Effect of Methanol Feeding Strategies on Production and Yield of Recombinant Mouse Endostatin From *Pichia pastoris*

L. B. Trinh, J. N. Phue, Joseph Shiloach

Biotechnology Unit, National Institutes of Health, NIDDK Building 6, Room B1-33, Bethesda, Maryland 20892-2715; telephone: 301-496-9719; fax: 301-451-5911; e-mail: yossi@nih.gov

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Abstract: *Pichia pastoris,* a methylotrophic yeast, is an efficient producer of recombinant proteins in which the heterologous gene is under the control of the methanol-induced *AOX1* promoter. Hence, the accepted production procedure has two phases: In the first phase, the yeast utilizes glycerol and biomass is accumulated; in the second phase, the yeast utilizes methanol which is used both as an inducer for the expression of the recombinant protein and as a carbon source. Since the yeast is sensitive to methanol concentration, the methanol is supplied gradually to the growing culture. Three methanol addition strategies were evaluated for the purpose of optimizing recombinant endostatin production. Two strategies were based on the yeast metabolism; one responding to the methanol consumption using a methanol sensor, and the other responding to the oxygen consumption. In these two strategies, the methanol supply is unlimited. The third strategy was based on a predetermined exponential feeding rate, controling the growth rate at 0.02 h^{-1}, in this strategy the methanol supply is limited. Throughout the induction phase glycerol, in addition to methanol, was continuously added at a rate of 1 g L h^{-1}. Total endostatin production was similar in all three strategies, (400 mg was obtained from 3 L initial volume), but the amount of methanol added and the biomass produced were lower in the predetermined rate method. This caused the specific production of endostatin per biomass and per methanol to be 2 times higher in the predetermined rate than in the other two methods, making the growth control strategy not only more efficient but also more convenient for downstream processing. **Biotechnol Bioeng* **82:** 438–444, 2003.
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INTRODUCTION

Pichia pastoris is an efficient producer of recombinant proteins (Cregg et al., 2000). This microorganism is a methylotrophic yeast (Faber et al., 1995) that utilizes methanol as its carbon source and grows to very high cell density (above 50% wet weight concentration). In many cases it secretes the produced protein to the culture media. The reason for the efficiency of this yeast as a producer of recombinant protein is the biosynthesis of alcohol oxidase (AOX). AOX is the first enzyme in the methanol oxidation pathway. The enzyme is produced in response to the methanol presence in the growth media. Methanol initiates the enzyme biosynthesis by activating the *AOX1* promoter, causing the enzyme accumulation to about 30% of the total yeast proteins. Therefore, the gene of choice is introduced downstream to the alcohol oxidase promoter and is expected to express the desired protein in an equivalent amount (Cereghino and Cregg, 2000).

Three types of recombinant Pichia can be obtained (Higgins and Cregg, 1998): Mut^+ where the two methanol oxidases genes *AOX1* and *AOX2* are intact; Mut^S, where only *AOX2*, which is responsible for 15% of the protein biosynthesis, is intact; and Mut^- where both *AOX1* and *AOX2* genes are disrupted. The Mut^+ strain is the most responsive to methanol concentration and is the recombinant strain most commonly used.

Alcohol oxidase promoter is activated by methanol, but it is also repressed by carbon source such as glycerol or glucose (Tschopp et al., 1987). In addition, the microorganism is sensitive to methanol, and concentrations above 0.4% inhibit growth and production (Katakuga et al., 1998; Zhang et al., 2000a). Hence, the accepted production procedure from the Mut^+ strain has two phases. In the first phase, the yeast utilizes glycerol and biomass is accumulated. In the second phase, the glycerol supply is terminated or reduced (Brierley et al., 1990; d'Anjou and Daugulis, 1997), methanol alone or in combination with lower concentration of glycerol (mixed feed) is added and the biosynthesis of the recombinant protein is initiated.

