Appl Microbiol Biotechnol (2003) 62:342–348
DOI 10.1007/s00253-003-1307-6

ORIGINAL PAPER

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Dissolved-oxygen-stat controlling two variables for methanol induction of rGuamerin in *Pichia pastoris* and its application to repeated fed-batch

Received: 15 January 2003 / Revised: 2 February 2003 / Accepted: 6 March 2003 / Published online: 29 April 2003

Abstract A DO-stat control strategy for two variables was introduced to the rGuamerin production process in *Pichia pastoris* and applied to repeated fed-batch culture. Two interrelated variables, namely the ratio of partial pressure of pure O_2 in the inlet air-stream and the methanol feed rate, were controlled simultaneously. By using this control strategy, methanol feeding for induction could be controlled automatically while efficiently controlling the dissolved oxygen level. As a result, the cell concentration reached more than 140 g l^{-1} and rGuamerin expression level 450 iu l^{-1}. rGuamerin was secreted into the culture medium and reached a level that was 40% higher than achieved in a fed-batch process using manual control of the methanol feeding rate. Repeated rGuamerin induction was achieved by repeating the methanol feeding and withdrawing the culture broth during extended production. During more than 250 h of culture, expression of rGuamerin was maintained at an average of about 430 iu l^{-1} (473 mg l^{-1}), without causing the cell density to decrease. In addition to the rGuamerin production process, the proposed control system might be applied to cultivation of other methylotrophic yeasts in the production of therapeutic proteins.

Introduction

Many proteins have been expressed efficiently in the *Pichia pastoris* (*P. pastoris*) expression system at a high level using the AOX1 promoter (Cregg et al. 1987, 1993; Sreekrishna et al. 1989; Buckholz and Gleeson 1991; Clare et al. 1991; Werten et al. 1999; Curvers et al. 2001; Minning et al. 2001). Its simplicity and scalability make the *Pichia* expression system an attractive candidate for the production of therapeutic proteins. To maximize the expression level of the target protein, the AOX promoter must be efficiently induced by methanol in combination with the proper feeding strategy, when it is used for large-scale cultivations. In the methanol-containing media, *P. pastoris* uses methanol by the oxidative pathway only in the presence of sufficient amounts of oxygen (Couderc and Baratti 1980), and consumes methanol as a carbon source and an inducer simultaneously. Thus, the oxygen level in the culture and the rate of methanol feeding are interrelated. In addition, these two variables must be simultaneously controlled; otherwise, the toxicity resulting from methanol accumulation fatally damages both cell growth and expression of the heterologous protein. For these reasons, the methanol-induction strategy associated with proper maintenance of dissolved oxygen (DO) concentration in production may be an important factor in the expression of protein using *P. pastoris*.

Previous reports have described various strategies for recombinant protein production in *P. pastoris* (Siegel and Brierley 1989; Brierley et al. 1990; Barr et al. 1992; d'Anjou and Daugulis 2001; Goodrick et al. 2001); however, these involved independent control of methanol induction and DO level. In such systems, the methanol feed rate was changed manually using the DO level as an indicator. DO was maintained at a certain level by manually manipulating agitation and the oxygen-mix in the inlet air-stream. However, in these systems it could be tedious to establish an optimal methanol feeding strategy suitable for optimal induction. In a recent report, methanol feed control in *P. pastoris* was carried out by monitoring and controlling the methanol concentration using a methanol sensor (Katakura et al 1998; Zhang et al. 2000, 2002).

rGuamerin is a novel elastase inhibitor that was isolated from a Korean leech (*Hirudo nipponia*) (Jung et al. 1995; Kim et al. 2000; Lim et al. 2000). Human neutrophil elastase (HNE) is involved in major tissue destruction during inflammation and is thus a contributing factor in the development of several diseases. As a result,

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HNE inhibitor sources have been under investigation as candidates for treatment of HNE-associated diseases (Janoff 1985; Fricker 1998). Secreted recombinant proteins from *Pichia* have been highly purified using a simple purification procedure (Kim et al. 2000; Whittaker et al. 2000). Nonetheless, the expression levels of rGuamerin and the methods of cultivating *P. pastoris* limit development of this drug as a therapeutic option because large amounts of candidate protein are generally required for drug development purposes.

