Appl Microbiol Biotechnol (2006) 72: 492–498
DOI 10.1007/s00253-005-0306-1

Narendar K. Khatri · Frank Hoffmann

Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with recombinant *Pichia pastoris*

Received: 4 November 2005 / Revised: 13 December 2005 / Accepted: 16 December 2005 / Published online: 11 March 2006

Abstract The yeast *Pichia pastoris* is a suitable production system for recombinant proteins due to its strong methanol-inducible *AOX1* promoter. A key parameter of the production process is the specific methanol uptake rate. To control the methanol uptake and simultaneously maintain a constant methanol concentration during the production phase, two strategies were developed to generate purposeful oxygen limitation and to feed-forward control the specific methanol uptake rate into the optimum range. First, the cell density at induction was adjusted by prolonged preinduction glycerol feeding. Alternatively, the airflow rate was restricted and increased in parallel with the biomass. While the product accumulation started 20 h earlier with the first approach, the specific production rate of a single-chain antibody fragment was three times higher in the latter case. After 70 h of production, both schemes yielded product concentrations in the gram-per-liter range. Moreover, they release the requirement for dosage of pure oxygen and thereby can facilitate the scale-up of the production process. The different production profiles indicate that the impact of specific methanol uptake rate on protein production by recombinant *P. pastoris* depends on the control mode.

Introduction

The methylotrophic yeast *Pichia pastoris* is an ideal expression system for many proteins (Lin Cereghino et al. 2002), because it combines advantages from eukaryotes, such as correct protein processing and efficient secretion, with those of unicellular microorganisms, such as ease of handling and growth on simple media to high cell densities. Although a variety of promoters have been successfully used for recombinant protein production (Goodrick et al. 2001; Hohenblum et al. 2003; Resina et al. 2005; Watermah et al. 1997), the strong *AOX1* promoter remains the most popular one. It is induced by change to methanol as carbon source.

Methanol metabolism requires high amounts of oxygen (Yurimoto et al. 2002). As low oxygen levels are generally recognized to interfere with the production of recombinant proteins (Bushell et al. 2003; Lee et al. 2003; Lin Cereghino and Cregg 2000), the oxygen demand is reduced using low cell densities, low cultivation temperature, or strains with slow methanol utilization (Chiruvolu et al. 1997; Cos et al. 2005; Curvers et al. 2001; Jahic et al. 2003). Most often, the methanol uptake is restricted by limiting methanol supply, either by preprogrammed feeding rates (Stratton et al. 1998) or controlled by the metabolic capacity of the cells, maintaining a constant dissolved oxygen saturation (DOstat principle) (Charoenrat et al. 2005; Jahic et al. 2003). The rate of methanol feeding has been identified as a major parameter to optimize protein production with recombinant *P. pastoris*, and small changes can have profound effects (Sinha et al. 2003).

The impact of the methanol supply rate can be described in terms of specific methanol uptake rate, specific growth rate, or dilution rate in chemostat cultures. These parameters are proportional and are also numerically similar, as the observed biomass yield coefficient is about 1 g g^{-1}. Comparing studies with a variety of systems, including limited feeding, chemostat and novel approaches, optimum production was often found with specific methanol uptake rates around 0.02–0.03 g g^{-1} h^{-1} (Table 1).

Limiting methanol feeding, however, is not suited for those recombinant proteins that get degraded or modified under these conditions (Jahic et al. 2003; Zhou and Zhang 2002). With a single-chain antibody fragment (scFv), maintaining a constant methanol concentration is required to avoid cleavage of its His_6 tag; the methanol-sufficient condition led to oxygen limitation after induction despite

N. K. Khatri · F. Hoffmann (✉)
Department of Biochemistry/Biotechnology,
Institute for Biotechnology,
Martin-Luther-University Halle-Wittenberg,
Kurt-Mothes-Straße 3,
06120 Halle, Germany
e-mail: f.hoffmann@biochemtech.uni-halle.de
Tel.: +49-345-5524934
Fax: +49-345-5527013

Table 1 Optimum process parameter for recombinant protein production

Mode	Optimum parameter	References
Limited feeding	μ_{set}=0.025→0.02 h^{-1}	Sinha et al. 2003
Limited feeding	μ_{set}=0.02 h^{-1}	Trinh et al. 2003
Fixed r_{MeOH}, CFW_{ind} adjusted	q_{MeOH}<0.026 g g^{-1} h^{-1}	Cunha et al. 2004
Cyclic fed-batch	q_{MeOH}=0.02 g g^{-1} h^{-1}	Bushell et al. 2003
Chemostat	D=0.01–0.03 h^{-1}	d'Anjou and Daugulis 2001
Chemostat	D=0.02 h^{-1}	Zhou and Zhang 2002
Constant c_{MeOH}	q_{MeOH} (c_{MeOH})=0.026 g g^{-1} h^{-1}	Zhang et al. 2000

