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Production process for recombinant human angiostatin in *Pichia pastoris*

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A pilot-scale production method of recombinant human angiostatin, a 38-kD fragment of plasminogen which has been reported to have antiangiogenic activity, has been successfully established by expressing the protein in the methylotrophic yeast *Pichia pastoris*. The secreted protein inhibited cultured endothelial cell proliferation *in vitro* and Lewis lung carcinoma growth in mice. The fermentation process was carried out using an on-line methanol controller, administering methanol to the growing culture and keeping its concentration under 2 g L^{-1}. The fermentation lasted 90 h, of which 70 h were growth on methanol. During growth on methanol the culture volume increased 64%, from 7 L to 11.5 L, producing 200 mg angiostatin and 5 kg of biomass. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 31–35.

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Introduction

There is cumulative evidence that angiogenesis, the process of blood vessel formation, is important for the development of solid tumors and their metastases [6,18]. As a result, there is an active search for compounds that can inhibit this process and consequently inhibit tumor growth. Angiostatin, a 38 K internal fragment of plasminogen, is such a compound [11]. Recombinant angiostatin, comprised of kringles 1–4 of human plasminogen, has been cloned and expressed in *Pichia pastoris* [13]. The antiangiogenic activity of this recombinant protein is similar to that of the protein described by O'Reilly *et al* [11], indicating that *P. pastoris* expresses and processes the protein correctly. To obtain the large amount of angiostatin required for clinical evaluation of its tumor inhibitory effect, a scale-up method for production of recombinant angiostatin is needed.

The methylotrophic yeast *Pichia pastoris* has been adapted as an efficient host for expression of heterologous genes. It can grow to very high cell densities (40–50% v/v) and secrete the recombinant proteins [12,14]. When the *Pichia* uses methanol for growth, methanol-oxidase, the first enzyme in the methanol-utilization pathway is activated. The fully activated enzyme can accumulate to 30% of the total yeast proteins. This enzyme is tightly regulated and is not expressed when *Pichia* is grown on glucose or glycerol; therefore, the methanol oxidase promoter (*AOX1*) has been chosen to construct an expression vector for production of a variety of recombinant proteins in *P. pastoris*. *AOX1* expression is strongly regulated at the transcriptional level by two mechanisms: repression by glucose or glycerol, and induction by methanol [16].

Typically, a two-stage strategy is used for production of recombinant proteins from *P. pastoris*: in the first stage (batch phase), glycerol is used as the carbon source to grow the culture to a high cell density; in the second stage (induction phase), glycerol is replaced by methanol and the heterologous protein is expressed. Since excess methanol is toxic to the culture [5,17], it is usually added at a constant, limiting rate. One problem associated with cultivation of *P. pastoris* on methanol, especially in large-scale processes, relates to the oxygen supply. The amount of oxygen required for biomass production on methanol is three to four times higher than the amount needed when the carbon source is glucose [10]. High agitation and high aeration, often with oxygen-enriched air, are required to keep the dissolved oxygen concentration at approximately 20%. Also, the large amount of oxygen consumed produces considerable heat, which can increase the growth temperature in the bioreactor. Another problem is the need to maintain methanol concentrations in a narrow range. Below 0.2 g L^{-1} (v/v) the *AOX1* promoter is turned off and above 2 g L^{-1} the methanol concentration inhibits cell growth. Therefore, extended *Pichia* cultivation requires that the methanol concentration is monitored and controlled, which can be accomplished with on-line sensors. While methanol can be measured using HPLC or GC, these techniques cannot conveniently be adapted to on-line operation. With the development of inexpensive alcohol sensors, on-line monitoring and tight control of methanol concentration can now be achieved [5,17], resulting in reproducible and efficient fermentation processes. An alternative method for controlling methanol concentrations is based on measuring the dissolved oxygen level. When an abrupt increase in the dissolved oxygen level occurs, due to exhaustion of available carbon source, methanol is added [3].

Recombinant protein expression in high cell density cultures of *P. pastoris* can also be accomplished by using mixed feed fermentations [1]. This strategy consists of feeding a limiting level of glycerol and an excess of methanol during the induction phase and has been used successfully for production of several recombinant proteins [2,9].

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For production of the sea raven type II antifreeze protein, a transition phase was added after the initial batch phase. In this transition stage, the amount of glycerol was gradually reduced to partially derepress the *AOX1* promoter in preparation for induction. The present report describes an efficient process for the production of active angiostatin from *P. pastoris* using the mixed feed and on-line methanol sensor.