Since the yeast is sensitive to methanol concentration, methanol needs to be supplied continuously to the growing culture keeping its concentration below 0.4%. Several fed-batch strategies for methanol addition have been established (Zhang et al., 2000b). These strategies can be metabolism related, based on parameters such as methanol consumption (Curvers et al., 2001; Guarna et al., 1997; Hellwig et al.,

Correspondence to: Joseph Shiloach

2000; Katakuga et al., 1998; Kobayashi et al., 2000), oxygen consumption (Byrne et al., 2000; Chung, 2000; Minning et al., 2001), pH control or CO_2 concentration. While implementing these strategies, the microorganism itself is controling the amount of methanol added as a response to its own metabolism, the methanol supply is practically unlimited and its concentration can have any value, usually not more than 4 g/L^{-1}. Growth strategies can also be based on predetermined methanol addition schemes at constant, linear, or exponential rates (Chauhan et al., 1999; Freyre et al., 2000; Inan et al., 1999; Marasugi et al., 2000, Zhang et al., 2000a). In these strategies the methanol feeding rate and not the microorganism is controling the growth, the actual methanol concentration is zero and its supply is limited.

It is very likely that the growth and production characteristics of the culture such as growth rate, production yield, and methanol consumption would depend on the fed-batch strategy. It is therefore important to evaluate the various fed-batch methods when the production process of a recombinant protein is being evaluated. In some cases, metabolism-related method is preferred (Curvers et al., 2001), and in other cases, nonmetabolism-related method, in which the growth rate can be controlled, is preferred (Zhang et al., 2000a). However, detailed comparisons of various fed-batch strategies for the production of a specific recombinant protein are very limited (Kupcsulik et al., 2001; Minning et al., 2001; Zhang et al., 2000b). Our purpose here is to evaluate several growth strategies for the production of extracellular murine endostatin. Endostatin, is a 20 KDa fragment of collagen XVIII, that had been shown to have an inhibitory effect on angionenesis and therefore can potentially be used as a tumor growth suppressor (Figg et al., 2002; O'Reilly et al., 1997). The compound is currently being tested in humans and different delivery strategies are being evaluated in mice. Gram quantities of the compound were needed and therefore efficient production procedure was required.

The growth strategies evaluation performed in this work can provide information not only on growth and production, but also on the specific production values of biomass in relation to methanol, and the specific production values of protein in relation to biomass and methanol.

MATERIAL AND METHODS

Yeast Strain and Fermentation Processes

Pichia pastoris strain GS 115 His^+ Mut^+ expressing mouse endostatin was prepared as described earlier (Boehm et al., 1999).

Bench-top fermentation was performed in a 7-L fermentor (New Brunswick Scientific, Edison, NJ) The fermentor is interfaced to an MD-Biostat System (B. Braun Biotech USA, Allentown, PA) furnished with an adaptive control algorithm (Hsiao et al., 1992) that maintained dissolved oxygen at 30% saturation by adjusting the agitation and air or oxygen. The fermentor was also equipped with a methanol sensor (Trinh et al., 2000; Wagner et al., 1997). Three liters BMGY media (peptone, 2%; yeast extract, 1%; YNB, 1.34%; glycerol, 3%; biotin, 4×10^{-5}%; and 100 m*M* potassium phosphate pH 6.0) was inoculated with 100 mL of overnight culture (O.D. ≈ 20 at 600 nm). The culture was grown in batch mode for approximately 16 h (O.D. ≈ 60 at 600 nm), after which fed-batch mode was started by adding a 50% glycerol solution at a flow rate of 18 mL L^{-1} h^{-1}; during this period, a dose of 0.3% (w/v) methanol was also pumped into the fermentor to calibrate the methanol sensor. After stabilization of the sensor signal (around 1 h), the glycerol feed rate was reduced gradually over a 1-hour period to 2 mL L^{-1} h^{-1}, allowing the culture to adapt smoothly to methanol. The reduced glycerol feed rate was kept constant throughout the methanol induction period (mixed feed). After the adaptation period, methanol was added according to the selected fed-batch strategy. The duration of the methanol addition phase was 48 h. During the fermentation the pH was kept at 6.5 using 50% NH_4OH solution, and foam was controlled by the addition of antifoam 289 (Sigma Chemical Co., St. Louis, MO.)—this antifoam did not affect growth or endostatin production.