CFW_{ind} cell density (cell fresh weight) at the time of induction, c_{MeOH} methanol concentration, D dilution rate, q_{MeOH} specific methanol uptake rate, r_{MeOH} volumetric methanol feeding rate, μ_{set} growth rate set by the methanol feeding rate

full aeration (Trentmann et al. 2004). Successful production under oxygen limitation has also been described for a number of other proteins (Charoenrat et al. 2005; Hellwig et al. 2001). Under oxygen limitation, the methanol consumption is determined by the oxygen transfer rate into the reactor. Therefore, with full aeration, the specific methanol uptake rate is high immediately after induction but can decline to the level of maintenance requirements when the cell density increases (Khatri and Hoffmann 2006).

In this study, the oxygen available per cell was reduced compared with the previous studies to generate a purposeful oxygen limitation and to feed-forward control the specific methanol uptake rate into the optimum range for efficient recombinant protein production. Two approaches are compared: first, the cell density is adjusted before induction by a prolonged glycerol feeding phase; second, the airflow rate is restricted and increased exponentially with time to adjust the specific uptake rate at low cell densities.

Materials and methods

Strain

An scFv against the fimbriae of enterotoxigenic *Escherichia coli* F4 (formerly called K88) was obtained by phage display screening of a library constructed from spleen cDNA of F4-challenged mice. From the phagemid vector of a high-affinity binder, the scFv sequence called BA11 was amplified, joined with sequences encoding the α-mating-factor secretion signal from *Saccharomyces cerevisiae* and a His_6 tag, and integrated in the genome of *P. pastoris* GS115 to give GS115:BA11 (Trentmann et al. 2004).

Media

Media were prepared according to the recommendation of the Invitrogen manual: YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] and BMGY [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6, 1.34% (w/v) yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 1% (v/v) glycerol]. To the basal salt medium (26.7 mL L^{-1} phosphoric acid, 14.9 g L^{-1} $MgSO_4.7H_2O$, 0.93 g L^{-1} $CaSO_4.2H_2O$, 18.2 g L^{-1} K_2SO_4, 4.13 g L^{-1} KOH, 40 g L^{-1} glycerol), 4 mL L^{-1} sterile-filtered trace element solution PTM1 (6 g L^{-1} $CuSO_4.5H_2O$, 0.08 g L^{-1} NaI, 3 g L^{-1} $MnSO_4.H_2O$, 0.2 g L^{-1} $Na_2MoO_4.2H_2O$, 0.02 g L^{-1} H_3BO_3, 0.5 g L^{-1} $CoCl_2$, 20 g L^{-1} $ZnCl_2$, 65 g L^{-1} $FeSO_4.7H_2O$, 0.2 g L^{-1} biotin, 5 mL concentrated H_2SO_4) was added after autoclaving (Zhang et al. 2002). The pH value was adjusted to 6 with 25% ammonium hydroxide solution.

Precultures

YPD (10 mL) was inoculated with a single colony of GS115:BA11 from an agar plate and incubated 24 h at 30°C and shaking at 160 rpm. Two times 200 mL BMGY in 1,000-mL baffled shake flasks were inoculated with 1% (v/v) of the first preculture and incubated 24 h as above. The reactor was inoculated with 5% (v/v) of the second preculture.

Bioreactor setup

Cultivations were carried out in a Biostat C 10 (BBI B. Braun Biotech International, Melsungen, Germany) at 30°C and pH 6, which was maintained by automatic addition of 25% ammonium hydroxide solution. The initial culture volume was 6 L. Antifoam was added automatically on demand. The dissolved oxygen (DO) concentration was maintained above 40% saturation by increasing the stirrer speed from 300 to 1,200 rpm, and afterward, the airflow rate from 2 to 16 L min^{-1}; when the oxygen transfer capacity was reached after induction, the DO decreased. Alternatively, for cultivations with restricted aeration, a predetermined profile was set for the airflow rate after induction: The airflow rate was increased exponentially with a time constant of 0.03 h, starting with 5 L min^{-1}, until the maximum airflow rate of 16 L min^{-1} was reached and maintained, unless otherwise stated in the "Results" section. The stirrer speed was kept at its maximum value after induction also in these processes.

After the batch phase, a glycerol feeding was activated with a rate $r_{glycerol}$ given by Eq. 1.

$$r_{glycerol} = \frac{\mu_{set}}{Y_{X/S}} \cdot X_f \cdot \exp\left[\mu_{set} \cdot (t - t_f)\right] \quad (1)$$

The parameter values used were μ_{set}=0.07 h^{-1} (set growth rate), $Y_{X/S}$=1 g g^{-1} (biomass yield coefficient on glycerol), and X_f=38.5 g L^{-1} (expected cell density at the time of feeding start t_f). Hence, the initial rate was 2.7 g L^{-1} h^{-1}.

