

Efficiencies of Growth and Angiostatin Expression in Cultures of *Pichia pastoris* Fed with Mixed Carbon Sources

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Different carbon sources, glycerol, sorbitol, acetate and lactate were added together with methanol in the induction phase of *Pichia pastoris* culture for angiostatin production in a 5-L fermentor. Mass balances were carried out for different processes, yield coefficients were discussed and stoichiometric coefficients were estimated to compare the efficiency of different carbon sources. Glycerol had the highest reduction degree among the four non-methanol carbon sources, as well as higher energetic yield coefficient for biomass production (η) and lower respiratory quotient (RQ), indicating that glycerol was a more efficient carbon source for biomass formation than the other three non-methanol carbon sources. Lactic acid, a non-repression carbon sources, had lower reduction degree, but achieved the highest energetic yield coefficient for angiostatin production (ξ_p).

Key words:

Mass balance, *Pichia pastoris*, glycerol, lactate, angiostatin

Introduction

The methylotrophic yeast *Pichia pastoris* is an effective host for the production of heterologous proteins.¹ *P. pastoris* is suited for foreign protein expression for three main reasons: it can be easily manipulated at the molecular level (e.g., gene targeting, high-frequency DNA transformation, cloning by functional complementation); it can express proteins at high levels intracellularly or extracellularly; and it can perform modification of many eukaryotic proteins, such as glycosylation, disulfide-bond formation, and proteolytic processing.² These advantages make *P. pastoris* a very attractive host for recombinant protein production.

An unusually efficient and tightly regulated promoter of *P. pastoris* from the alcohol oxidase 1 (AOX1, EC 1.1.3.13) gene is used to express foreign genes.³ Through gene disruption,⁴ the “AOX1 promoter – gene of interest” expression cassette can be inserted in the genome.^{2,5,6} Depending on the mode of insertion, three different phenotypes of *P. pastoris* are generated: Mut⁺ (wild AOX phenotype, where the two alcohol oxidase genes *AOX1* and *AOX2* are intact), Mut^S (methanol utilization slow, where *AOX1* is disrupted while *AOX2*, which is responsible for only 15 % of the total AOX activity, is intact), and Mut⁻ (methanol utilization negative,

in which both genes *AOX1* and *AOX2* are disrupted). For Mut^S strains, due to their slow utilization of methanol, a mixed feed of glycerol and methanol is commonly employed in the induction phase of fermentation.⁷ With this strategy, expression of various proteins has been successfully enhanced in either fed-batch or continuous culture of Mut^S strains.^{8–11} However, with the volumetric productivity enhanced, the specific productivity of foreign protein may be lower because excess glycerol represses the *AOX1* promoter and limits the expression of foreign genes.⁹

In the present study, the same strategy was used in fed-batch fermentation for the production of a 38-kDa heterologous protein, angiostatin, using *P. pastoris* with a phenotype of Mut^S. We have tried some other carbon sources, sorbitol, acetate and lactic acid, to replace glycerol in the induction phase. Sorbitol is a widely accepted non-repressive carbon source for gene expression controlled by *P. pastoris* *AOX1* promoter,^{12,13} and the results of our fermentation, in which sorbitol was added together with methanol in the induction phase, supported this concept. Acetate has been reported to be a repressor in the fermentation using recombinant *P. pastoris* with a phenotype of Mut⁺ or Mut⁻,¹⁴ and the results of our work indicated that acetate was not good for angiostatin production. Lactic acid was used as a substrate for the production of pyruvic acid with the *P. pastoris* cells as a cellular

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catalyst, in which the glycolate oxidase (EC 1.1.3.15) gene from spinach and an endogenous catalase (EC 1.11.1.7) gene were expressed.^{15,16} A notable discovery of our previous work was that lactic acid was a non-repressive carbon source for the expression of angiostatin, while the highest angiostatin level and the highest productivity were achieved in the fermentation fed with lactic acid and methanol in the induction phase.¹⁷ The objective of the present study was to analyze the utilization efficiencies of the carbon sources based on the material and energy balances.

Material and energy balances are widely used in analysis of processes including fermentation.¹⁸ However, publications are few on metabolic balance for fermentation using *P. pastoris* with methanol as the sole carbon source and inducer.^{19,20} This paper describes the material and energy balances in cultures of the Mut^S *P. pastoris* strain producing angiostatin on mixed carbon sources for angiostatin production, and the efficiencies of different carbon sources for cell growth and angiostatin expression are evaluated, based on our previous experimental work of Xie et al.¹⁷

Materials and methods

Organism

Pichia pastoris GS115 (*his4*) (Invitrogen, San Diego, CA, USA) was transformed with plasmid pPIC9k (Invitrogen) containing the gene encoding angiostatin obtained by RT-PCR from a human HepG2 hepatoma cell line.²¹ The pPIC9K plasmid contains the bacterial kanamycin gene (*kan* from Tn903) that confers resistance to Geneticin® in *Pichia*. The pPIC9k plasmid that contains the *his4* gene for selection of His⁺ clones was integrated by homologous recombination into the *AOX1* gene site of the chromosome DNA. A transformant with a Mut^S phenotype was selected, and angiostatin was secreted into the fermentation broth. This strain was cultured in YPD medium at 30 °C and 250 rpm for 12 h, and mixed with an equal volume of 50 % sterile glycerol and stored at –20 °C.