In this report, we used a DO control strategy, achieved by automatically manipulating the ratio of partial pressure of pure O_2 in the inlet air-stream (O_2^{PR}) and the methanol feed rate (MFR) during the induction of recombinant *P. pastoris*. This induction strategy protected cells from both oxygen deficiency and excess methanol supply by linking the methanol feed rate and the DO level. Finally, we successfully operated long-term high-cell-density fed-batch cultures repeatedly by using the proposed DO-stat control strategy and periodical removing culture broth.

Materials and methods

Strain

rGuamerin-producing recombinant *P. pastoris*, in which the structural gene for Guamerin (GenBank accession no. U38282) fused with an α-factor leader sequence containing the KEX2 cleavage site was integrated into chromosomal DNA, was used for this study. The detailed procedures used to construct the expression plasmid pGS29A and the rGuamerin-expressing strain were described previously (Kim et al. 2000; Lim et al. 2000). The host (*Pichia pastoris* GS115 (*his*4, Mut$^+$)) and the vector system (pPIC9) used for strain construction were purchased from Invitrogen (San Diego, Calif., USA).

DO-stat fed-batch culture and methanol induction

For rGuamerin production, a DO-stat fed-batch culture and the following methanol induction system were adopted. Seed flask culture of rGuamerin-producing recombinant *P. pastoris* was carried out in 100 ml of YPD medium (1% glucose, 1% yeast extract, 2% peptone) and incubated with shaking (30°C, 150 rpm) for 20 h . For DO-stat fed-batch culture, a 2.5-l Bioflow III bioreactor (New Brunswick Scientific, N.J., USA) was used with an initial culture volume of 1.0 l. The medium consisted of glycerol, 40 g l^{-1}; H_3PO_4, 27 ml l^{-1}; $CaSO_4{\cdot}2H_2O$, 0.9 g l^{-1}; K_2SO_4, 18.0 g l^{-1}; $MgSO_4$, 10.24 g l^{-1}; KOH, 4.13 g l^{-1}; NH_4OH, 30 ml l^{-1}; trace metal solution ($CuSO_4{\cdot}5H_2O$, 6.0 g l^{-1}; KI, 0.09 g l^{-1}; $MnSO_4{\cdot}H_2O$, 3.0 g l^{-1}; $NaMo{\cdot}H_2O$, 24.0 g l^{-1}; H_3BO_4, 0.02 g l^{-1}; $CoCl_2$, 0.5 g l^{-1}; $ZnCl_2$, 20.0 g l^{-1}; $FeSO_4{\cdot}H_2O$, 65.0 g l^{-1}; biotin, 0.2 g l^{-1}; H_2SO_4, 20.0 ml l^{-1}), 4.4 ml l^{-1}. Temperature was maintained at 30°C, and pH was controlled at 5.0 by using 15% (v/v) NH_4OH and 30% (v/v) H_3PO_4. DO was maintained at above 30% air-saturation by automatic adjustment of the agitation speed and the O_2^{PR}, controlled using a two-gas mixer (New Brunswick Scientific). Cells were allowed to grow for ca. 15 h in the batch culture until the glycerol was completely exhausted. At this point, DO-stat fed-batch culture was started and carried out for 13 h in glycerol feed medium (80% (w/v) glycerol and 12 ml trace metal solution l^{-1}). The DO set-point for DO-stat fed-batch culture was 50% of air-saturation under the atmospheric pressure at 30°C. After ca. 500 ml of the feed medium had been supplied, the feed was stopped and the culture starved for 0.5 h. Methanol containing 12 ml trace metal solution l^{-1} was used to induce rGuamerin. The methanol feed rate in the conventional methanol induction method was increased manually from 1.63 to 15.38 g h^{-1} in a stepwise fashion.