For the induction of recombinant gene expression, the glycerol feeding was stopped, and the methanol concentration was increased to 0.1, 0.3, 0.5, 1, 2, and 3%(v/v) with complete consumption of methanol between the steps; the concentration afterward was controlled to 3% by the Alkosens system (Frings, Bonn, Germany) as described (Trentmann et al. 2004). The substrate reservoirs were placed on balances to keep track of the substrate consumption.

Analysis and calculations

The product concentration was determined by ELISA assay in microtiter plates coated with F4 fimbriae using monoclonal anti-His_6 antibodies for detection as described (Trentmann et al. 2004). Product was purified and lyophilized as described (Trentmann et al. 2004). Known product quantities reconstituted in PBS (10 mM phosphate buffer pH 7.4, 150 mM NaCl) served as standard for product quantification.

For cell density determination, 1.5 mL of culture was centrifuged in preweighed Eppendorf tubes; fresh weight was determined after removal of supernatant. One gram fresh weight corresponds to 0.3 g dry weight. Additionally, the cell fresh weight was estimated from the amount B of base consumed for the maintenance of the pH value and initial culture volume V_0 and mass of added glycerol $m_{glycerol}$ and methanol m_{MeOH} as described (Khatri and Hoffmann 2006; Eq. 2).

$$X \approx \frac{B \cdot Y_{X/B}}{V_0 + m_{\text{glycerol}} + m_{\text{methanol}}} + X_0 \qquad (2)$$

The methanol uptake rate was calculated as the slope of the methanol consumption over time. The specific methanol uptake rate q_{MeOH} is the ratio of methanol uptake rate to biomass, i.e., cell fresh weight multiplied by culture volume taken from online estimates. For the calculation of the specific production rate q_P, the product concentration was multiplied by the supernatant volume $V(1-X/\rho)$ with a biomass density of ρ=1,000 g L^{-1} and represented by a first- or second-order linear regression as appropriate. The slope of the regression curve was divided by biomass to give the specific production rate.

Results

Production of a scFv with recombinant *P. pastoris*

An scFv was produced with recombinant *P. pastoris* using the methanol-inducible *AOX1* promoter. After the batch phase on glycerol, additional glycerol was fed at limiting rate for 4 h. For the induction of recombinant protein production, the glycerol feeding was stopped, and the methanol concentration was increased stepwise; this procedure serves to adapt the cells and to calibrate the methanol sensor. Despite full aeration, the dissolved oxygen (DO) saturation decreased to limiting levels after induction (Trentmann et al. 2004). To ensure efficient production under oxygen limitation, a high methanol concentration was important. With a methanol concentration of 3% (v/v), a final product concentration of 350 mg L^{-1} was obtained (Khatri and Hoffmann 2006).

The volumetric methanol uptake rate was constant, as it was determined by the oxygen transfer capacity of the reactor. Therefore, with increasing cell densities, the specific methanol uptake rate q_{MeOH} decreased from more than 0.05 g g^{-1} h^{-1} after the adaptation phase to 0.02 g g^{-1} h^{-1}