Media

The BMGY medium for inoculum culture contained (per liter): yeast extract (Oxoid, UK), 10 g; Polypeptone (Daigo Eiyo, Japan), 20 g; KH₂PO₄/K₂HPO₄ buffer (pH 6.0), 100 mmol; Yeast Nitrogen Base without amino acid (Difco, USA), 13.4 g; (NH₄)₂SO₄, 5 g; biotin, 400 μg; glycerol, 10 ml. The BSM fermentation medium contained (per liter): glycerol, 40 g; 85 % (w/w) H₃PO₄, 26.7 mL; CaSO₄, 0.93 g; K₂SO₄, 18.2 g; MgSO₄·7H₂O,

14.9 g; KOH, 4.13 g; trace salts (PTM₁, see below), 4.35 mL; pH 5.0 adjusted with 28 % (w/w) NH₄OH. This medium except for the trace salts was sterilized at 120 °C for 30 minutes. The PTM₁ trace salts solution contained (per liter): CuSO₄, 6.0 g; KI, 0.08 g; MnSO₄, 3.0 g; Na₂MoO₄, 0.2 g; H₃BO₃, 0.02 g; CoCl₂, 0.5 g; ZnCl₂, 20.0 g; FeSO₄·7H₂O, 65.0 g; biotin, 0.2 g; 98 % (w/w) H₂SO₄, 5 mL. This solution was filter sterilized and stored at 4 °C.

Fermentation

Fermentation performance can be seen in the previous work of Xie et al.¹⁷ The primary inoculum culture was prepared by transfer of 0.7 mL glycerol stock to 25 mL BMGY medium in a 250-mL flask and incubation for 14 h at 30 °C and 250 rpm. The secondary inoculum was obtained by distributing the primary inoculum culture to three 500-mL flasks each containing 50 mL BMGY, and incubation at 30 °C and 250 rpm for 7.5 h. All the secondary cultures were combined and inoculated into 2.5 L BSM fermentation medium in a 5-L fermentor (Model RIBE-5, ECUST, China).

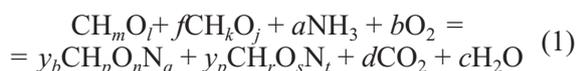
The 5-L fermentor was controlled by a personal computer with a software program (TopHawk Fermentation Control System, National Center for Biochemical Engineering Research, Shanghai, China), and the on-line and off-line data were collected in the PC. The fermentation conditions were: temperature 30 °C; impeller speed 450–1100 rpm; aeration 4 L min⁻¹; pH 5.0 controlled with 5 M KOH as the cell density was lower than 54 g L⁻¹ and with 7.2 M NH₄OH for the rest period of fermentation. Dissolved oxygen was measured by a sterilizable electrode (Mettler-Toledo, Moburn, MA, USA) and maintained above 20 % of air saturation by manual adjustment of the agitation speed.

The fermentation experiments began with a batch growth phase on glycerol for approximately 17 h when the initial glycerol was exhausted and the cell density reached around 30 g L⁻¹. A solution of 50 % (w/w) glycerol supplemented with PTM₁ (12 mL per liter) was then continuously added to initiate the fed-batch growth phase. The initial flow rate of glycerol was 1.1 g h⁻¹, which was gradually increased to 5.3 g h⁻¹ to obtain approximately exponential growth. The induction phase was started at a cell density of about 60 g L⁻¹. During this period, methanol feeding was automatically executed with a peristaltic pump controlled by a methanol monitor and control system. This system was composed of a methanol collector inserted into the culture broth, an alcohol detector, and a feeding controller. Methanol in the culture broth diffused across a silicon membrane of the methanol collector into a stream of air, and was brought to the alcohol detector con-

taining a semiconductor element to produce an electrical signal,²² which was used to control the pump for feeding of methanol in a proportional regulation mode. The residual methanol concentration in the culture was maintained at 5 g L⁻¹ in all the fed-batch cultures. Different carbon sources, i.e., glycerol (in experiment F2), sorbitol (in experiment F3), acetic acid-ammonium acetate (in experiment F4), and lactic acid (in experiment F5) were respectively used. The feeding rates were manually adjusted to maintain continuous oscillation of the dissolved oxygen concentration. During the induction phase of experiment F2, the initial feeding rate of the glycerol feed was 1.1 g h⁻¹, which was gradually increased to 4.8 g h⁻¹. A 70 % (w/w) sorbitol solution (containing 12 mL PTM₁ per liter) was added at an initial flow rate of 3.5 g h⁻¹ and gradually increased to 9.1 g h⁻¹ in the induction phase of F3. A solution of 28.6 % (w/w) ammonium acetate (containing 12 mL PTM₁ per liter) was continuously added at an initial flow rate of 1.2 g h⁻¹, and then gradually increased to 2.8 g h⁻¹ by the end of the induction phase of F4. The pH of the fermentation broth rose due to consumption of acetate, and a solution of 50 % (v/v) acetic acid (containing 12 mL PTM₁ per liter) was automatically added to maintain the culture pH at 5.0. A solution of 51.4 % (w/w) lactic acid (containing 12 mL PTM₁ per liter) was used as the non-methanol carbon source in the experiment F5. The initial feeding rate of lactic acid was 2.6 g h⁻¹, and then gradually increased to 11.3 g h⁻¹ by the end of fermentation. For feeding of the non-methanol carbon sources, the activation period of the feeding pump was fixed to 1 second, and by changing the non-activation period of the peristaltic pump, the feeding rate can be changed according to a pre-determined flow rate relationship. The feed reservoirs of methanol and the second carbon source were respectively weighed as the sample was withdrawn from the fermentor to calculate the real amount of added carbon sources. Casamino Acids (Bacto, France) was supplemented into the fermentor at a concentration of 1 g L⁻¹ every 24 h to suppress the hydrolysis of angiostatin caused by extracellular proteases.²³