Pilot-scale fermentation was performed in 80-L fermentor (Bioflo 5000, New Brunswick Scientific, Edison, NJ) equipped with the same control and data acquisition as the bench top.

Methanol Addition Strategies

Fed-Batch Fermentation Based on On-Line Methanol Sensor

After the adaptation period, the addition of methanol containing 12 mL/L^{-1} PTMI solution (per L: cupric sulphate-$5H_2O$ 6.0 gm; sodium iodide 0.08 gm; manganese sulphate-H_2O 3.0 gm; sodium molibdate-$2H_2O$ 0.2 gm; boric acid 0.02 gm; cobalt chloride 0.5 gm; zinc chloride 20.0 gm; ferrous sulphate-$7H_2O$ 65.0 gm; biotin 0.2 gm; sulfuric acid 5.0 gm) was initiated. Methanol concentration was controlled at 3.0 g/L^{-1} by an on-line monitoring and control device described previously (Trinh et al., 2000; Wagner et al., 1997).

Fed-Batch Fermentation Based on Dissolved Oxygen Signal

After the adaptation period, methanol solution containing 12 mL/L^{-1} PTMI was added into the fermentor when the dissolved oxygen signal spiked 10% above the set-point. Each time methanol was added to adjust the methanol concentration to 0.3%.

Fed-Batch Fermentation Based on Predetermined Exponential Feed Rate

After the adaptation period, methanol was pumped to the culture by a ChemTec pump (Scilog, Middleton, WI) at a

rate based on the following relationship (Zhang et al., 2000a), $F = 0.962\ \mu X_0 V_0 e^{\mu t}$ where F is the methanol flow rate (g/L^{-1}), X_0 is the biomass-wet weight at induction ($gWCW/L^{-1}$), V_0 is culture volume at induction (L), μ is culture growth rate under methanol (h^{-1}).

The methanol flow rate was ramped exponentially to keep the culture growth rate at 0.02 h^{-1}.

Analytical Methods

Off-Line Measurements

Off-line methanol measurements were made using YSI Biochemistry Analyzer (Yellow Spring Instruments, Yellow Spring, OH).

Endostatin Determination

Samples taken during the course of induction were centrifuged at 4000*g* for 40 min. The supernatant was diluted to adjust the conductivity to 5.5 mS/cm and the pH to 6.0 and was loaded on a 5 mL HiTrap™ SP HP (Amersham Pharmacia Biotech AB, Piscataway, NJ) at a ratio of 20 mg total protein per mL packed resin. The column was washed with 10 column volumes of 20 m*M* phosphate buffer pH 6.0 containing 50 m*M* NaCl buffer, and the endostatin was eluted with 3 column volumes of 20 m*M* phosphate containing 1.5*M* NaCl. Endostatin was analyzed by loading the various samples and increasing amount (0–8.6 μg) of known endostatin standard on 4–20% Tris-glycine SDS PAGE under reducing conditions and running at 125 volts for 90 min. After staining with Simply Blue™ SafeStain (Invitrogen, Carlsbad, CA) the amount of endostatin was determined by digitising and calculating with NIH image analysis software (NIH, Bethesda, MD).

Dry Weight Determination

Dry weight determination was done by drying cell paste in a moisture determination balance (Ohaus MB 200, NJ) for 3 h at 95°C.

Statistical Analysis

Each of the methanol feeding strategies was tested in three repetitions. Standard deviation was calculated for the average values of the three experiments from each feeding strategy.