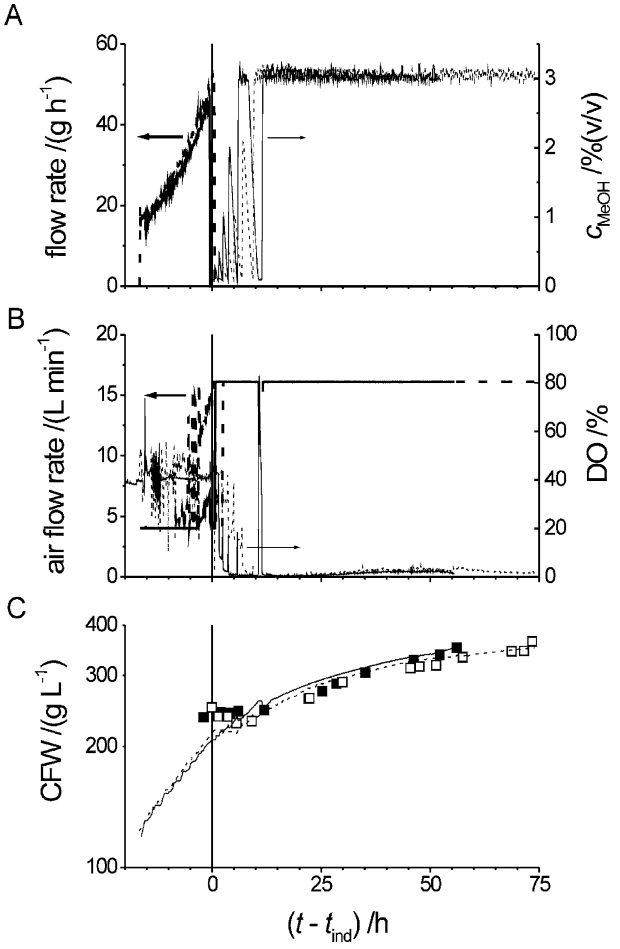

Fig. 1 Cultivation of *P. pastoris* with extended glycerol feeding phase. After batch and 16-h fed-batch phase on glycerol, recombinant protein production was induced by methanol. **a** The glycerol flow rate (*thick lines*) was determined gravimetrically. The methanol concentration (*thin lines*) was measured and controlled by a methanol sensor in the culture broth. **b** The DO saturation (*thin line*) was measured by a Clark electrode (Ingold). The airflow rate (*thick line*) was controlled to its maximum value during the production phase. **c** The cell fresh weight (*CFW*) was determined gravimetrically (*symbols*); additionally, CFW was estimated from online data (Eq. 2, *lines*). Time is given relative to the time of induction t_{ind} (indicated by *vertical lines*). *Solid* and *dashed lines*, *open* and *solid symbols* present two independent cultivations

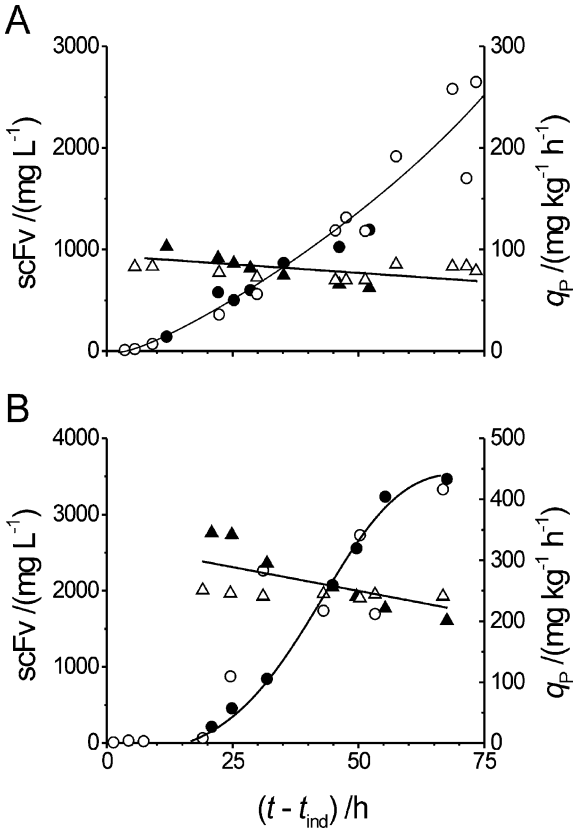

Fig. 2 Production of scFv under deliberate oxygen-limited control of the specific methanol uptake rate. The concentration of the recombinant scFv in the culture supernatant was determined by quantitative ELISA (*circles*). Specific production rates q_P (*triangles*) were determined as described in "Materials and methods." **a** Production after extended glycerol feeding phase in the cultivations of Fig. 2. **b** Production with restricted air supply in the cultivations of Fig. 4. *Lines* are drawn to interpret the trend. Time is given relative to the time of induction t_{ind}. *Open* and *solid symbols* present two independent cultivations

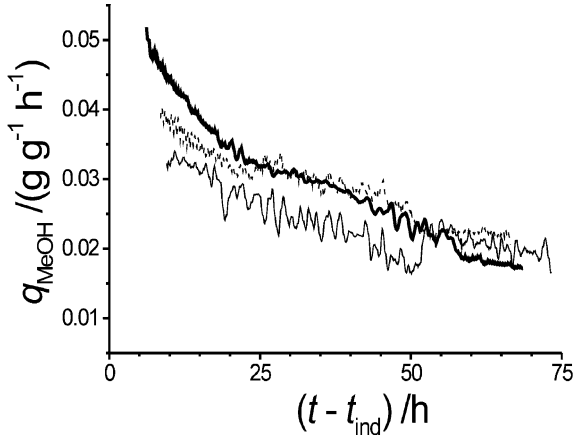

Fig. 3 Actual specific methanol uptake rates. Specific methanol uptake rates are compared for cultivations with short glycerol feeding and full aeration throughout the production phase (*thick lines*; Khatri and Hoffmann 2006), with prolonged glycerol feeding (*thin solid lines*; Fig. 2), and with restricted air supply (*thin dashes lines*; Fig. 4). Time is given relative to the time of induction t_{ind}

(Fig. 1). The product accumulated only after a lag phase; the start of product accumulation coincided with a q_{MeOH} of 0.02–0.03 g g^{-1} h^{-1} (Khatri and Hoffmann 2006). Therefore, in this study, the methanol uptake rate was lowered to this range early after induction. Two approaches to control the specific uptake rate were examined.