Mass and energy balances in fermentation fed with mixed carbon sources

The mass-energy balance method employed in this study was based on the approach proposed by Erickson et al.¹⁸ The microbial growth in a chemically defined medium containing two carbon sources can be illustrated by the equation:



where CH_mO_l stands for methanol ($m = 4$ and $l = 1$), and CH_kO_j for the second carbon source. y_b and y_p are the stoichiometric coefficients of the biomass and product, angiostatin, respectively, based on consumed methanol. The reduction degree, γ_s , γ_b and γ_p , is defined as the moles of available electrons per mole of substrate, biomass and product, respectively.¹⁸ The elemental reduction degree is 4 for carbon, 1 for hydrogen, -2 for oxygen, and -3 for nitrogen, thus the reduction degree is 0 for CO₂, H₂O and NH₃.¹⁸ The reduction degrees of the carbon sources 1 (methanol, γ_{s_1}) and 2 (the non-methanol carbon source, γ_{s_2}), biomass (γ_b), and product (γ_p) are:

$$\gamma_{s_1} = 4 + m - 2l \quad (2)$$

$$\gamma_{s_2} = 4 + k - 2j \quad (3)$$

$$\gamma_b = 4 + p - 2n - 3q \quad (4)$$

$$\gamma_p = 4 + r - 2s - 3t \quad (5)$$

The oxygen requirement is directly related to available electrons transferred to oxygen, and a balance based on the available electrons is:

$$\gamma_{s_1} + f\gamma_{s_2} + b(-4) = y_b\gamma_b + y_p\gamma_p \quad (6)$$

Thus, the stoichiometric coefficient for oxygen in equation (1), b , is given by the following:

$$b = \frac{1}{4}(\gamma_{s_1} + f\gamma_{s_2} - y_b\gamma_b - y_p\gamma_p) \quad (7)$$

Equation (7) can be rearranged to reflect the balance of fractions of available electron in the carbon sources:

$$\frac{4b}{\gamma_{s_1} + f\gamma_{s_2}} + \frac{y_b\gamma_b}{\gamma_{s_1} + f\gamma_{s_2}} + \frac{y_p\gamma_p}{\gamma_{s_1} + f\gamma_{s_2}} = 1 \quad (8)$$

The first term on the left side of equation (8) is the fraction of available electrons in the carbon sources transferred to oxygen. Since this part of energy finally releases as heat, it gives the fraction of energy contained in the carbon sources generating heat. If the heat evolved from 1 mole available electrons transferred to oxygen is Q_o (113.1 kJ mol⁻¹), the heat evolved, Q , by 1 mol carbon source 1 and f mol carbon source 2, is:

$$Q = 4Q_o b \quad (9)$$

The second term on the left side of equation (8) is the fraction of available electrons in the carbon sources transferred to the biomass:

$$\eta = y_b \left(\frac{\gamma_b}{\gamma_{s_1} + f\gamma_{s_2}} \right) \quad (10)$$

which is the energy yield coefficient for biomass production, because it is the ratio of the heat derived from oxidation of the produced biomass to that from oxidation of the utilized carbon sources.

The third term on the left side of equation (8) is the fraction of available electrons transferred to the product:

$$\xi_p = y_p \left(\frac{\gamma_p}{\gamma_{s_1} + f\gamma_{s_2}} \right) \quad (11)$$

The energy yield coefficient of biomass can be mathematically related to the mass yield coefficient based on consumed oxygen, $Y_{X/O}$. The oxygen requirement in equation (1) is b mol O_2 or $32b$ g O_2 , while the produced biomass contains y_b moles of carbon which corresponds to $12y_b/\sigma_b$ g biomass. From equations (7), (10) and (11), the dimensionless biomass yield coefficient based on consumed O_2 , y_O can be written as

$$y_O = \frac{y_b}{b} = \frac{4y_b}{(\gamma_{s_1} + f\gamma_{s_2})(1 - \eta - \xi_p)} \quad (12)$$

and, the mass yield coefficient of biomass based on consumed oxygen,

$$Y_{X/O} = \frac{3y_b}{2\sigma_b(\gamma_{s_1} + f\gamma_{s_2})(1 - \eta - \xi_p)} \quad (13)$$

Analytical procedures

Cell concentration was estimated from the optical density of appropriately diluted culture sample measured at 600 nm (OD_{600}). An OD_{600} of 1 was equivalent to dry cell weight of 0.36 g L^{-1} and all cell densities are reported as dry cell weight per liter in this paper. Glycerol was determined by using an enzymatic assay kit (Jiemen Co., Shanghai, China) containing glycerol kinase (EC 2.7.1.30), ATP, glycerol phosphate oxidase (EC 1.1.3.21), and peroxidase (EC 1.11.1.7). Glycerol concentration was estimated according to the red color generated by a reaction with formed hydrogen peroxide.²⁴ Lactic acid was determined by a lactate assay kit (Jiancheng Co. Ltd, Nanjing, China) containing lactate dehydrogenase (EC 1.1.1.27) and NAD according to NADH formed. Methanol and acetate were measured by gas chromatography equipped with a column packed with Chromosorb 101 (Dikma, Lampoc, CA, USA) and detected by an FID detector. The flow rates of nitrogen and hydrogen were respectively 1 and 30 mL min^{-1} , but the column temperatures were different, i.e., 180 °C for methanol and 230 °C for acetate. Sorbitol was measured by HPLC (Waters, Milford, MA, USA) using a Sugar-Pak I column at 90 °C. The mobile phase

was double distilled water, and the eluted sorbitol was detected with a Waters 2410 differential refractometer.

