Methanol induction strategy using the two-loop control-based DO-stat and its application to repeated induction in methylotrophic yeast *Pichia pastoris*

최승진, 임형권, 우성환, 정정환*
(재)목암생명공학연구소
전화 (031)260-9841, FAX (031)260-9808

ABSTRACT

A simple control strategy of DO-stat was introduced to the recombinant rGuamerin production process in *Pichia pastoris*. This induction strategy consisted of two interrelated control loops, by which oxygen ratio of inlet gas and methanol feeding rate was controlled. Using this control strategy, over-feeding or under-feeding of methanol could be avoided in concomitance with the efficient control of dissolved oxygen level. As a result, the cell concentration reached 130 g/L and rGuamerin expression level was 450 iu/L, which was more than 40% increased result comparing with the fed-batch process using manual control of methanol feeding rate.

INTRODUCTION

In the *pichia pastoris* expression system, many proteins have been expressed efficiently at a high level using AOX1 promoter.(1,2) The simplicity and easy scalability make *Pichia* expression system the attractable candidate for secretion of therapeutic proteins activity alive. For maximizing the expression level of target protein, the AOX promoter must be efficiently induced by methanol with the proper feeding strategy when it is used for the large-scale fermentation. Methanol is consumed as a carbon source and an inducer simultaneously. When *Pichia pastoris* is grown in the methanol-containing media, the oxidative pathway can utilize methanol only if there are sufficient oxygen molecules. Thus, the oxygen level in the culture and the rate of methanol feeding interrelated with each other. And also these two parameters must be controlled at the same time. Otherwise, the toxicity accompanying by accumulation of methanol could exert fatal damage to both the cell growth and the expression of heterologous protein. For these reasons, methanol induction strategy associated in proper maintenance of dissolved oxygen (DO) concentration in production phase is the most important factor in *Pichia pastoris* expression system.

MATERIALS ANS METHODS

Guamerin-producing recombinant *Pichia pastoris*, in which the structural gene for guamerin (GenBank Accession No. U38282) fused with α-factor leader sequence containing KEX2 cleavage site was integrated into chromosomal DNA was used for this study. The detailed procedures for the constructions of expression plasmid (pGS29A) and recombinant guamerin-expressing strain were described in previous publications. (3,4,5)

To improve the methanol induction procedure, DO-stat control mode was used, in which DO was controlled by both the oxygen partial pressure of inlet air and the methanol feeding rate. DO set-point was controlled at the range of 40 to 45% air-saturation. The first DO control loop was as follows; when DO reached below lower set-point (40%, DO_{low}), the pure oxygen ratio in gas mixer increased by 1% and when DO reached above upper set-point (45%, DO_{up}), the pure oxygen ratio decreased by 0.5%. The second DO control loop, simultaneously performed with the first control loop, was as follows; when DO reached below DO_{low}, the methanol feeding rate decreased by 0.5% and, when DO reached above DO_{up}, the methanol feeding rate increased by 1%. Those loops were executed every 30 seconds.

RESULT AND DISCUSSION

The result of the proposed scheme was compared to the results of the fed-batch of which feed rate was controlled manually. As shown in **figure 1**, proposed two control loop were operated simultaneously and well suited for on-line computer applications. After a short period of adaptation, methanol feeding rate was sharply increased to 20.3 g/h. The plateau was formed in methanol feeding rate during the middle of the methanol feeding period because of the oxygen transfer limitation. Actually, 100% pure oxygen was supplied during this period. After this period, methanol feed rate was slowly decreased.

Culture profile of the improved DO-stat was shown in **figure 3**. The final cell growth and the expression level of the rGuamerin after 24 hr of induction were 140g/L and 450 iu/L, respectively, which was 12% and 40% higher than the final results from the conventional fed-batch, which were 125 g/L and 320 iu/L after 40 hr of induction (**figure 2**). This was due to the efficient methanol feeding rate control via interdependent two loop-control system. In special, early stage of the post-induction fed-batch phase was more efficiently controlled by a two loop-control system comparing to the conventional fed-batch results. This means that in this stage, methanol feeding rate was higher in two loop control system than in the conventional fed-batch system without accumulation of methanol in the culture media. As mentioned above, early stage of the post-induction phase is very important for the cell growth and rGuamerin production. Thus, efficient methanol feeding in the crucial early stage of post-induction phase resulted in higher cell growth rate and rGuamerin expression rate. The specific productivity of the rGuamerin production in induction phase was 0.134 iu/g/h, which was more than twice the result in the conventional fed-batch, 0.064 iu/g/h.

In this case, western blot analysis showed that little amount of rGuamerin was remained in the cell during the production stage.(data not shown) That is, rGuamerin was secreted into the medium immediately after expression even though there was high level expression of rGuamerin proteins.

Experimentation with and application of more sophisticated algorithms, particularly adaptive ones, may contribute to further improvement in the control quality. However, the accuracy

achieved by the algorithms currently used was sufficient and there was no practical reason for replacing them by more complicated ones.