Prolonged glycerol feeding

First, the specific methanol uptake rate was controlled by the amount of biomass present at the time of induction. The glycerol feeding phase before induction was extended to 16 h (Fig. 2a), which resulted in a cell density of 245 g L^{-1} at the time of induction (Fig. 2c). After induction, the DO saturation fell to limiting levels within 5 h despite full aeration (Fig. 2b). The specific methanol uptake rate showed a declining trend due to a continued increase of the cell

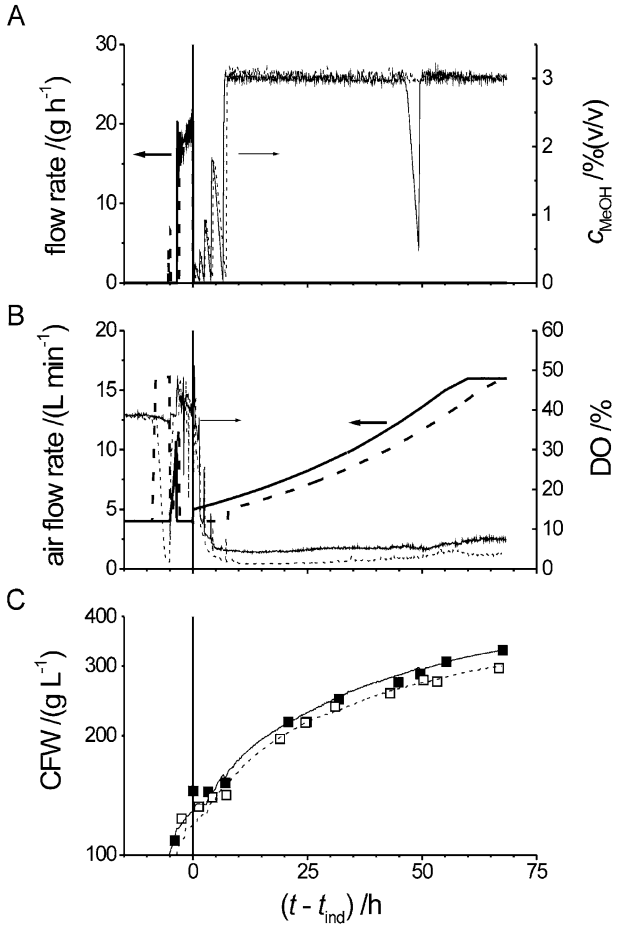

Fig. 4 Cultivation of *P. pastoris* with restricted air supply. After batch and 4-h fed-batch phase on glycerol, recombinant protein production was induced by methanol. **a** The glycerol flow rate (*thick lines*) and methanol concentration (*thin lines*) were measured as in Fig. 2. **b** DO saturation (*thin line*) was measured as in Fig. 2. The airflow rate (*thick line*) was controlled to a predetermined profile as described in "Materials and methods." **c** *CFW* was determined gravimetrically (*symbols*) and estimated from Eq. 2 (*lines*). Time is given relative to the time of induction t_{ind} (indicated by *vertical lines*). *Solid* and *dashed lines*, *open* and *solid symbols* present two independent cultivations