DO-stat controlling two variables for methanol induction

To improve the methanol induction procedure, a DO-stat control mode system was used that involved controlling DO by using both O_2^{PR} and MFR. The DO set-point was controlled in the range 40–45% of air-saturation. The first DO control loop was as follows; when DO was just below the lower set-point (40%, DO_{low}), O_2^{PR} was increased by 1%, and when DO was above the upper set-point (45%, DO_{up}), O_2^{PR} was decreased by 0.5%. The second DO control loop was carried out at the same time as the first as follows; when the DO was below DO_{low}, MFR was decreased by 0.5%, and when DO was above DO_{up}, MFR was increased by 1%. These loops were executed every 30 s.

Repeated methanol induction

After methanol induction using the DO-stat controlling two variables, 1.0 l of the culture broth was withdrawn and methanol induction was resumed for 20 h. This "induction and withdrawal" procedure was carried out repeatedly. Before the induction, if necessary, KH_2PO_4, $MgSO_4$, and $CaSO_4{\cdot}2H_2O$ were added to a final concentration of 10.0 g l^{-1}, 10.0 g l^{-1}, and 1.0 g l^{-1}, respectively, in the culture broth.

Analytical methods

Dry cell weight was determined after removing moisture from the culture broth using an electronic moisture analyzer (Sartorius MA 40, Germany). Elastase inhibition activity in the culture medium was measured as described previously (Kim et al. 2000; Lim et al. 2000), and the amount of rGuamerin was expressed in inhibition unit (iu). Glycerol and methanol concentrations were measured by gas chromatography (HP5890 series II, Hewlett Packard, USA) using OV-17 (6 ft, WHP 100/120, 19001A-B12) and 10% degs (6 ft, WHP 100/120, 19001a-L12) columns for glycerol and methanol, respectively.

To determine the secretion efficiency of rGuamerin, cell lysate and the culture supernatant were analyzed by Western blot. After separation by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane. The membrane was treated with glutaraldehyde to cross-link the rGuamerin with itself and to enhance the detection sensitivity (Karey and Sibasku 1989). Rabbit antisera against rGuamerin and horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, Mich., USA) were used as the first and the second antibodies, respectively.

Results

Manual methanol feeding for rGuamerin induction

rGuamerin expression using a fed-batch process may be divided into three phases: batch, pre-induction fed-batch, and induction fed-batch (Fig. 1). The methanol feed rate was increased stepwise manually for induction beginning 30 h after cell growth reached about 140 g l^{-1}. The methanol feed pump was turned on when the DO level was above the DO set-point and was turned off below this set-point. The rGuamerin expression level increased up to 320 iu l^{-1} in the culture medium after 37 h induction, and cell growth decreased slightly after induction. As shown in Fig. 2, control of the DO level was closely related to the MFR. These two variables, MFR and DO, influenced each other in a complicated manner. Therefore, in order to

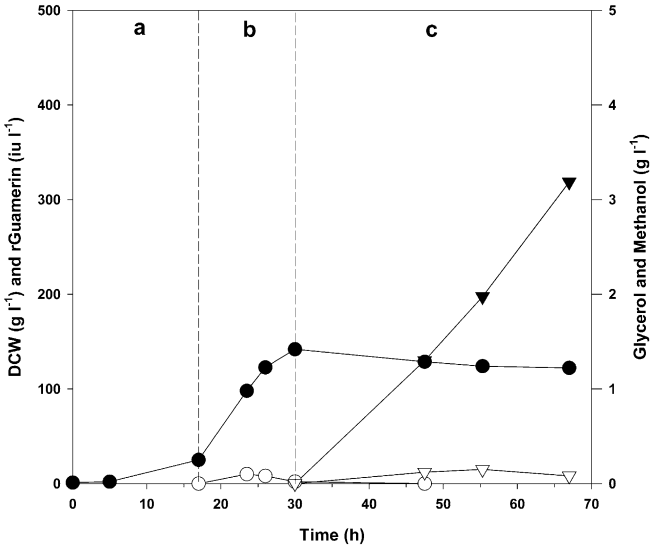


Fig. 1 Time courses of various parameters in conventional rGuamerin fed-batch culture. Dry cell weight (●), rGuamerin activity (▼), residual glycerol concentration (◯), residual methanol concentration (▽). **a**, **b**, and **c** indicate the batch phase, the pre-induction phase, and the induction phase, respectively

optimize the methanol induction strategy, the proposed control structure was designed as follows.