Angiostatin was measured by ELISA. The supernatant of culture samples was reacted at 37 °C for 2 h with goat anti-human plasminogen polyclonal antibody, then reacted at 37 °C for 1 h with rabbit anti-goat antibody coupled with horseradish peroxidase. The absorbance was then measured at 450 nm and compared with those of angiostatin standards.²¹

The elemental composition (C, H, O, N) of biomass was determined at the Chemical Analysis Center of ECUST with a VARIO EL III elemental analyzer (Elementar, Germany). The ash content of biomass was determined through heating the biomass at 1000 °C for 4 h (Chinese National Standard, GB7531).

Results and discussion

Fermentation fed with different carbon sources in the induction phase

Zhang et al. have reported that the methanol metabolism in a Mut^S *P. pastoris* strain expressing the heavy-chain fragment C of botulinum neurotoxin serotype C is affected by the gene insertion event.²⁵ The specific growth rate of Mut^S *P. pastoris* strains growing on methanol was once reported between 0.01 and 0.04 h^{-1} .²⁶ However, in a more recent research,²⁵ it could be as low as 0.008 h^{-1} . In the present work, the capacity of methanol utilization was investigated using a methanol monitor and control system to well control the methanol concentration in the fermentation broth. We tried several methanol levels in the induction phase and a methanol concentration of 5 g L^{-1} was adopted due to the higher cell growth rate and angiostatin productivity at this level (data not shown). Mut^S strains can use methanol as sole carbon source at lower concentration than a Mut⁺ but at higher concentrations than a Mut⁻ strains.²⁷

During the expression phase, the methanol concentration was automatically controlled at 5 g L^{-1} by the methanol measurement and control system and no other carbon source was delivered (experiment F1). At 49 h past induction, the final cell and angiostatin concentrations were 83.6 g L^{-1} (Fig. 1) and 30.4 mg L^{-1} (Fig. 2), respectively. The overall specific growth rate and specific methanol uptake rate during the induction phase were 0.006 h^{-1} and 0.016 g $g^{-1} h^{-1}$, respectively. In order to improve cell growth and angiostatin production, a second carbon source was added together with methanol in the induction phase. Methanol addition was automatically executed by the methanol mea-

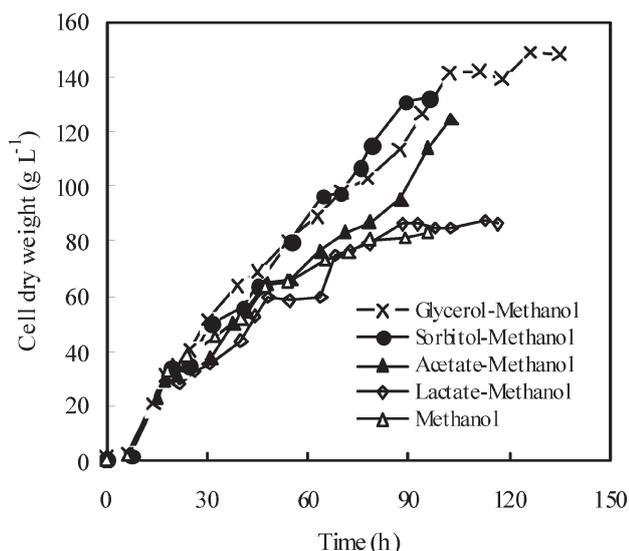


Fig. 1 – Time courses of cell growth in the fermentations fed with different carbon sources

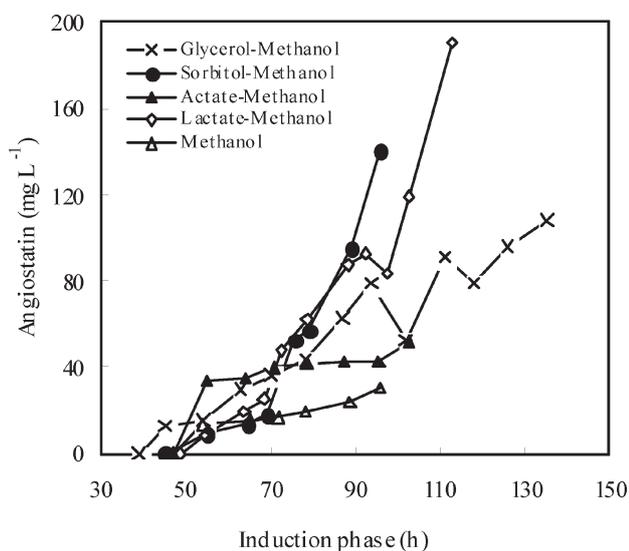


Fig. 2 – Time courses of angiostatin concentration in the fermentations fed with different carbon sources in the induction phase

surement and control system, and the non-methanol carbon source was added by manual adjustment of the feeding rate. Glycerol was firstly considered, since it is the most generally used carbon source added simultaneously with methanol.²⁸ In establishing the glycerol feeding protocol, dissolved oxygen level was used as an indicator to avoid over-feeding of glycerol that represses the *AOXI* promoter,²⁹ and continuous oscillations in dissolved oxygen concentration were realized, which indicated a glycerol-limitation condition was maintained, and the residual glycerol was lower than 0.078 g L^{-1} . After an induction period of 96 h, during which the average specific growth rate was 0.012 h^{-1} , the final cell concentration was 150 g L^{-1} (Fig. 1),

and the angiostatin concentration was 108 mg L^{-1} (Fig. 2).