Materials and methods

Preparation of Pichia pastoris strains expressing angiostatin

Human angiostatin cDNA was PCR-cloned into pPIC9K (Invitrogen Corp, San Diego, CA, USA) and then expressed in *Pichia pastoris* (GS115 His^+ Mut^+) as follows: human plasminogen cDNA (ATCC, Manassas, VA, USA) was used as a template for PCR amplification of the human angiostatin fragment. The 5′ primer was 5′-AAAT ACGTAGTGTATCTCTCAGAGTGC-3′ and the 3′ primer was 5′-AAACGCGGCCGCTCACGCTTCTGTTCCTGA GCA-3′. The resulting cDNA fragment was comprised of human plasminogen cDNA encoding amino acids 98–459 with a *Sna*BI site at the 5′ end and a *Not*I site at the 3′ end. This fragment was then cloned into the *Sna*BI and *Not*I sites of pPIC9K. This human angiostatin construct was transformed into GS115 and KM71 host strains and high producers were selected per the manufacturer's recommendations [8]. Angiostatin expression was detected by Western blot using a conformation-dependent monoclonal antibody against kringles 1–3 of human plasminogen (Enzyme Research Laboratories, South Bend, IN, USA). A strain with the phenotype GS115 His^+ Mut^+ showed the highest expression level, and was chosen for further investigation.

Fermentation conditions and metabolite determination

A modified version of the growth medium recommended by Invitrogen Corp [8] was used. It consisted of yeast extract (10 g L^{-1}), peptone (20 g L^{-1}), glycerol (30 ml L^{-1}), KH_2 PO_4 (13.6 g L^{-1}), yeast nitrogen base (13.4 g L^{-1}) and biotin (0.4 mM). PTM1 trace salts were added according to the protocols mentioned above. Two 500-ml flasks (Tunair Inc, Detroit, MI, USA) with 150 ml medium each were inoculated from a frozen *Pichia* stock and incubated at 30°C and 250 rpm for 20 h. Bench-top fermentation was performed in a 15-L Magnaferm fermentor (New Brunswick Scientific, Edison, NJ, USA) with a 10-L working volume. The fermentor was interfaced to a MD-Biostat system (B Braun Biotech USA, Allentown, PA, USA) equipped with a data acquisition and control system. An adaptive control algorithm [7] maintained dissolved oxygen levels at 30% of saturation by adjusting the agitation rate and the supply of enriched air. House air was enriched to 50% oxygen using an Avir Gas Separation unit (A/G Technology Corp, Needham, MA, USA). The methanol concentration was controlled using an on-line monitoring and control device described previously [17]. Off-line methanol measurements were performed using a GC (Hewlett Packard, Atlanta, GA, USA). pH was controlled at 5.5 by the addition of 7 M NH_4OH.

After inoculation, the culture was grown in a batch mode for approximately 17 h (OD ~100 at 600 nm), after which fed batch addition of a 50% glycerol solution was initiated at a flow rate of 18 ml L^{-1} h^{-1}. After another hour (OD ~150 at 600 nm), the glycerol feed rate was reduced to 2 ml L^{-1} h^{-1} and addition of a methanol supplement commenced at a set point of 1 g L^{-1}. After another 3 h, the methanol set point was increased to 2 g L^{-1} and was maintained at this level for the remainder of the process (another 65 h).

Glycerol measurements were done using a glycerol determining kit (Boehringer Mannheim, IN, USA, No. 148270).

Angiostatin determination and recovery

Samples taken during the course of the fermentation were centrifuged at $4100 \times g$ for 40 min to remove the biomass. Supernatant (100 ml) was dialyzed against 2 L of 50 mM potassium phosphate buffer pH 7.4 (equilibration buffer) at 4°C using 6000 MWCO dialysis tubing. Lysine-Sepharose 4B (2 ml) (Pharmacia Biotech, Piscataway, NJ, USA) was equilibrated with 10 column volumes (CV) of equilibration buffer (50 mM phosphate buffer, pH 7.4). The dialyzed sample was then loaded onto the resin using gravity flow. The resin was next washed with 10 CV of equilibration buffer followed by 5 CV of 0.5 M NaCl, 50 mM phosphate buffer pH 7.4. Bound protein was eluted using 5 CV of 0.2 M ϵ-amino-N-caproic acid pH 7.4. Elution fractions were characterized using SDS-PAGE and quantified using the modified bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA).