DO-stat controlling two variables for methanol feed

The proposed control structure was designed to consist of two interrelated loops, as shown in Fig. 3. DO was controlled with a narrow window about a set-point (ca. 40–45%). The first loop controlled DO by manipulating the oxygen transfer rate (OTR), which was varied by altering O_2^{PR}. When the DO value dropped to below the lower limit of the DO set-point (DO_{low}), O_2^{PR} was increased. When the DO value rose above the upper limit of the DO set point (DO_{up}), O_2^{PR} was decreased. At the same time, the second loop controlled the DO value by manipulating the oxygen uptake rate (OUR) by varying the MFR. When the DO value dropped to below the DO_{low}, which meant that, in order to maintain the optimal methanol concentration, methanol feeding should stop or the feed rate should be decreased, MFR was reduced.

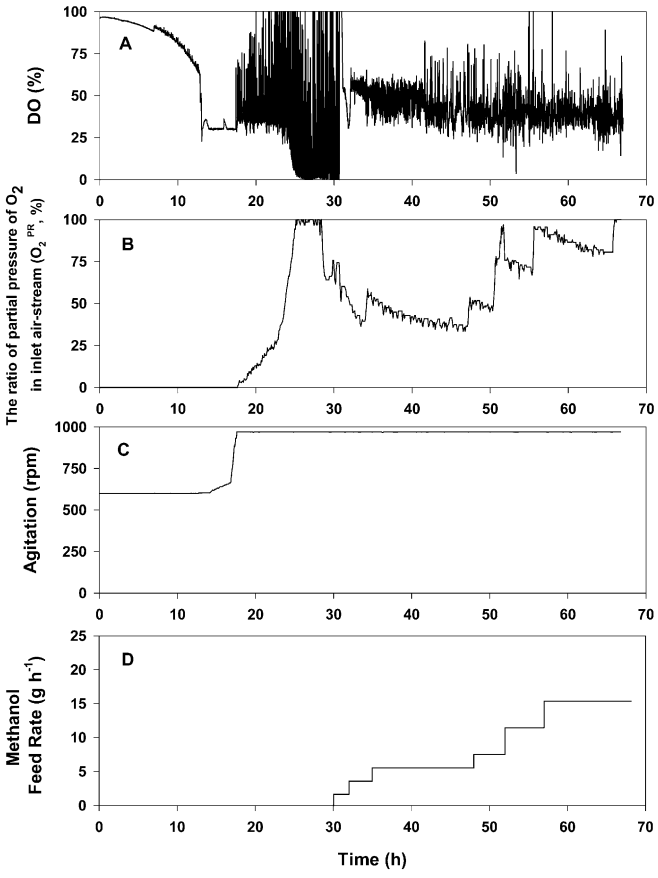
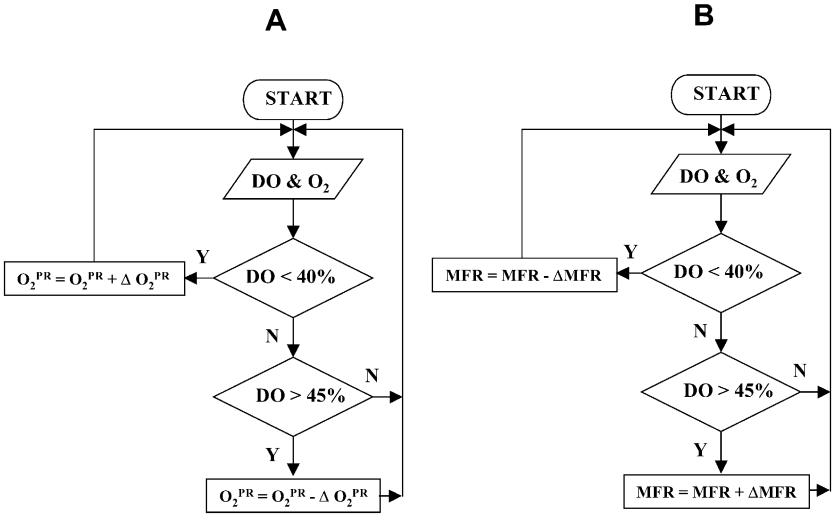