Because of the repressive effect of glycerol on the *AOXI* promoter and the possible problem to quickly distribute the added concentrated glycerol to the whole fermentation broth especially in large reactors, exploitation of more suitable carbon sources is critical for efficient heterologous protein production by *P. pastoris*. Sorbitol was tried (experiment F3) because it is a well-accepted, non-repressive carbon source to the *AOXI* promoter.^{8–11,26} After an induction period of 51 h, the angiostatin concentration reached 141 mg L^{-1} (Fig. 2) at a cell density 132 g L^{-1} (Fig. 1). The residual sorbitol was not detected in the fermentation broth except at 10 h past induction, when the residual sorbitol concentration was 0.4 g L^{-1} . Acetate was used as the second carbon source in the experiment F4. During the induction phase, the measured residual acetate concentration was below 0.05 g L^{-1} . The cell density achieved 125 g L^{-1} (Fig. 1) while the angiostatin concentration was only 52 mg L^{-1} after an induction period of 55 h (Fig. 2).

An unexpected result came out in the experiment F5, in which lactate acid was used as the second carbon source. After an induction period of 64.5 h, the angiostatin concentration reached 191 mg L^{-1} (Fig. 2). In addition, residual lactic acid reached 0.6 g L^{-1} at 6 h after the start of induction and then gradually increased up to 6.3 g L^{-1} at the end of fermentation. Since angiostatin concentration increased progressively and achieved a high level, this phenomenon suggested that lactic acid could be a non-repressive carbon source. However, the cell density at the end of fermentation was as low as 87.5 g L^{-1} (Fig. 1).

The parameters of the fermentation experiments are summarized in Table 1. When methanol was used as the sole carbon source (F1), the overall specific growth rate in the induction phase was 0.006 h^{-1} and the overall specific angiostatin productivity was $0.008 \text{ mg (g cell)}^{-1} \text{ h}^{-1}$. Obviously, the very low capability of methanol assimilation of the present strain ($0.016 \text{ g (g cell)}^{-1} \text{ h}^{-1}$) seriously limited cell growth and angiostatin expression. When another carbon source was supplied together with methanol, although the specific methanol utilization rates were similar (F4 and F5) or even lower (F2 and F3), the specific growth rates were greatly improved. The biomass concentration was also well elevated due to addition of the non-methanol carbon sources, indicating that the non-methanol carbon sources largely contributed to biomass formation. However, the higher specific cell growth rate did not necessarily lead to a higher specific protein production rate.^{29,30} When a repressive carbon source (such as glycerol) was used, the excessive

cell growth rate was accompanied with successive exposure of the cells to that carbon source thus bringing about lower productivity.²⁹ Acetate has been reported as a repressive carbon source,¹⁴ so the lower angiostatin productivity in experiment F4 might be caused by the high specific growth rate of 0.02 h⁻¹ resulting from a high ammonium acetate feeding rate.

In the processes F2, F3, F4 and F5, the real cell yields based on methanol or the non-methanol carbon source could not be estimated directly. However, the specific uptake rates of the non-methanol carbon sources could indicate the efficiency of carbon sources transformed to biomass. Through comparison of the specific uptake rates of the non-methanol carbon sources under the condition that the specific uptake rate of methanol was less different among all the processes, the conclusion could be drawn that the cell yield coefficient on glycerol was the highest among all the non-methanol carbon sources used in this work, followed by sorbitol and lactate, and acetate had the lowest efficiency for cell growth. Another way was to compare the overall cell yields based on molar carbon consumed (Table 1), which also indicate that the feeding of glycerol and methanol supported cell growth the best. Glycerol not only supported the growth of *P. pastoris*, but also remarkably improved angiostatin production as shown by the enhanced specific productivity (Table 1). Feeding of sorbitol and lactate in the induction phase could also enhance the angiostatin production significantly; however, the biomass formations were less than that fed with glycerol. A lower cell density means that more supernatant of fermentation broth could be obtained and more angiostatin could be harvested. Thus, glycerol was an efficient carbon source for the growth of *P. pastoris*, while for the angiostatin separation after the fermentation, fewer cells with a high angiostatin expression level could bring about more protein harvest, and sorbitol and lactate would be preferred.

Table 1 – Characteristic variables in fed-batch fermentations conducted in a 5-L fermenter using different carbon source combinations during the induction phase

	F1	F2	F3	F4	F5
Average specific growth rate during the induction phase (h ⁻¹)	0.006	0.012	0.018	0.020	0.011
Average angiostatin productivity (mg L ⁻¹ h ⁻¹)	0.62	1.66	2.76	0.95	2.96
Average specific angiostatin productivity (mg (g cell) ⁻¹ ·h)	0.008	0.019	0.030	0.011	0.044
Average cell yield on molar carbon (g cell (mol carbon) ⁻¹)	3.62	14.3	11.8	6.38	6.61
Specific uptake rate of non-methanol carbon source (g (g cell) ⁻¹ h)	–	0.016	0.028	0.078	0.034
Specific uptake rate of methanol (g (g cell) ⁻¹ h)	0.016	0.011	0.009	0.016	0.017
Uptake of non-methanol carbon source (w) : methanol (w)	–	1.50	3.50	5.10	2.20

The reduction degree and combustion heat of the carbon sources could theoretically well support the above discussion about the carbon source efficiency, since carbon source with different reduction degree and combustion heat is degraded and assimilated in different pathways, and thus displays different efficiencies for growth and product formation.³¹ Table 2 shows the elemental composition, reduction degree and the standard molar combustion heat of the five carbon sources used in this study. The reduction degree and combustion heat indicate the energetic potential of a particular substrate. By taking into account the thermodynamic properties indicated in Table 2 alone, methanol should be a highly efficient energy source due to the high reduction degree and combustion heat, but methanol utilization of the Mut^S strain was upset because of the destroyed AOX1 activity. It can be seen in Table 2 that methanol is followed by glycerol, sorbitol, lactate and acetate. Although acetate and lactate have the same reduction degree, the molar combustion