As shown in **figure 3**, rGuamerin expression was consistently increased even at the end of fermentation process. Thus, extension of culture time was challenged to investigate the stability of the long-term operation. Cyclic fed-batch, in which the methanol feeding and withdrawal of culture broth was repeated, was applied for the long-term culture. As shown in **figure 4**, this procedure could also be applied for the long-term stable culture system for the *Pichia pastoris* expression system. rGuamerin expression level was reached their maximum value, 590 iu/L, after 2nd cycle and slowly decreased thereafter. Cell density was also maintained more than 120 g/L all through the 290 hr cultivation. More that ten cycles of the cyclic fed-batch were accomplished without decrease of either expression level or cell growth.

REFERENCES

1. Buckholz, R. G. and Gleeson, M. A. G. 1991. Yeast systems for the commercial production of heterologous proteins. Bio/Technology 9: 1067-1072

2. Clare, J. J., Romanos, M. A., Rayment, F. B., Rowedder, J. E., Smith, M. A., Payne, M. M., Sreekrishna, K. and Henwood, C. A. 1991. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. Gene 105: 205-212

3. Jung, H. I., Kim, S. I., Ha, K. S., Joe, C. O. and Kang, K. W. (1995) Isolation and Characterization of Guamerin, a New Human Leukocyte Elastase Inhibitor from *Hirudo nipponia*. *J. Biol. Chem.* 270, 13879-13884.

4. Kim, K.-Y., Lim, H.-K., Lee, K.-J., Park, D.-H., Kang, K.-W., Chung, S.-I. and Jung, K.-H. 2000. Production and characterization of recombinant guamerin, and elastase-specific inhibitor, in methylotrophic yeast *Pichia pastoris*. Protein Expression Purif. 20, 1-9.

5. Lim HK, Kim KY, Lee KJ, Park DH, Chung SI, & Jung KH (2000) Genetic stability of integrated structural gene of guamerin in recombinant *Pichia pastoris* J. Microbiol. Biotechnol. 10(4), 470-475

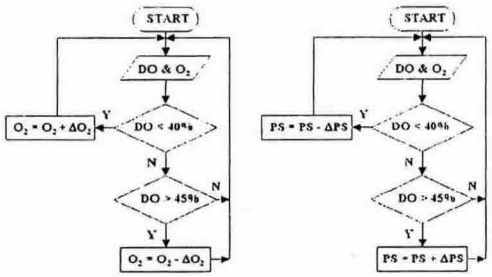

Figure 1. Algorithms of the balanced DO-stat: (a) algorithm for control of DO by agitation; (b) algorithm for control of DO by methanol feed rate.

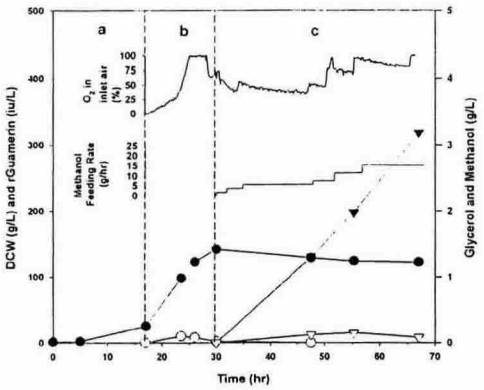

Figure 2. (A) Time courses of the conventional rGuamerin production process: dry cell weight (●); rGuamerin activity (▼); residual glycerol concentration (O); residual methanol concentration (▽).

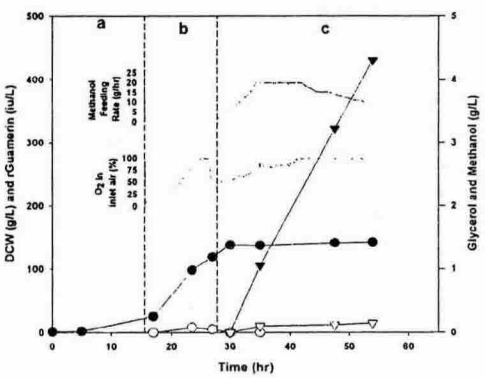

Figure 3. (A)Time courses of the improved rGuamerin production process: dry cell weight (●); rGuamerin activity(▼); residual glycerol concentration(O); residual methanol concentration(▽).

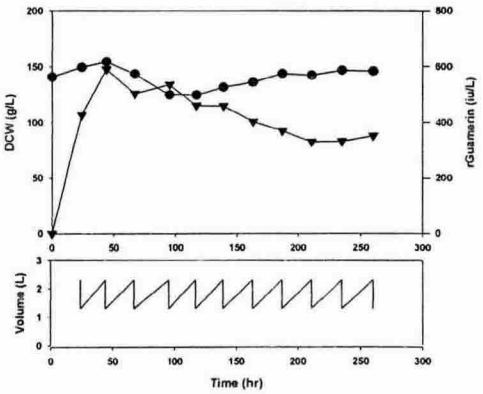

Figure 4. (A) Time courses of the cyclic fed-batch of rGuamerin production process: dry cell weight (●); rGuamerin activity (▼). (B) Time courses of the culture volume in the cyclic fed-batch of rGuamerin production process.