Fig. 2A–D Time courses of the conventional rGuamerin production process. **A** DO; **B** ratio of partial pressure of pure O_2 in inlet air-stream (O_2^{PR}); **C** agitation speed; **D** methanol feed rate (MFR)

Fig. 3 **A** Algorithm for the control of DO using the O_2^{PR}. **B** Algorithm for the control of DO using the methanol feed rate (*MFR*).



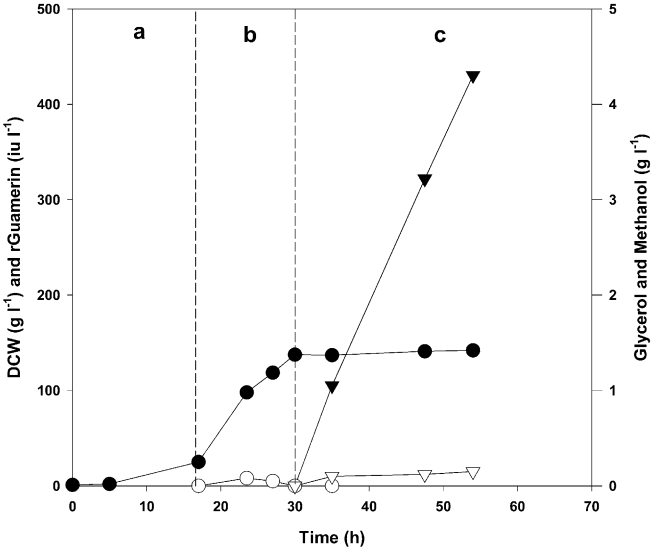


Fig. 4 Time course of dry cell weight (●), rGuamerin activity (▼), residual glycerol concentration (◯), and residual methanol concentration (▽) in fed-batch culture using the DO-stat controlling two variables. **a**, **b**, and **c** indicate the batch phase, the pre-induction phase, and the induction phase, respectively

When the DO value rose above DO_{up}, which meant that the methanol feeding should start or the feed rate should be increased, MFR was increased.

The results obtained using this control system are shown in Fig. 4. The profiles are almost the same as before induction (compare with Fig. 1). After 24 h induction, the rGuamerin expression level in the culture medium was 450 iu l^{-1}, which was 40% higher than that obtained from the conventional fed-batch culture (Fig. 1). The specific productivity of rGuamerin production in the induction phase was 0.13 iu g^{-1} h^{-1}, which was twice as high as that of the conventional fed-batch system, 0.06 iu g^{-1} h^{-1}. As shown in Fig. 5, after a short period of adaptation, the methanol feed rate increased rapidly to 20.3 g h^{-1} by an automatic MFR control using the two-variable control system. The MFR was maximal at the middle of the methanol-feeding period because of oxygen transfer limitations. Actually, pure oxygen was supplied after MFR reached a plateau. After this period, MFR decreased slowly.

Based on Western blot analysis of cell lysates and culture supernatants of rGuamerin-producing *P. pastoris*, it was demonstrated that rGuamerin was secreted into the medium immediately after its expression, although there was a non-specific reaction with the non-purified rabbit anti-sera as well as a smeared band of rGuamerin due to its molecular features (Fig. 6).