Table 2 – Elemental composition (w/w), reduction degree γ_s , standard molar combustion heat ΔH_c° and mass combustion heat of carbon sources used in the present work³⁴

Carbon sources	C (%)	H (%)	O (%)	γ_s		ΔH_c°	
				(mol electron mol ⁻¹)	(mol electron g ⁻¹)	(kJ mol ⁻¹)	(kJ g ⁻¹)
Methanol (CH ₄ O)	37.5	6.25	50	6.0	0.188	-727.6	-22.7
Glycerol (CH _{2.667} O)	39.1	8.7	52.2	4.7	0.152	-552.6	-18.0
Sorbitol (CH _{2.333} O)	39.6	7.7	52.7	4.2	0.143	-468.3	-15.4
Lactic acid (CH ₂ O)	40.0	6.7	53.3	4.0	0.133	-456.4	-15.2
Acetic acid (CH ₂ O)	40.0	6.7	53.3	4.0	0.133	-437.9	-14.6

heat of acetate is lower. The descending alignment of the reduction degree and combustion heat based on the 4 non-methanol carbon sources in Table 2 coincides well with the cell yield shown in Table 1.

Stoichiometric coefficients

The stoichiometric coefficients in equation (1) in the expression phase were estimated according to the elemental composition of the carbon sources (Table 2), biomass (with the molecular composition of $\text{CH}_{1.898}\text{O}_{0.627}\text{N}_{0.152}$ without including sulfur and phosphorous, γ_b of 4.188, assumed to be constant independent of the carbon source), and angiostatin ($\text{CH}_{1.493}\text{O}_{0.343}\text{N}_{0.285}\text{S}_{0.018}$ with the γ_P of 3.916, based on the amino acid sequence of angiostatin with kringles 1–4) (Table 3).³² The energy yield coefficients and mass yield coefficients were also estimated according to equations (10) and (12), and are also shown in Table 3.

Methanol consumed by the cells is first oxidized to formaldehyde in a reaction catalyzed by alcohol oxidase (AOX), and the H_2O_2 formed is degraded to O_2 and H_2O by catalase (CAT) in the peroxisomes. These two reactions in the initial oxidation of methanol can be combined to give



According to the model proposed by Jahic,¹⁹ the flux of methanol consumption is divided into one for anabolism and one for energy production. In the anabolism of methanol, oxygen is consumed only in the initial oxidation, and the oxygen demand is insignificant while the coefficient for oxygen consumption per mol methanol used for anabolism in this reaction is 0.5 mol O_2 (mol methanol)⁻¹. For energy production, formaldehyde is further oxidized to CO_2 and water with molecular oxygen as the ultimate electron acceptor:



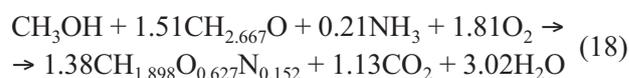
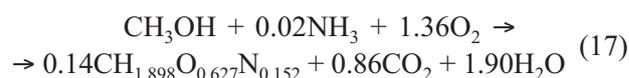
Thus, the overall stoichiometry for methanol oxidation in the energy metabolism is,



and the oxygen demand per mol methanol used for energy production, $Y_{O/M}$, is 1.5 mol O_2 (mol methanol)⁻¹.

For instance, in experiment F1, angiostatin formation could be neglected due to the very low yield coefficient of angiostatin (0.66×10^{-3} mol (mol methanol)⁻¹, or 18.4×10^{-3} g (mol methanol)⁻¹) compared with $Y_{X/M}$ (0.11 g cell (g methanol)⁻¹, or 3.65 g biomass (mol methanol)⁻¹, or 0.14 mol biomass (mol methanol)⁻¹). Therefore, when 1 mol methanol was used, 0.14 mol was used for growth and the rest 0.86 mol was driven to the energy production pathway, whereas the oxygen consumed for energy metabolism and anabolism was 1.29 mol and 0.07 mol, respectively according to equations (14) and (16). The oxygen demand for 1 mol consumed methanol, $Y_{O/M}$, based on Jahic's model,¹⁹ can be calculated to be 0.14 (the fraction for anabolism) \times 0.5 mol + 0.86 (the fraction for energy production) \times 1.5 mol = 1.36 mol, which is the same as that calculated by the stoichiometric coefficients for this instance (Table 3), and supports the validity of Jahic's model.

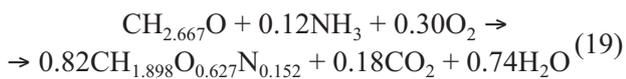
The equations describing cell growth on methanol and methanol plus glycerol can be written as equations (17) and (18), respectively, based on Table 3:



Assuming the cell growth on two carbon sources is additive, the contribution of glycerol to growth can be shown as follows:

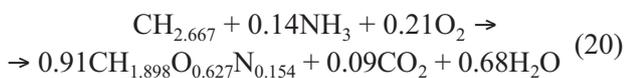
Table 3 – Estimated parameters in the mass and energy balances of the fermentation fed with different carbon sources in the induction phase