For large-scale recovery, biomass was removed using a continuous flow centrifuge (Sharples AS-16, Alfa-Laval Sharples, Wage, PA, USA). The supernatant was diafiltered three times against an equal volume of equilibration buffer using a 10 000 MWCO tangential flow filtration device (Millipore, Bedford, MA, USA). The supernatant was then loaded onto a Lysine-Sepharose 4B resin using the conditions described above.

Bovine capillary endothelial cell proliferation assay

Bovine capillary endothelial cells were obtained and maintained as previously described [4]. Briefly, cells dispersed with 0.05% trypsin, 0.53 mM EDTA were plated onto gelatinized 24-well culture plates (Difco) at 12 500 cells per well, in DMEM containing 10% bovine calf serum (BCS), and incubated for 24 h at 10% CO_2. The medium was replaced with 0.25 ml DMEM containing 5% bovine calf serum and either buffer or buffer-containing test samples were added. After 20 min incubation, the same medium supplemented with bFGF (basic fibroblast growth factor) (Scios Nova, Mountainview, CA, USA) was added to a final volume of 0.5 ml and 1 ng ml^{-1} bFGF. After 72 h, the cells were counted using a Coulter Counter. Cell numbers are reported as the means of triplicate determinations.

Treatment of Lewis lung carcinoma primary tumors in mice

Animal studies were carried out at the animal facility of Children's Hospital (Boston, MA, USA) in accordance with institutional guidelines. Tumors (Lewis lung carcinoma, 1 million cells per mouse) were inoculated into the subcut-

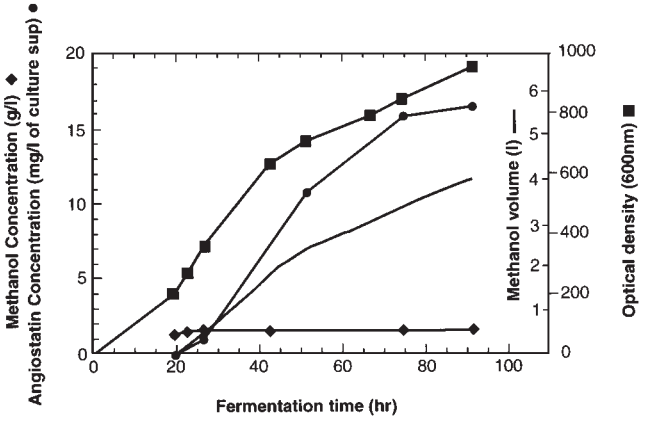

Figure 1 Fermentation of *P. pastoris* for production of angiostatin, using methanol as the inducer. Methanol feeding was under control of the methanol sensor. *Pichia* concentration, OD 600 nm (■); angiostatin concentration in the supernatant medium, mg ml^{-1} (●); methanol concentration, g L^{-1} (◆); methanol added, liters (—).

aneous mid-dorsum of C57B1/6J mice (Rosco Jackson Laboratory, Bar Harbor, ME, USA), as previously described [11]. Treatment was commenced when the tumors reached 150–200 mm^3. Four mice received 10 mg (per kg weight) angiostatin, administered subcutaneously every 12 h, four mice received 50 mg angiostatin every 12 h and four mice treated with phosphate-buffered saline served as the control group.

Results

Angiostatin production

Several key parameters of the fermentation process for angiostatin production from *P. pastoris* are described in Figures 1 and 2. The fermentation was a fed-batch process, in which the culture volume increased from 7 L to 11.5 L as a result of continuous methanol addition. *Pichia* concentration (OD), angiostatin concentration in the culture supernatant (mg L^{-1}), total amount of added methanol (L) and methanol concentration (g L^{-1}) are described in Figure 1; total biomass (kg), total angiostatin (mg) and culture volume are described in Figure 2. In this fermentation procedure, there was a considerable accumulation of biomass and considerable increase in culture volume, but the volume of the workable supernatant (approximately 7 L after biomass removal) hardly changed during the fermentation process (Figure 3).