Repeated fed-batch culture for the prolonged production of rGuamerin

As shown in Fig. 4, rGuamerin expression increased consistently throughout the cultivation. Thus, we exam-

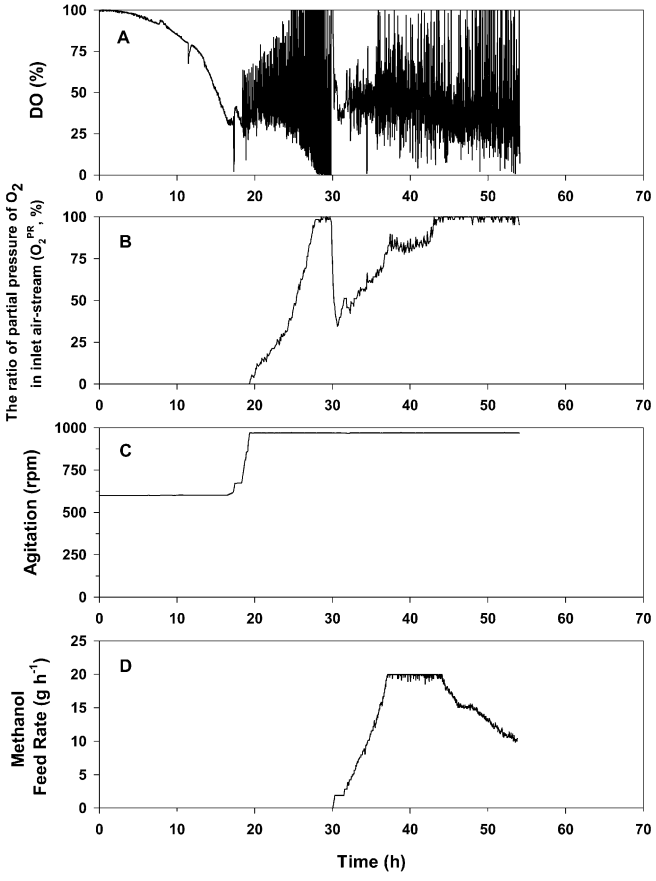


Fig. 5A–D Time courses of the rGuamerin production process using the DO-stat controlling two variables. **A** DO; **B** O_2^{PR}; **C** agitation speed; **D** MFR

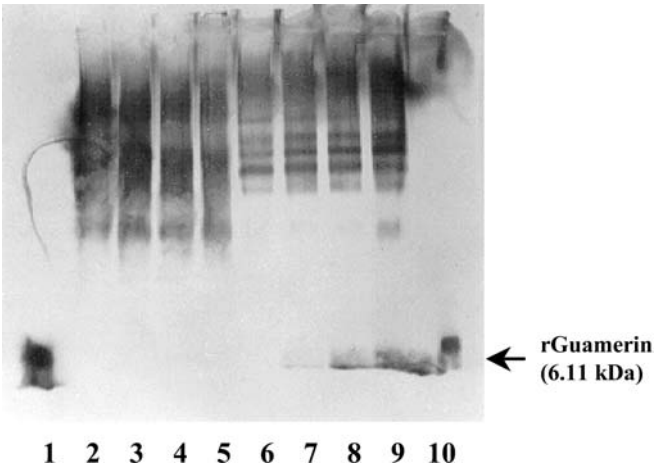


Fig. 6 Western blot of intracellular and extracellular rGuamerin after induction. *Lanes 1,10* Standards for purified rGuamerin; *lanes 2, 3, 4, 5* total cell lysates 0, 6, 18, and 25 h after induction, respectively; *lanes 6, 7, 8, 9* culture supernatants 0, 6, 18, and 25 h after induction, respectively. *Arrow* rGuamerin

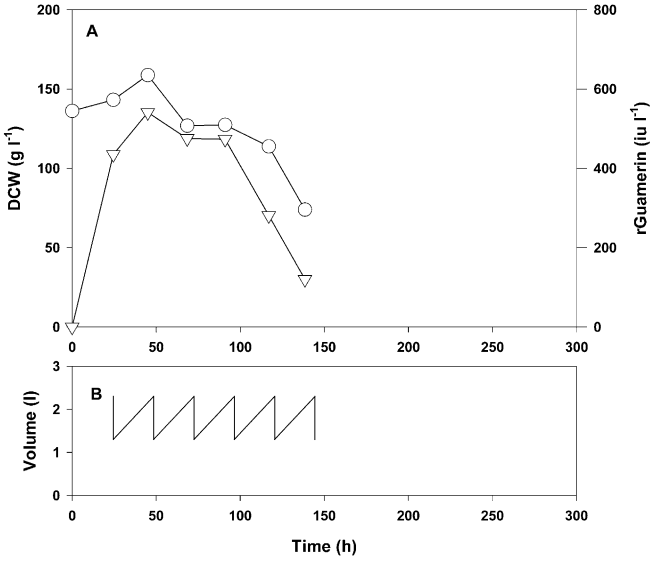


Fig. 7A, B Time courses in rGuamerin production by repeated fed-batch without the addition of salts. **A** Dry cell weight (◯); rGuamerin activity (▽). **B** Culture volume