	F1	F2	F3	F4	F5
Formed Biomass (g)	16.2	188.0	219.4	255.0	125.3
Formed angiostatin (mg)	69	243	393	161	582
Consumed non-methanol carbon source (g)	–	242.0	438.0	1002.6	390.9
Consumed methanol (g)	143.0	167.0	134.0	210.5	189.8
f	–	1.51	3.45	5.08	2.20
b	1.36	1.81	3.14	5.02	2.85
a	0.02	0.21	0.30	0.23	0.12
y_b	0.14	1.38	2.00	1.49	0.81
$y_P \times 10^3$	0.66	1.98	3.99	1.04	4.17
d	0.87	1.13	2.45	4.59	2.39
c	1.90	3.02	4.57	6.00	3.61
Q (kJ mol ⁻¹)	547.5	864.5	1461.6	2221.9	1312.3
RQ	0.64	0.64	0.80	0.91	0.85
η	0.091	0.41	0.38	0.22	0.22
$\xi_P \times 10^4$	4.43	6.08	7.83	1.59	11.34
y_O	0.10	0.76	0.64	0.30	0.28
$Y_{X/O}$ (g cell (g O_2) ⁻¹)	0.083	0.62	0.52	0.24	0.23



According to equation (19), the calculated cell yield on glycerol ($Y_{X/G}$) at an overall specific growth rate of 0.012 h^{-1} , should be $0.70 \text{ g cell (g glycerol)}^{-1}$ ($18.2 \text{ g cell (mol carbon)}^{-1}$). The $Y_{X/G}$ in the fed-batch phase on glycerol, where glycerol was limitedly added as a single carbon source (glycerol fed-batch phase of the whole fermentation), was $0.74 \text{ g cell (g glycerol)}^{-1}$ at the specific growth rate of about 0.029 h^{-1} (obtained from 12 batches of fermentation with a standard deviation of ± 0.03 , data not shown), and $Y_{X/G}$ in the batch phase on glycerol at the specific growth rate of about 0.2 h^{-1} was 0.73 g g^{-1} (summarized from about 19 batches of fermentation with a standard deviation of ± 0.01 , data not shown). This hinted that the glycerol metabolism had not changed greatly whether it was used as either a single carbon source or together with methanol. The coefficient for oxygen consumption per mol glycerol, $Y_{O/G}$, was $0.30 \text{ mol O}_2 \text{ (mol glycerol)}^{-1}$, less than $Y_{O/M}$, because the reductance degree of glycerol, 4.7, is lower than that of methanol, 6.0. In high cell density cultures, oxygen mass transfer limitation is likely to occur due to limited oxygen transfer capacity of the bioreactor. Feeding of the carbon source must then be carefully controlled on the basis of the oxygen availability. Due to the low $Y_{O/G}$ value, mixed feeding of glycerol and methanol is an effective way to lessen oxygen limitation in the induction phase.

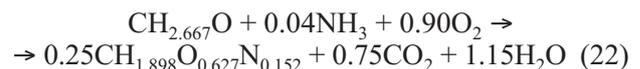
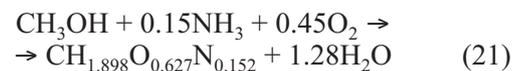
In the culture on glycerol and methanol, there are two other possibilities. The first one is that methanol is all used for energy metabolism, and 1.5 mol O_2 were used to oxidize 1 mol methanol, and the remaining 0.31 mol O_2 was used to oxidize 1.51 mol glycerol to form biomass. In such a case, equation (18) can be divided into two reactions, (16) and (20),



and the calculated cell yield on glycerol at the specific growth rate of 0.012 h^{-1} was $0.77 \text{ g cell (g glycerol)}^{-1}$ ($23.8 \text{ g cell (mol carbon)}^{-1}$), higher than that in the fed-batch phase on glycerol. In fact, more glycerol should be used for cell maintenance at lower specific growth rates. In the present hypothesis, methanol was used to supply all energy including maintenance, thus the calculated $Y_{X/G}$ was higher than that of the glycerol fed-batch phase. The coefficient for oxygen consumption per mol glycerol, $Y_{O/G}$, was $0.21 \text{ mol O}_2 \text{ (mol glycerol)}^{-1}$, less than $0.30 \text{ mol O}_2 \text{ (mol glycerol)}^{-1}$ (where methanol was used for both anabolism and energy

metabolism), since less glycerol was completely oxidized for energy production.

The second possibility is that methanol was all used for anabolism. When 1 mol methanol formed 1 mol biomass, 0.45 mol O_2 was required to maintain the mass balance (equation 21), and one fourth of consumed glycerol was also used for cell growth, and the rest was used for catabolism, as shown in equation (22), to make up the whole reaction (18). The calculated cell yield on glycerol at the specific growth rate of 0.012 h^{-1} was $0.21 \text{ g cell (g glycerol)}^{-1}$ ($6.51 \text{ g cell (mol carbon)}^{-1}$), the cell yield on methanol was $0.81 \text{ g cell (g methanol)}^{-1}$ ($26.1 \text{ g cell (mol carbon)}^{-1}$), and the demand for oxygen per mol glycerol, $Y_{O/G}$, was $0.90 \text{ mol O}_2 \text{ (mol glycerol)}^{-1}$, more than $0.30 \text{ mol O}_2 \text{ (mol glycerol)}^{-1}$ (methanol was used for both anabolism and energy metabolism, equation (18)), due to more glycerol being completely oxidized for energy.