RESULTS

Effect of the Various Fed-Batch Strategies on Growth Rate, Biomass Accumulation, and Methanol Supply

Growth rate and biomass accumulation during the three different fed-batch strategies are shown in Figure 1 and Table I. The growth rate during the predetermined methanol addition strategy was kept constant at a value of 0.02 h^{-1}, but the growth rates during the DO control strategy and the methanol sensor control strategy followed different patterns. The rate was 0.06 h^{-1} and 0.05 h^{-1}, respectively 10 h after the induction, and the rate continuous to decline throughout the induction phase which lasted 48 h. The biomass accumulation rate was corresponded to the growth rate. It accumulated at a constant rate during the predetermined addition strategy reaching a final level of 434 g DCW (dry cell weight). During the DO control strategy and the methanol sensor strategy, the biomass accumulation was much faster at the beginning of the induction phase, and slowed down towards the end of the induction period, reaching a value of 986 and 988 gDCW, respectively, which is over 2 times as much as accumulated in the predetermined growth rate strategy.

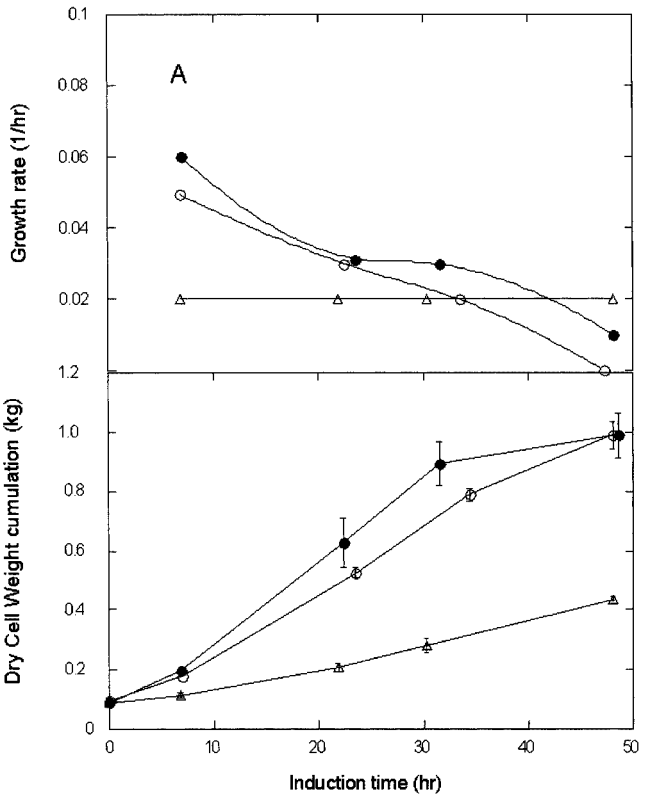

Figure 1. *Pichia pastoris* growth in response to different methanol supplies strategies. On line sensor (○), DO signal (●), predetermined rate (△). (Time zero indicates the initiation of the methanol induction phase). (A) Growth rate, (B) Biomass accumulation. Fermentation was conducted in a 7-L fermentor with initial volume of 3.0 L. Methanol addition was started when the biomass concentration was between 100 and 120 gram per liter (wet weight).

Methanol addition is described in Figure 2 and Table I. When the methanol concentration in the media was controlled by an online sensor, the total amount added was 3.23 L, compared with 2.25 L added in response to the dissolved oxygen concentration and to less than 1 L when the methanol was added using the predetermined rate.

Table I. Comparison of the various methanol feeding strategies. Fermentations were conducted in a bench top fermentor with initial volume of 3 L, the numbers reflect the values after 48 h induction.

