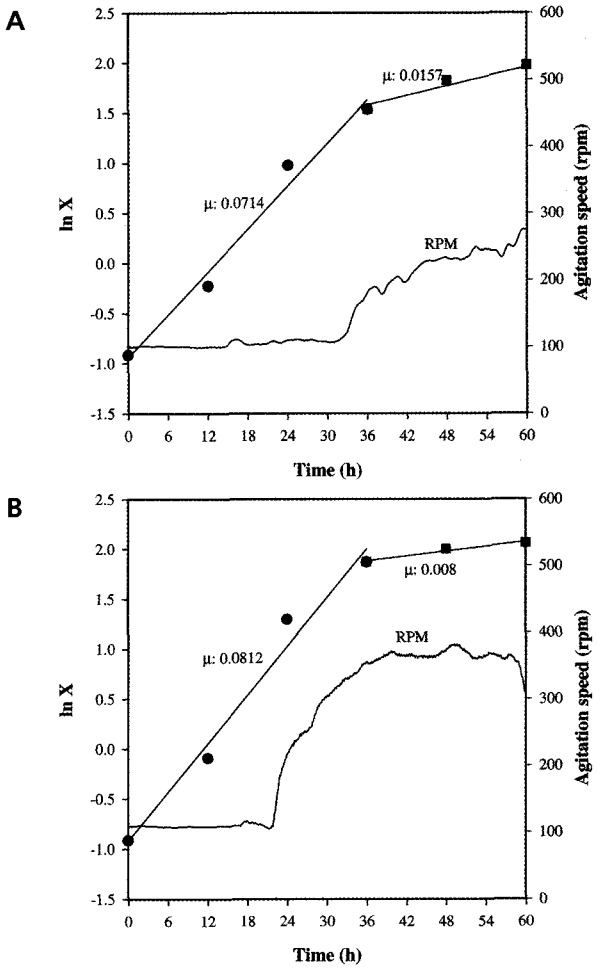

Fig. 2. The specific growth rate and agitation speed at various DOC levels. (A) 10% and (B) 20%.

abrupt decrease in oxygen demand at about 60 h (>60 h, Phase IV). In this phase, a significant cell lysis was observed. In this final phase, DOC could not be regulated at the set point, since the agitation speed had to be maintained at a minimal level of 100 rpm for sufficient mixing of the culture broth, even with no cellular oxygen demand. In practice, the culture would be terminated before reaching the last phase. Therefore, only the first three phases were considered in the development of our estimation algorithm.

Fig. 2 illustrates the profiles of the specific growth rate and the agitation speed at different DOC levels of 10 and 20% air saturation. In both cases, two distinct growth phases represented by different specific cell growth rates were observed, which was quite similar to the pattern of the agitation speed. Such results implied that the dynamics of agitation speed could be an indicator of the metabolic state, including the cell growth rate, and that an estimation method of cell mass concentration could be developed based on agitation speed data.

In our previous work [7], the specific growth rate and, thus, the cell mass concentration in DO-stat culture of *B. ketoglutamicum* were estimated from agitation speed data, with an assumption that the yield coefficient was constant, and that the initial cell mass was essentially zero and could be neglected. In a preliminary study, however, the above estimation algorithm was found to be too primitive to be applied to *A. blazei* cultures in which changes in the yield coefficient was significant and the initial transient period was relatively long. Therefore, the development of a more sophisticated method for estimating the cell mass concentration was necessary.

The time-change in DOC in the bioreactor may be represented by:

$$\frac{dC_{O_2}}{dt} = OTR - OUR \tag{1}$$

where Co_2, *OTR*, and *OUR* denote DOC, oxygen transfer rate, and oxygen uptake rate, respectively. *OTR* and *OUR* may be represented by:

$$OTR = k_L a'\left(C_{O_2}^{*} - C_{O_2}\right) \tag{2}$$

$$OUR = \frac{\mu X}{Y_{X/O_2}} \tag{3}$$

with an assumption that the oxygen requirement for cell maintenance is negligible. $k_L a'$, $C_{O_2}^{*}$, and Y_{X/O_2} denote the volumetric oxygen transfer coefficient, the saturated oxygen concentration, and the cellular oxygen yield at the given conditions.