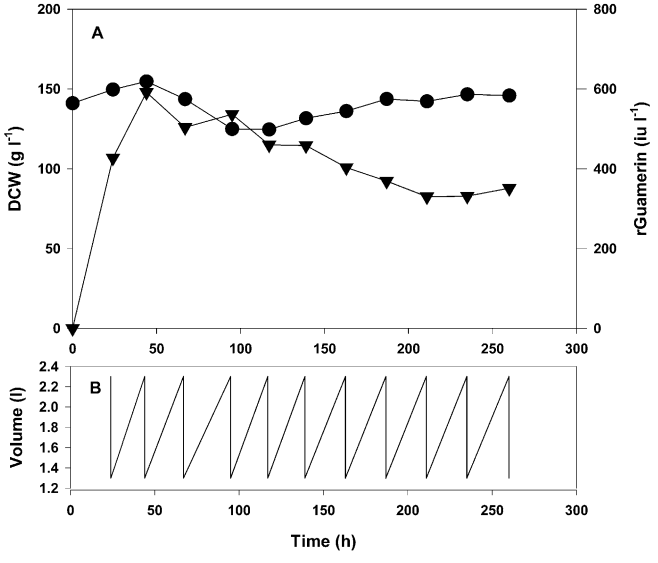


Fig. 8A, B Time courses in rGuamerin production by repeated fed-batch with the addition of salts. **A** Dry cell weight (●); rGuamerin activity (▼). **B** Culture volume

ined extended culture times to investigate the stability of the long-term operation. Repeated fed-batch was applied to a long-term culture strategy. As shown in Fig. 7, rGuamerin secretion into the culture medium reached a maximum value of 540 iu l^{-1} after the second cycle and decreased thereafter. Cell density decreased gradually after the second cycle of fed-batch. On the fifth cycle of methanol induction, rGuamerin production decreased steeply. To maintain both the production level and cell growth over a prolonged period, salts were added at the initiation of induction (as described in Materials and