Methanol control strategy	Final culture volume (L) V_0 = 3.0 L	Endostatin (mg/L culture)	Endostatin Total (mg)	Biomass (gDCW/L)	Biomass Total g DCW	Methanol consumed (L)	Base consumed (L)
Fed-batch using D.O. signal	6.09	66 ± 4	402	162 ± 2.6	986	2.25 ± 0.04	0.84
Fed-Batch using online gas sensor	7.06	67 ± 19	470	140 ± 2.7	988	3.23 ± 0.15	0.83
Fed-batch using predetermined feed rate	4.25	96 ± 16	408	102 ± 1.7	434	0.90 ± 0.11	0.34

Effect of the Various Fed-Batch Strategies on Endostatin Production

Total and volumetric endostatin productions during the different fed batch strategies are described in Figure 3 and Table I. The total amount of endostatin produced was similar in the fed-batch strategies using the dissolved oxygen signal and the predetermined feed rate. Approximately 400 mg were accumulated using these two strategies. Higher endostatin, 470 mg, was accumulated using the on-line sensor fed-batch strategy. Endostatin concentration was higher in the predetermined methanol feeding strategy compared with the dissolved oxygen control and the methanol sensor strategies. Final concentration of 96 mg/L was detected with the predetermined feed rate, compare with concentrations of 66 mg/L and 67 mg/L in the other two strategies.

Specific Production Values, Specific Consumption Values, and Production Rates in the Three Different Growth Strategies

Specific production of endostatin based on methanol utilization, biomass production based on carbon utilization (methanol and glycerol), and specific production of endostatin based on biomass are summarized in Table II. Specific endostatin production on methanol with the predetermined feeding rate was much higher than it was with the two other fed-batch strategies. It was 489 mg/L of methanol with the predetermined feeding rate compared with 160 mg/L, and 116 mg/L with the dissolved oxygen controlled strategy and the methanol sensor strategy, respectively. 0.72 mg endostatin per gram dry cell weight were produced with the predetermined feeding rate strategy compared with 0.31 and 0.33 mg endostatin per gram dry cell weight with the two other feeding strategies. The specific biomass production was 1.67 gram dry cell weight per gram carbon used (methanol and glycerol) with the predetermined feeding rate and 1.34 and 0.96 gram dry cell weight per gram carbon used, at the dissolved oxygen-controlled and the sensor-controlled strategies, respectively.

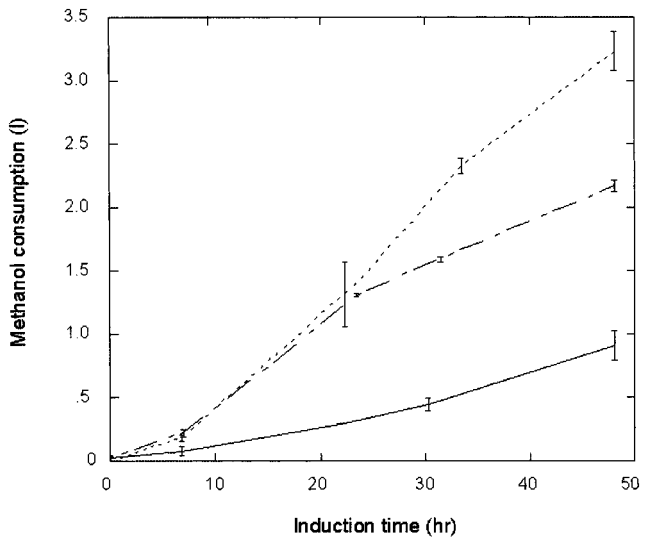

Figure 2. Methanol consumption in response to different supply strategies. On line sensor (– – – –), DO signal (— - — –), predetermined rate (—-). (Time zero indicates the initiation of the methanol induction phase).