By assuming that $k_L a'$ is a linear function of the agitation speed, as previously reported [13], Eq. (2) may be recast as:

$$OTR = \alpha \cdot RPM\left(C_{O_2}^{*} - C_{O_2}\right) \tag{4}$$

where α is an appropriate constant and *RPM* denotes the agitation speed. Eqs. (5) and (6) can be derived from Eqs. (1)~(4).

$$\mu X = \frac{dX}{dt} = Y_{X/O_2}\left(\alpha \cdot RPM\left(C_{O_2}^{*} - C_{O_2}\right) - \frac{dC_{O_2}}{dt}\right) \tag{5}$$

$$dX = Y_{X/O_2}\left(\alpha \cdot RPM\left(C_{O_2}^{*} - C_{O_2}\right)dt - dC_{O_2}\right) \tag{6}$$

By the integration of Eq. (6), Eq. (7) can be obtained;

$$X = X_0 + \int \alpha Y_{X/O_2} RPM\left(C_{O_2}^{*} - C_{O_2}\right)dt - Y_{X/O_2}\left(C_{O_2} - C_{O_2,t=0}\right) \tag{7}$$

A discrete version of Eq. (7) is:

$$X(N) = X(N-1) + \alpha Y_{X/O_2} RPM(N)\left(C_{O_2}^{*} - C_{O_2}(N)\right) - Y_{X/O_2}\left(C_{O_2}(N) - C_{O_2}(N-1)\right) \tag{8}$$

where N denotes the N^{th} sampling time.

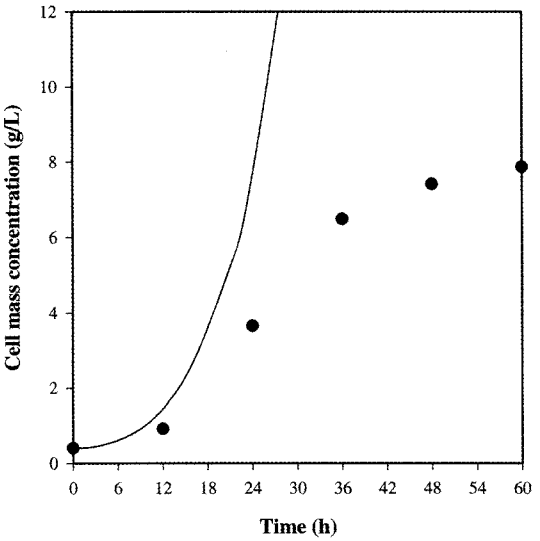

Fig. 3. Estimation result with yield coefficient to be assumed constant.

Fig. 3 shows the estimation results when the yield coefficient was assumed to be constant. In the later part of the culture, the cell concentration tended to be overestimated. The main reason for this was believed to be that, contrary to our assumptions, the oxygen requirement for cell maintenance was significant during this period. To accommodate such a phenomenon, the yield coefficient was assumed to change over time. As demonstrated in Fig. 2, cell growth was severely retarded when the agitation speed no longer significantly increased in the later part of the culture, implying a reduced cellular yield during this period. This can be more clearly understood by examining the cell growth pattern in Fig. 1. If the yield coefficient had been constant, the cell growth would have shown a linear pattern of increase. Instead, cell growth