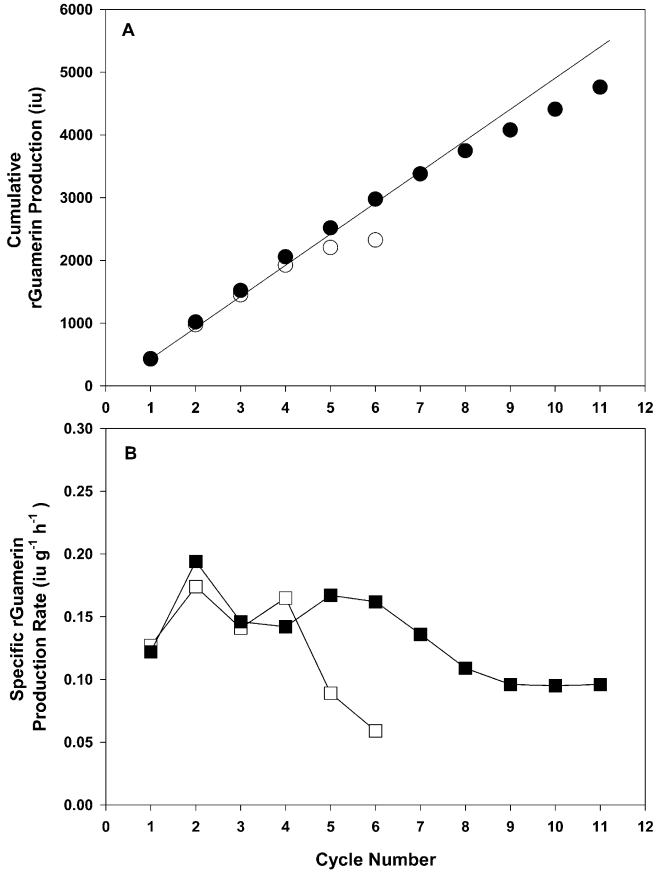


Fig. 9 Cumulative production (**A**) and specific production rate (**B**) of rGuamerin according to the repeated fed-batch cycle number when the culture was replenished with salts (●, ■) and when salts were not added (◯, ❑)

methods). As a result, more than ten cycles of the repeated fed-batch were accomplished without a steep decrease in either rGuamerin expression or cell growth (Fig. 8). Although eventually there was a gradual decrease in rGuamerin expression, from 590 to 330 iu l^{-1}, operation could be extended over 250 h. Comparing the results shown in Fig. 7 and Fig. 8 in terms of cumulative production and the specific production rate shows that cumulative rGuamerin production increased linearly and the specific rGuamerin production rate was maintained up to the seventh cycle (repeated induction over 150 h) when salts were added for replenishing, whereas both parameters decreased after the fourth cycle when salts were not added (Fig. 9).

Discussion

In our preliminary tests, the initial stage of the induction fed-batch phase was the most important phase in terms of inducing high-levels rGuamerin expression. During the early stage of induction, cells were prone to damage by the increased methanol concentration, which rose to above inhibitory levels, before adapting to this new

carbon source. Furthermore, when the amount of methanol fed at this stage was less than optimal, the rGuamerin expression rate and the productivity of the entire process decreased. For these reasons, efficient control of the MFR, especially early in the induction fed-batch phase, was found to be crucial for maximizing rGuamerin expression. The higher expression level and the higher specific productivity of rGuamerin by the interdependent two-variable control system were due to the higher MFR in the early induction stage. As a result of increased MFR without an accumulation of methanol, the cell growth rate and rGuamerin expression rate were higher than that obtained by conventional DO-stat fed batch culture.

The OUR is closely related to the nutrient consumption rate (Konstantinov et al. 1990). When the latter is high, especially when methanol is used as the carbon source, the OUR may be maintained at a high level. However, when DO is controlled by altering the OTR only via agitation speed, the nutrient feed rate and the OUR are restricted by limitations of OTR. Eventually, cell growth becomes linearly dependent on the OTR value, because the OUR and the nutrient uptake rate converge to certain values. In the rGuamerin production process, cell density reached about 140 g l^{-1} at the initiation of the methanol feed for induction. At this stage, oxygen demand was so large that OTR could not meet the O_2 demand of of the cells even at the maximum agitation speed. For this reason, the O_2^{PR} and the MFR were automatically changed to meet the OTR and the OUR. To overcome this constraint, it would be necessary to increase OTR by designing more efficient agitation/aeration fermentation systems and to decrease OUR by controlling the cell density at a lower level.

The secretion of proteins using the α-mating factor as a signal sequence does not always yield adequate results in the *Pichia* expression system due to the large molecular weight of the heterologous protein, defects in signal sequence cleavage affected by the surrounding amino acid sequences, and other unknown factors (Martinez-Ruiz et al. 1998). It seems that the efficient secretion of rGuamerin is due to its low molecular mass (6,110 Da) (Jung et al. 1995; Kim et al. 2000). However, Western blot analysis of rGuamerin is very tedious because of its many disulfide bonds and its rigid and compact structure (Jung et al. 1995; Kim et al. 2000).

We successfully operated long-term high-cell-density fed-batch culture repeatedly using the proposed DO-stat and periodically removing culture broth. With this approach, rGuamerin production was maintained at an average level of about 430 iu l^{-1}, which corresponds to 473 mg l^{-1} (Lim et al. 2000), and about 4,761 iu (5.2 g) of rGuamerin could be produced in the culture medium.

Acknowledgements This work was supported by a grant (961-33-1) from the Ministry of Commerce, Industry and Energy, Korea.

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