The initial carbon source was glycerol, added first as a batch, and later (after 18 h) at a constant rate of 2 g L^{-1} h^{-1} throughout the rest of the fermentation. The residual glycerol concentration in the culture was between 0.01 and 0.05 g L^{-1}, a range that does not affect the AOX1 promoter [13]. When the culture density reached approximately 150 OD at 600 nm (10% v/v), about 20 h after starting the fermentation, methanol addition was commenced and its concentration in the culture was kept at 2 g L^{-1} using the described methanol sensor. Although the methanol controller displayed oscillatory behavior and was sensitive to the culture temperature, the methanol level was maintained successfully at 2 (±0.5) g L^{-1}. It was important to begin induction with methanol at a cell density between 150 and 200 OD (at 600 nm). Inducing the culture at higher cell densities resulted in significant problems with maintaining the desired temperature and dissolved oxygen level because the high amounts of oxygen, needed for methanol oxidation, generate a large amount of heat during the exponential growth phase on methanol. Higher temperatures affect the growth of *Pichia*, the stability of the product and the calibration of the methanol sensor. During the fermentation, 1120 g of glycerol, 1.15 L of 7 M ammonium hydroxide and 3.8 L of methanol were added. After 90 h of fermentation, of which 70 h was the duration of growth on methanol, 200 mg of angiostatin and 5 kg of biomass were produced.

Since a high percentage of the culture is biomass, the appropriate way to express angiostatin production is in mg per liter supernatant instead of mg per liter culture, the average production (based on five consecutive runs) was 20 (±5) mg L^{-1} supernatant. A slightly lower concentration has been claimed in a recent patent application [15].

Effect of recombinant angiostatin on capillary endothelial cell proliferation

The effect of recombinant angiostatin on capillary endothelial cell proliferation is seen in Figure 4. The results are presented as percent inhibition of proliferation driven by

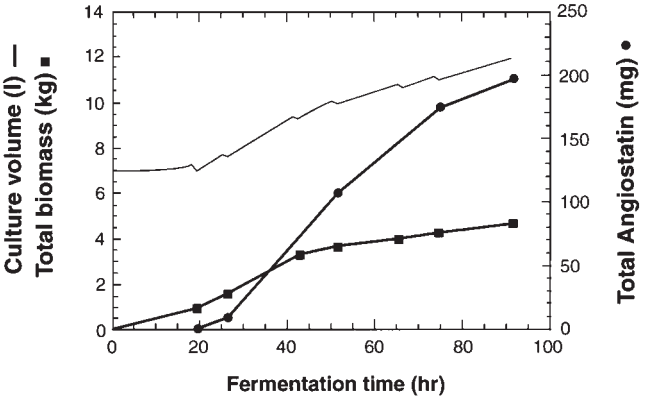

Figure 2 Accumulation of biomass and angiostatin during the fermentation process of *P. pastoris*. Pichia biomass, kg (■); angiostatin, mg (●); culture volume, L (—).

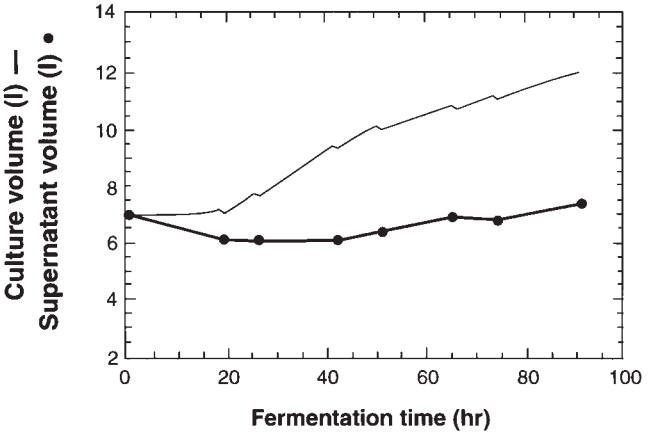

Figure 3 Total volume and supernatant volume during *P. pastoris* fermentation process. Total volume, liter (—); supernatant volume, liter (●).

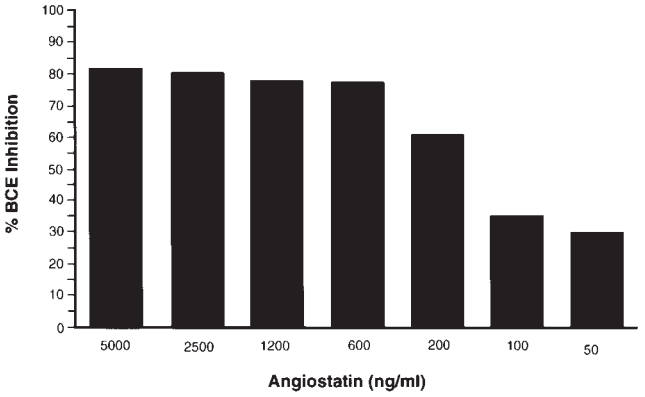

Figure 4 Effect of recombinant angiostatin on bovine capillary endothelial cell proliferation (BCE).