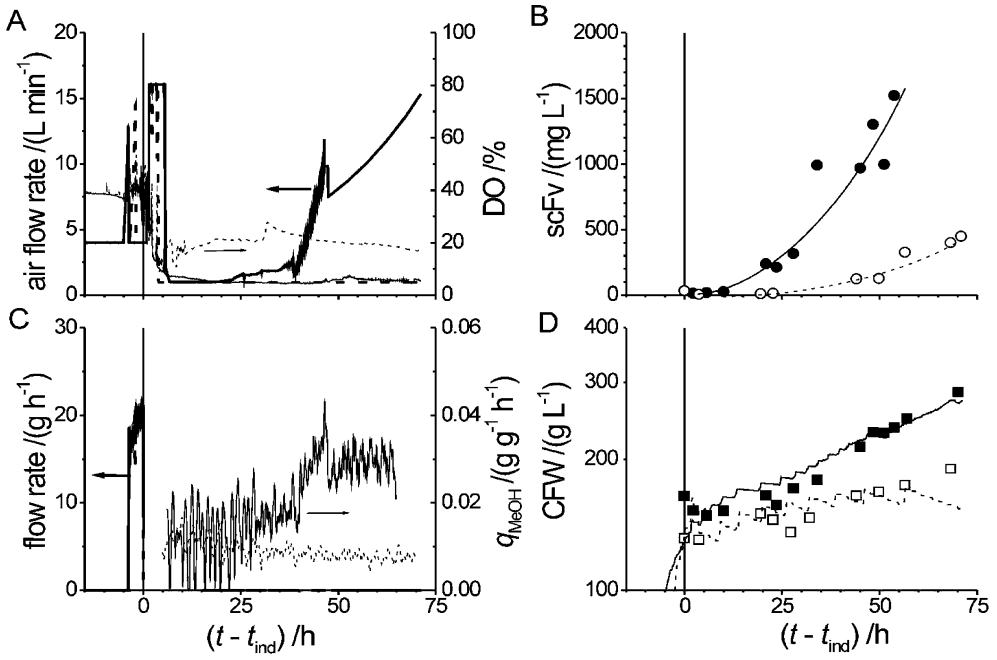

Fig. 5 Lower limits of air supply rates. Cultivations with restricted air supply during the production phase were performed as above. After the adaptation phase, the airflow rate (*thick lines*) was controlled manually to an increasing profile (*solid lines*, *solid symbols*) or maintained at 1 L min^{-1} (*dashed lines*, *open symbols*) (**a**). The DO saturation (*thin lines*), the product concentration in the culture supernatant (symbols) (**b**), the glycerol flow rates (*thick line*), the specific methanol uptake rate (*thin line*) (**c**), and cell density (*CFW*) (**d**) were determined as before. Time is given relative to the time of induction t_{ind} (indicated by *vertical lines*)

density to 345 g L^{-1} but remained close to 0.02 g g^{-1} h^{-1} during the whole production phase (Fig. 1).

In contrast to cultivations with shorter glycerol feeding, product accumulation started immediately after the addition of methanol (Fig. 3a). The specific production rate was constantly 80 mg kg^{-1} h^{-1} throughout the whole production phase (Fig. 3a). After 3 days of production, 2,500 mg L^{-1} of scFv per culture supernatant was obtained (Fig. 3a).

Restricted air supply

As an alternative strategy to control the specific methanol uptake rate during production at low cell densities, the airflow rate was set to 0.8 vvm upon induction and was increased with an exponential profile proportional to exp (0.03 h^{-1}(t–t_{ind})) (Fig. 4b). The DO consequently was below 10% during the entire production phase (Fig. 4b). The cell density increased from 135 g L^{-1} at induction to 300 g L^{-1} after 70 h of production (Fig. 4c). The specific methanol uptake rates decreased with increasing cell densities from 0.04 g g^{-1} h^{-1} immediately after the adaptation phase, which took 8 h with restricted air supply, to 0.02 g g^{-1} h^{-1} (Fig. 1).

The recombinant scFv accumulated in the culture supernatant only after a lag phase of 20 h after induction (Fig. 3b). The specific production rate was almost constant during the next 50 h of production, averaging at 250 mg g^{-1} h^{-1} (Fig. 3b). This is five to eight times faster than in the culture with full aeration, in which the scFv accumulated with an average specific production rate of q_P=30–50 mg kg^{-1} h^{-1} (data not shown). Likewise, q_P was two to three times faster as in the cultivations with long glycerol feeding (Fig. 3a). After 70 h of production under restricted air supply, final product concentrations of 3,500 mg L^{-1} were reached (Fig. 3b).

Lower limits of the airflow rate

To test whether even lower airflow rates can ameliorate the production further and to determine the lowest airflow rate that gives efficient protein production, the airflow rate was maintained at 0.16 vvm or increased manually starting from this value (Fig. 5a), resulting in a specific methanol uptake rate in the range of the maintenance requirements, 0.01 g g^{-1} h^{-1} (Khatri and Hoffmann 2006), or in an increase from 0.01 to 0.03 g g^{-1} h^{-1} (Fig. 5c). Growth and production were slower than with the previous profile (Fig. 5b,d), but with the manually controlled profile, the product concentration 50 h after induction was still three times higher than with full aeration. The cell density with an airflow rate of 0.16 vvm was almost constant; nevertheless, 500 mg L^{-1} of scFv was produced. Thus, even very low airflow rates could sustain recombinant protein production at the high methanol concentration used in this study, although less product was obtained than with the optimum aeration profile (Fig. 4b).