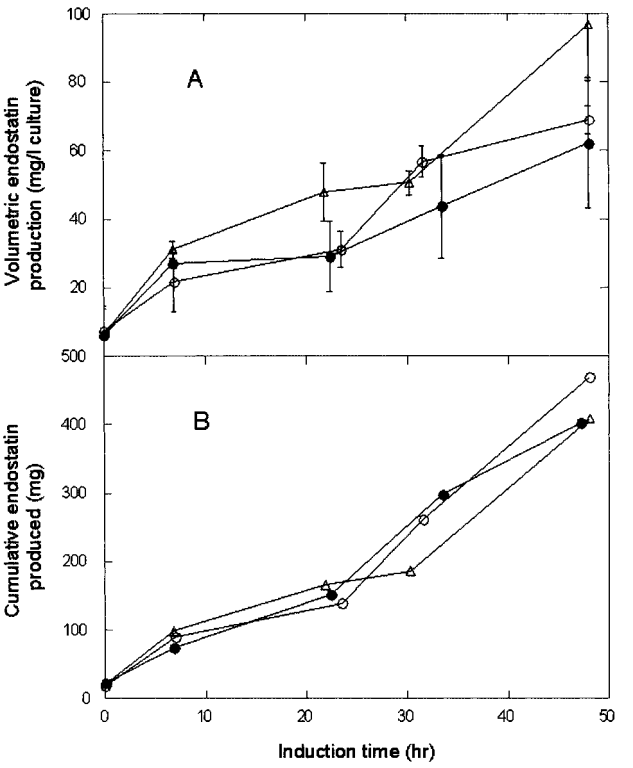

Figure 3. Endostatin production in response to different methanol supplies strategies. On-line sensor (○), DO signal (●), predetermined rate (△). (Time zero indicates the initiation of the methanol induction phase). (A) Total endostatin production, (B) volumetric endostatin production.

Table II. Specific endostatin and biomass production at different methanol feeding strategies. The yield constants reflect average values during the last 24 h of the induction phase.

Methanol control strategy	Specific endostatin production (mg Endostatin/ 1 methanol)	Specific endostatin production (mg Endostatin/ g DCW)	Specific biomass production (g DCW/g carbon)[a]
Fed-batch using D.O. signal	160 ± 6	0.31 ± 0.03	1.34 ± 0.11
Fed-batch using on-line gas sensor	116 ± 15	0.33 ± 0.09	0.96 ± 0.07
Fed-batch using predetermined feed rate	489 ± 8	0.72 ± 0.01	1.67 ± 0.16

[a]Specific biomass production was based on the total utilization of carbon in the methanol and glycerol.

Pilot production of endostatin using predetermined feeding rate

Based on the above information, a pilot-scale production (10 times bigger) was conducted using the preferred growth conditions. Typical process is shown in Figure 4; after 48 h induction 6.8 L of methanol were added, producing 2.7 g endostatin and 9.3 kg biomass (wet weight). Specific endostatin production yield was 25% lower than the value obtained in the bench-top fermentor, total consumption of methanol and total biomass production were also 25% lower.

DISCUSSION

Due to the sensitivity of *P. pastoris* to methanol concentration, production of recombinant proteins under the control of the *AOX1* promoter is done by implementing different fed-batch strategies controling the methanol concentration. Growth characteristics can be different at the various strategies, and therefore, recombinant protein production can also be affected. In addition, different values of metabolic indicators (e.g., methanol concentration, dissolved oxygen concentration) and different methanol feeding rates and profiles can be selected. These parameters can potentially affect growth and recombinant protein production within each strategy. Establishing optimal protein expression protocols should therefore include the evaluation of various growth and induction strategies in addition to other parameters such as pH and temperature. In this work, we examine the effect of three fed-batch strategies on the production of recombinant murine endostatin, evaluating not only the amount of endostatin produced but also the methanol consumption, biomass production, and the specific production values. Two strategies were based on the metabolic activity of the yeast (methanol and oxygen consumption) and one was based on supplying methanol at a predetermined exponential rate (limited methanol supply). The total production of endostatin with the three growth strategies was similar. Approximately 400 mg of endostatin were produced from a 3 L initial volume, but the amount of methanol used and the biomass produced were different, affecting final volume and specific production values of both endostatin and biomass.