basic fibroblast growth factor (bFGF). Angiostatin inhibited the bovine cell proliferation in a dose-dependent and saturable manner. At a concentration of 50 ng ml^{-1}, it inhibited endothelial cell proliferation by 30%; and at 1 mg ml^{-1} it completely inhibited cell proliferation. The median effective dose (ED-50) was approximately 100 ng ml^{-1}, which is as good or even better than angiostatin produced by elastase digestion of plasminogen. The inhibitory activity of the protein could be completely diminished by pre-incubating the sample with lysine-Sepharose, which depleted angiostatin with high affinity (results not shown).

Effect of recombinant angiostatin on the growth of Lewis lung carcinoma in mice

The effect of recombinant human angiostatin on the growth of Lewis lung carcinoma in mice was tested by subcutaneous administration of the protein to mice bearing Lewis lung carcinomas of at least 150–200 mm^3. After 10 days of daily injections, there was no sign of skin inflammation at the site of injection. In all experiments, treated mice gained weight normally. As indicated in Figure 5, the growth of Lewis lung primary tumors was potently suppressed by systemic therapy with recombinant angiostatin. Increasing the dose of angiostatin was associated with improved efficacy. Compared to control mice, which were treated with phosphate-buffered saline alone, tumor growth was inhibited by 46% when the mice were treated with 10 mg kg^{-1} every 12 h. When the dose was increased to 50 mg kg^{-1} every 12 h, tumor growth was inhibited by 70% (treated/control = 0.3, $P < 0.001$). Similar results were obtained in two separate experiments. These results are consistent with those observed using other forms of human angiostatin to treat Lewis lung primary tumors. To obtain anti-tumor activity that was similar to murine angiostatin injected once daily, it was necessary to inject human protein every 12 h.

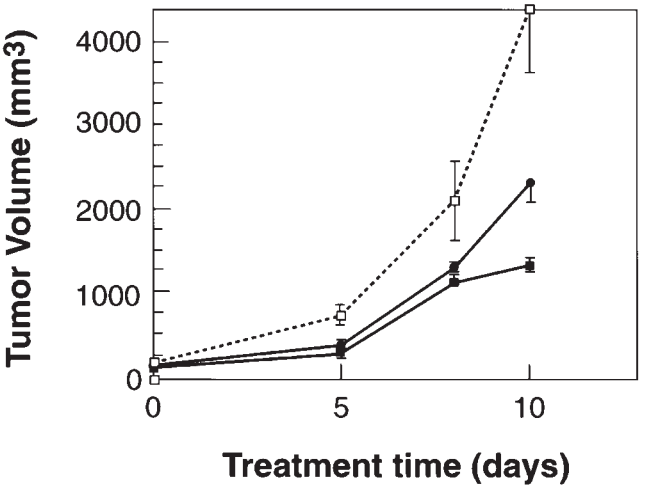

Figure 5 Effect of recombinant human angiostatin on growth of Lewis lung carcinoma in three groups of mice. Untreated (□); treated with 10 mg angiostatin per kg every 12 h (●); and treated with 50 mg angiostatin per kg every 12 h (■).

Discussion and conclusions

The produced angiostatin was active *in vitro* and *in vivo*. It completely inhibited endothelial cell proliferation at a concentration of 1 mg ml^{-1}, and it inhibited tumor growth in mice by 70% when the mice were treated with 50 mg kg^{-1} every 12 h.

The production process is comprised of two phases; in the first phase the yeast grows on glycerol and in the second phase it grows on methanol. Because of the sensitivity of the yeast to the concentration of methanol, which serves as both carbon source and inducer, it was added continuously in response to an on-line methanol sensor, keeping its concentration at around 2 g L^{-1}. Compared to other methanol addition strategies, such as continuous addition at a predetermined rate, or addition based on response to changes in pH or changes in dissolved oxygen concentration, this strategy resulted in a relatively constant concentration of methanol throughout the fermentation. In addition to the methanol supply, the culture was also supplied with glycerol at a constant rate of 1 g L^{-1} h^{-1}. At this rate, the residual glycerol concentration was below 0.05 g L^{-1}, therefore not affecting protein expression. At the end of the fermentation, the culture volume was up 60%, however the workable supernatant volume was slightly lower than the starting volume, indicating a substantial biomass buildup that can cause difficulties in the angiostatin initial recovery steps.

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