Considering the fact that 1 mol methanol for anabolism in the AOX pathway needs 0.5 mol O_2 based on Jahic's model, there might be another pathway for methanol anabolism simultaneously worked into the *P. pastoris* strain of the present experiments, for instance, methanol dehydrogenase (MDH, alcohol dehydrogenase, EC 1.1.1.1) pathway in which oxygen is not necessary. The presence of alcohol dehydrogenase to oxidize alcohols to the corresponding aldehydes in methanol utilizing yeasts has been reported, and the activity of the alcohol dehydrogenase is observed in cell-free extract in all methanol-utilizing yeasts. In some methanol-utilizing yeasts, such as *Candida boidinii* and *Pichia pinus*, the alcohol dehydrogenase is NAD-dependent,³³ and in crude extract of *P. pastoris*, the activity of an NAD-dependent secondary alcohol dehydrogenase has been detected.³⁴ However, when grown on methanol, the special activity of methanol oxidase is much higher than methanol dehydrogenase (about 200:1) in cell extract of *Candida boidinii*.³⁵ Accordingly, methanol metabolism was mostly via the AOX pathway and a small portion of the methanol was transformed in the MDH pathway (equation 23) to result in 0.45 mol O_2 but not 0.5 mol O_2 required per mol methanol used.



For process F3, F4 and F5, similar stoichiometric analysis can also be performed as in the above discussion.

b is the stoichiometric coefficient of oxygen consumed according to the oxygen balance of equation (1), and reflects the oxygen requirement in the fermentation. In the fermentation processes F2, F3, F4 and F5, b was a total estimation of oxygen required for metabolism of methanol and the non-methanol carbon source. Owing to the three possible ways to metabolize methanol, oxygen required for methanol could not be determined according to the value of b . Further analysis could be done combined with the coefficient y_O or $Y_{X/O}$. As y_O or $Y_{X/O}$ (Table 3) was lower when methanol was used as the single carbon source, the oxygen requirement to reach a fixed amount of biomass was higher. Methanol metabolism requires high amounts of oxygen,³⁶ the oxygen requirement in F1 was higher than that in the processes where a second carbon source was used. Therefore, addition of the non-methanol carbon source reduced the specific oxygen consumption. Thus, the dissolved oxygen level could be maintained above 20 % by simply adjusting the agitation speed of the fermentor, even though the cell dry weight achieved as high as about 150 g L⁻¹ when supply of glycerol was limited, or even the non-methanol carbon source was not limited in F4 and F5. The y_O and $Y_{X/O}$ in F4 and F5 were much less than those in F2 and F3 due to lower reduction degree of acetate and lactate. The energy yield coefficient for biomass production, η , related with y_O and $Y_{X/O}$; higher level of η corresponded to higher efficiency of biomass formation from the carbon source(s), and glycerol was an efficient carbon source for biomass formation. However, for a process of secretory recombinant protein production, high protein production (represented by ξ_P) is even more important than biomass production. Therefore, among the five processes, F5 was the most efficient because of the highest ξ_P and ease of feeding control.

The respiratory quotient (RQ) is a variable that reflects the utilization efficiency of carbon source. Lower level of RQ indicated less carbon source was transformed to CO₂ and higher efficiency of assimilation. The RQ calculated on the basis of $RQ=d/b$ is shown in Table 3, also indicating that glycerol was an efficient carbon source for biomass while acetate was the worst. RQ is related to the reductance degree of the carbon source, therefore, the higher the γ_S , the higher the growth efficiency.

Conclusion

Mass and energy balances indicated that among the non-methanol carbon sources used in the present study, glycerol was the best one for cell growth of Mut^S *P. pastoris*, and mixed feeding of glycerol-methanol, sorbitol-methanol and lactate-methanol could re-

markably improve angiostatin expression compared to the process with methanol as the sole carbon source in the induction phase. However, based on the high ξ_P and nonrepressive characteristic to the *AOXI* promoter, lactate was the best carbon source for angiostatin production. In addition, the supply of a non-methanol carbon source reduced the specific oxygen requirement, and is expected to alleviate oxygen limitation in high cell density culture.

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Nomenclature

- a, b, c, d, f, y_b, y_p – stoichiometric coefficients
 CH_mO_l – molecular formula of carbon source 1 (methanol)
 CH_kO_j – molecular formula of carbon source 2 (non-methanol carbon source)
 $CH_pO_nN_q$ – molecular formula of biomass
 $CH_rO_sN_t$ – molecular formula of product (angiostatin)
 Q – heat evolved from complete oxidation of carbon source based on carbon, kJ (mol C)⁻¹
 $Y_{X/S}$ – biomass yield coefficient based on consumed carbon source(s), g biomass (g substrate)⁻¹ or g biomass (mol C)⁻¹
 $Y_{X/M}$ – biomass yield coefficient based on consumed methanol, g biomass (g methanol)⁻¹ or g biomass (mol C)⁻¹
 $Y_{X/G}$ – biomass yield coefficient based on consumed glycerol, g biomass (g glycerol)⁻¹ or g biomass (mol C)⁻¹
 $Y_{X/O}$ – biomass yield coefficient based on consumed oxygen, g biomass (g oxygen)⁻¹
 $Y_{O/G}$ – coefficient of oxygen consumption per mol glycerol consumed, mol O₂ (mol glycerol)⁻¹
 $Y_{O/M}$ – coefficient of oxygen consumption per mol methanol consumed, mol O₂ (mol methanol)⁻¹
 y_O – dimensionless biomass yield coefficient based on consumed O₂
 γ_S – reduction degree of carbon source, mol electron mol⁻¹
 γ_b – reduction degree of biomass, mol electron mol⁻¹
 γ_P – reduction degree of product, mol electron mol⁻¹
 σ_b – weight fraction of carbon in biomass
 σ_S – weight fraction of carbon in carbon source
 σ_P – weight fraction of carbon in product
 η – energetic yield coefficient for biomass production, fraction of the chemical energy of carbon source conserved in biomass
 ξ_P – energetic yield coefficient for product formation, fraction of the chemical energy of carbon source conserved in product

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