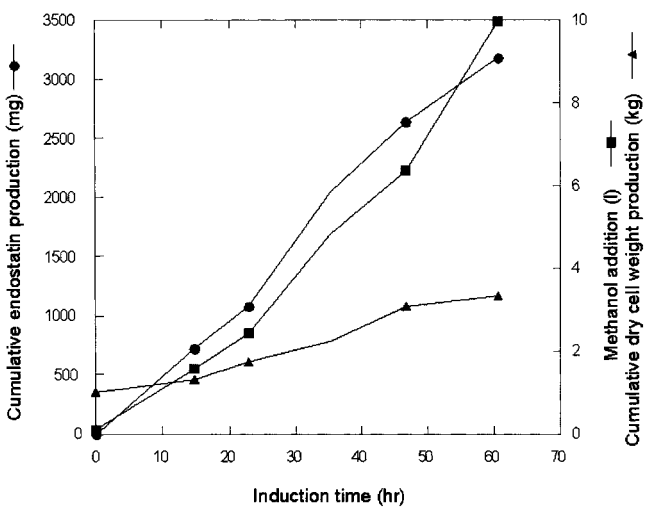

Figure 4. Pilot scale production of endostatin using predetermined methanol addition strategy: Endostatin production (●), methanol addition (■), biomass production (▲). (Time zero indicates the initiation of the methanol induction phase).

Feeding the methanol at a predetermined exponential profile and controling the growth rate at 0.02 h^{-1} was more efficient than the other two strategies. The amount of methanol used and the biomass produced were lower, causing the specific production of endostatin per biomass unit to be over 2 times higher than the dissolved oxygen controlled strategy or the sensor-based controlled strategy. Endostatin production per methanol used was almost 3 times higher in the predetermined strategy compared with the dissolved oxygen controlled strategy or the sensor strategy. A similar trend was observed with the specific biomass production. It was higher with the predetermined rate strategy than with the other two.

Methanol, the main carbon source is used both for energy generation and anabolism (Jahic et al., 2002). The higher efficiency of methanol utilization at the predetermined exponential rate suggests that the methanol is directed mainly towards energy generation, and that only a small portion is directed to biomass production. Since endostain biosynthesis is controlled by the same promoter responsible for the synthesis of the methanol oxidase, the amount of endostatin produced should be related to the methanol oxidase produced and should not be affected by the low methanol uti-

lization. It was also mentioned (Cregg, 1999) that when cells are fed methanol at a growth-limiting rate, the *AOX1* is induced to levels 3 to 5 times higher than in cells growing in excess methanol. Methanol consumption and biomass production were lower in the dissolved oxygen-controlled strategy than in the sensor-controlled strategy. This fact supports the above explanation since the methanol supply is not continuously unlimited as it is in the sensor controlled strategy.

Throughout the induction phase glycerol was supplied continuously to the culture at the rate of 1 gram per liter per hour. At this rate there was no glycerol accumulation. This supplemental carbon source is channelled directly to anabolism, (Jahic et al., 2002) and therefore compensate for possible shortage in carbon source as a result of methanol utilization for energy. While the glycerol supply may not be essential for the culture grown at excess methanol, it may be essential for the culture grown at limited methanol supply.

The results obtained from this comparison indicate that a growth strategy based on predetermined exponential feeding of methanol has an advantage for the production process of recombinant endostatin. The amount of methanol used and the biomass accumulation are lower than the values obtained using the dissolved oxygen or the methanol sensor methods, simplifying the endostatin recovery. In addition, the controlled growth rate limited the oxygen consumption and the heat production. It seems that these advantages are not necessarily specific for recombinant endostatin production. It is expected that any recombinant protein under the control of the *AOX1* promoter should respond in the same manner.

The high biomass concentration, above 50% wet weight, produced during the *Pichia pastoris* fermentation interferes with the recovery of the recombinant protein produced (Thommes et al., 2001; Trinh et al., 2000), especially when the protein is secreted to the media. In this aspect the predetermined addition methanol strategy offers another advantage, in addition to the high productivity, it simplifies the recovery process of the secreted endostatin due to the lower biomass produced and the lower overall volume.

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