Discussion

An scFv was produced and secreted by recombinant *P. pastoris*. In previous work, limiting methanol feeding was found to result in product modification, whereas the full length product was obtained maintaining a constant methanol concentration (Trentmann et al. 2004). While this results in oxygen limitation despite full aeration—

conditions that are generally regarded as unfavorable (Bushell et al. 2003; Lee et al. 2003; Lin Cereghino and Cregg 2000)—efficient production was obtained under this condition using high methanol concentrations (Khatri and Hoffmann 2006). The product accumulation started after a lag phase of 20 h, when the specific uptake rate has declined to 0.02–0.03 g g^{-1} h^{-1}, a range that optimizes protein production in many variant producing systems (Bushell et al. 2003; Cunha et al. 2004; d'Anjou and Daugulis 2001; Sinha et al. 2003; Trinh et al. 2003; Zhang et al. 2000; Zhou and Zhang 2002). Therefore, in this study, purposeful oxygen limitation was generated to feed-forward control the specific methanol uptake rate to lower values immediately after induction. Two approaches were compared, and both resulted in high product yields. They differed, however, with respect to the production kinetics.

Three advantages stand out from the practical point of view: First, scFv titers in the gram-per-liter range were obtained, as in few other studies (Damasceno et al. 2004; Freyre et al. 2000), whereas scFv usually reach product concentrations in the range of 50–250 mg L^{-1} (cf., compilation in Fischer et al. 1999). The specific production rate was increased to 250 mg kg^{-1} h^{-1}, and the final product concentrations reached 3.5 g L^{-1}, which is ten times higher than with previous approaches (Khatri et al., submitted for publication; Khatri and Hoffmann 2006). Second, the methanol uptake can be controlled even with products that suffer from degradation or modification during limiting methanol feeding (Jahic et al. 2003; Trentmann et al. 2004; Zhou and Zhang 2002). Alternatively, methanol uptake can be restricted by reducing the maximum growth rates as a function of the cultivation temperature; this strategy improves recombinant protein production compared to methanol-limited DOstat control threefold (Jahic et al. 2003). Because the specific growth rate at 12°C is still as high as 0.04 h^{-1} (Jahic et al. 2003), however, the temperature-limited fed-batch is not ideal to control the low methanol uptake rates used in this study. Third, problems related to insufficient oxygen transfer capacities in large scale that cannot meet the high oxygen demand during protein production on methanol (Curvers et al. 2001) can impede production (Bushell et al. 2003; Lee et al. 2003; Lin Cereghino and Cregg 2000); the drawback of low oxygen transfer rate can be circumvented by using deliberate oxygen limitation to control the production process. Experiences with controlled oxygen limitation, e.g., in conjunction with a hypoxia-induced promoter (Görgens et al. 2005; Klinner et al. 2004) or the production of PHB granula (Vijayasankaran et al. 2005), gave no indication of general problems of *P. pastoris* under oxygen-limited conditions. Actually, oxygen limitation may be superior to methanol limitation with respect to release of host proteins even if air is provided with maximum rate (Charoenrat et al. 2005). Thus, oxygen-limited control of the methanol uptake is a versatile tool to optimize secreted protein production with recombinant *P. pastoris*.

An open question remains, that is, the mechanism by which the specific methanol uptake rate influences recombinant protein production. Limiting methanol supply has been found to induce the *AOX1* promoter more strongly than methanol-sufficient conditions (Cregg 1999). In addition, lower methanol uptake rate can reduce accumulation of proteases (Sinha et al. 2003). However, as the two approaches compared here yielded different production kinetics despite similar q_{MeOH} profiles, additional factors will be involved. Long glycerol feeding did also shorten the lag phase between induction and first product accumulation under oxygen-sufficient conditions, however without influencing the subsequent production rate (Khatri et al., submitted for publication). Adaptation during prolonged carbon limitation may facilitate subsequent product secretion after the switch to methanol. In addition, in another study, the production rate was higher, the longer the glycerol feeding phase before induction was; this was, however, attributed to the lower q_{MeOH} (Cunha et al. 2004).

In conclusion, restricted air supply was successfully used to control the methanol uptake during protein production with recombinant *P. pastoris*. This process is advantageous to scale up as it relieves the requirement for dosage of pure oxygen and also reduces heat generation. The simple feed-forward control strategy is easy to implement.

References

Bushell ME, Rowe M, Avignone-Rossa CA, Wardell JN (2003) Cyclic fed-batch culture for production of human serum albumin in *Pichia pastoris*. Biotechnol Bioeng 82:678–683

Charoenrat T, Ketudat-Cairns M, Stendahl-Andersen H, Jahic M, Enfors S-O (2005) Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. Bioprocess Biosyst Eng 27:399–406

Chiruvolu V, Cregg JM, Meagher MM (1997) Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations. Enzyme Microb Technol 21:277–283

Cos O, Serrano A, Montesinos JL, Ferrer P, Cregg JM, Valero F (2005) Combined effect of the methanol utilization (Mut) phenotype and gene dosage on recombinant protein production in *Pichia pastoris* fed-batch cultures. J Biotechnol 116:321–335

Cregg JM (1999) Expression in the methylotrophic yeast *Pichia pastoris*. In: Fernandez JM, Hoeffler JP (eds) Gene expression systems: using nature for the art of expression. Academic, San Diego, pp 157–191

Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJT (2004) Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. Biotechnol Bioeng 86:458–467

Curvers S, Brixius P, Klauser T, Thömmes J, Weuster-Botz D, Takors R, Wandrey C (2001) Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing. Part 1. Fermentation. Biotechnol Prog 17:495–502

d'Anjou MC, Daugulis AJ (2001) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnol Bioeng 72:1–11

Damasceno LM, Pla I, Chang H-J, Cohen L, Ritter G, Old LJ, Batt CA (2004) An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. Protein Expr Purif 37:18–26

Fischer R, Drossard J, Emans N, Commandeur U, Hellwig S (1999) Towards molecular farming in the future: *Pichia pastoris*-based production of single-chain antibody fragments. Biotechnol Appl Biochem 30:117–120

Freyre FM, Vázquez JE, Ayala M, Canaán-Haden L, Bell H, Rodríguez I, González A, Cintado A, Gavilondo JV (2000) Very high expression of an anti-carcinoembryonic antigen single chain Fv antibody fragment in the yeast *Pichia pastoris*. J Biotechnol 76:157–163

Goodrick JC, Xu M, Finnegan R, Schilling BM, Schiavi S, Hoppe H, Wan NC (2001) High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. Biotechnol Bioeng 74:492–497

Görgens JF, Passoth V, van Zyl WH, Knoetze JH, Hahn-Hägerdal B (2005) Amino acid supplementation, controlled oxygen limitation and sequential double induction improves heterologous xylanase production by *Pichia stipitis*. FEMS Yeast Res 5:677–683

Hellwig S, Emde F, Raven NPG, Henke M, van der Logt P, Fischer R (2001) Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. Biotechnol Bioeng 74:344–352

Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D (2003) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnol Bioeng 85:367–375

Jahic M, Wallberg F, Bollok M, Garcia P, Enfors S-O (2003) Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. Microbial Cell Factories 2:6

Khatri NK, Hoffmann F (2006) Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant *Pichia pastoris*. Biotechnol Bioeng (in press)

Klinner U, Fluthgraf S, Freese S, Passoth V (2004) Aerobic induction of respiro-fermentative growth by decreasing oxygen tensions in the respiratory yeast *Pichia stipitis*. Appl Microbiol Biotechnol 67:247–253

Lee CY, Lee SJ, Jung KH, Katoh S, Lee EK (2003) High dissolved oxygen tension enhances heterologous protein expression by recombinant *Pichia pastoris*. Proc Biochem 38:1147–1154

Lin Cereghino J, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66

Lin Cereghino GP, Lin Cereghino J, Ilgen C, Cregg JM (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichiapastoris*. Curr Opin Biotechnol 13:329–332

Resina D, Cos O, Ferrer P, Valero F (2005) Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated FLD1 Promoter. Biotechnol Bioeng 91:760–767

Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C-P, Meagher MM (2003) Improved production of recombinant ovine Interferon-τ by Mut^{+} strain of *Pichia pastoris* using an optimized methanol feed profile. Biotechnol Prog 19:794–802

Stratton J, Chiruvolu V, Meagher M (1998) High cell-density fermentation. In: Higgins DR, Cregg JM (eds) *Pichia* protocols. Humana, Totowa, pp 107–120

Trentmann O, Khatri NK, Hoffmann F (2004) Reduced oxygen supply increases process stability and product yield with recombinant *Pichia pastoris*. Biotechnol Prog 20:1766–1775

Trinh LB, Phue JN, Shiloach J (2003) Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from *Pichia pastoris*. Biotechnol Bioeng 82:438–444

Vijayasankaran N, Carlson R, Srienc F (2005) Synthesis of poly[(*R*)-3-hydroxybutyric acid) in the cytoplasm of *Pichia pastoris* under oxygen limitation. Biomacromolecules 6:604–611

Watermah HR, Digan ME, Koutz PJ, Lair SV, Cregg JM (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186:37–44

Yurimoto H, Sakai Y, Kato N (2002) Methanol metabolism. In: Gellisen G (eds) *Hansenula polymorpha*. Biology and applications. Wiley-VCH, Weinheim, pp 61–75

Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM (2000) Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of Botulinum neurotoxin, Serotype A. Biotechnol Bioeng 70:1–8

Zhang W, Smith LA, Plantz BA, Schlegel VL, Meagher MM (2002) Design of methanol feed control in *Pichia pastoris* fermentations based upon a growth model. Biotechnol Prog 18: 1392–1399

Zhou X-S, Zhang Y-X (2002) Decrease of proteolytic degradation of recombinant hirudin produced by *Pichia pastoris* by controlling the specific growth rate. Biotechnol Lett 24:1449–1453