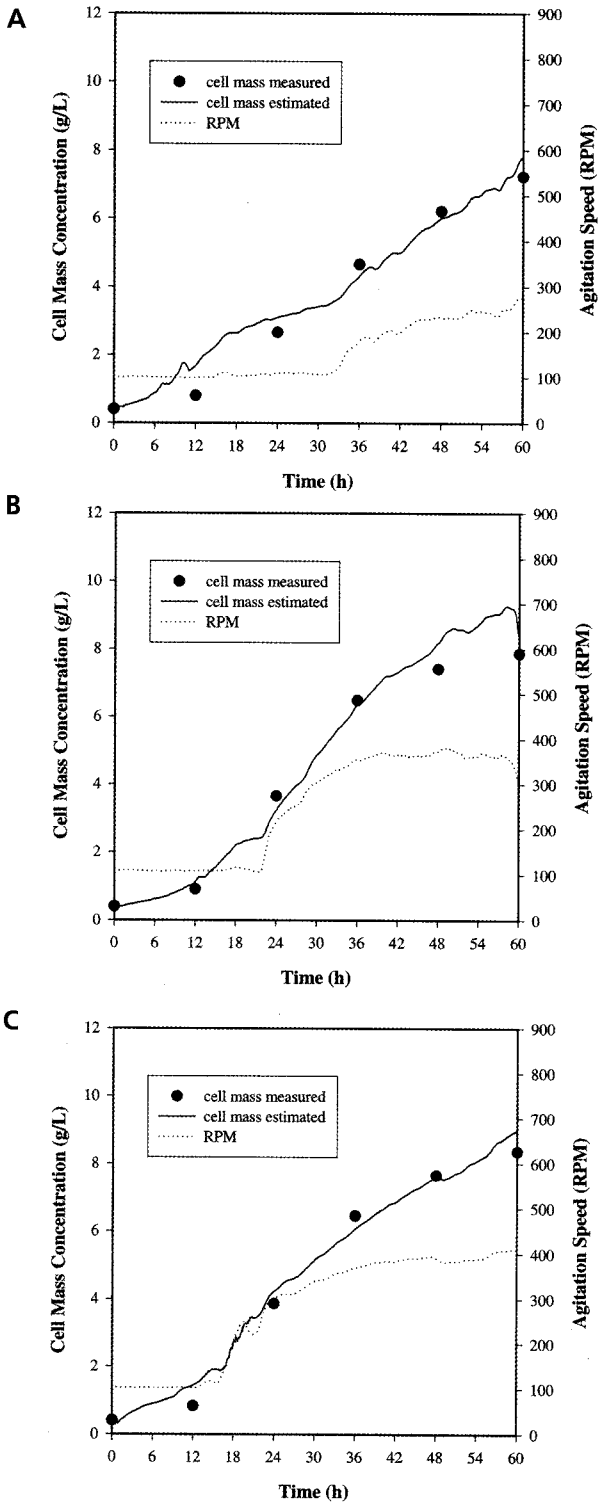

Fig. 4. Estimation results using Eq. (10) at various DOC levels. (A) 10%, (B) 20%, and (C) 40%.

was much lower than expected. According to this rationale, the yield coefficient was assumed to be a saturated function of the time-derivative of the agitation speed.

$$Y_{X/O_2} = Y_{X/O_2,m}\left(k_1 + \frac{RPM'(N)}{K_s + RPM'(N)}\right) \tag{9}$$

where RPM' denotes the time-derivative of RPM and K_s is a saturation constant to be specified.

By combining Eqs. (8) and (9), Eq. (10) can be obtained;

$$\begin{aligned} X(N) &= X(N-1) + Y_{X/O_2,m}\left(k_1 + \frac{RPM'(N)}{K_s + RPM'(N)}\right) \\ &\quad \left(\alpha RPM(N)\left(C_{O_2}^* - C_{O_2}(N)\right) - \left(C_{O_2}(N) - C_{O_2}(N-1)\right)\right) \\ &= X(N-1) + \left(k_1 + \frac{RPM'(N)}{K_s + RPM'(N)}\right) \\ &\quad \left(k_2 RPM(N)\left(C_{O_2}^* - C_{O_2}(N)\right) - k_3\left(C_{O_2}(N) - C_{O_2}(N-1)\right)\right) \end{aligned} \tag{10}$$

where k_i' s are appropriate constants to be specified. k_1, k_2, and k_3 denote a constant which takes into consideration the cell growth at initial and latter stages, a lumped constant of the maximum yield constant and a factor for OTR conversion, and the maximum oxygen yield constant, respectively.

The values of k_1, k_2, k_3, and k_s which were estimated from the experimental data at 20% DOC using the least square error method were k_1=0.072, k_2=0.00006, k_3=0.3, and k_s =92.

Fig. 4 shows the estimation results at various DOC levels. Although the parameters estimated from the data at 20% DOC were used, the estimator could appropriately predict the cell mass concentration regardless of DOC levels, showing a good extrapolation capability.

In most DO-stat cultures of a bacterial strain, the transient period in the beginning, during which DOC approaches the set point from its initial level, is very short. In this circumstance, DOC may be assumed to be maintained at the set point for the entire culture period without causing any serious errors in estimation. In this study, however, the metabolic rate of *A. blazei* was very slow and it took about 24 h for DOC to reach the set point. Since such a long initial transient period was not negligible compared with the entire duration of the approximately 60 h culture, it could not be assumed that DOC was maintained at the set point for the entire culture period. Moreover, the oxygen consumption for cell maintenance was significant in the later part of the culture. To accommodate for these problems, the dynamic change of oxygen concentration and the change in yield were considered in the development of the estimation algorithm. As a result, the proposed method accurately estimated the time courses of the cell mass concentration.

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