Biotechnology Journal

DOI 10.1002/biot.201000193

Biotechnol. J. 2011, 6, 452–462

Research Article

Single-chain antibody fragment production in *Pichia pastoris*: Benefits of prolonged pre-induction glycerol feeding

*Narendar K. Khatri[1,2], Dörte Gocke[1]**, Oliver Trentmann[3]***, Peter Neubauer[2,4] and Frank Hoffmann[1]**

[1] Institute for Biotechnology, Martin-Luther-University Halle-Wittenberg, Halle, Germany
[2] Bioprocess Engineering Laboratory, Department of Process and Environmental Engineering and Biocenter Oulu, University of Oulu, Oulu, Finland
[3] Novoplant GmbH, Gatersleben, Germany
[4] Department of Bioprocess Engineering, Institute of Biotechnology, Technische Universität Berlin, Berlin, Germany

Secretory production of a single-chain antibody fragment (scFv) by recombinant *Pichia pastoris* using the methanol inducible *AOX1* promoter is limited biochemically by retarded secretion, and economically by the high demand for pure oxygen. To address the problem, the adaptation phase with growth-limiting feeding of glycerol before the production phase was optimized. In a standard procedure with a short glycerol-feeding phase before induction, scFv accumulated in the supernatant only after 15 h. Conversely, scFv started to appear immediately in the medium upon methanol induction when the glycerol-feeding phase was extended to 18 h. Interestingly, despite a significantly lower cell density in the cultivation with extended glycerol feeding, the same amount of functional product of 300 mg/L was obtained about 30 h after the start of glycerol feeding with both methods. mRNA analysis revealed that the higher and faster production of the product was related to longer lasting induction of the scFv mRNA. Additional effects of a better adaptation of the secretion machinery may be suggested by higher expression of unfolded protein response-related genes *KAR2* and *PDI*. A clear benefit of the longer glycerol-feeding phase was a 75% reduction of the consumption of both pure oxygen and methanol, and a significantly lower cell density, which would be beneficial for down-stream purification of the product.

Received 4 June 2010
Revised 7 December 2010
Accepted 9 December 2010

Keywords: *AOX1* promoter · *Pichia pastoris* · Process optimization · Recombinant protein production · Single-chain antibody fragment

1 Introduction

Highly efficient protein secretion is a major advantage of *Pichia pastoris* for recombinant protein production. Product concentrations close to 15 g/L in the culture supernatant have been obtained [1], and also single-chain antibody fragments (scFv) can be produced up to the gram per liter scale [2, 3].

Recombinant protein production by the methylotrophic yeast *P. pastoris* benefits from the tightly controlled *AOX1* promoter, which is strongly induced during growth on methanol [4]. The drawback of methanol induction is the high oxygen demand [5] that may result in low oxygen concentra-

Correspondence: Dr. Peter Neubauer, Department of Bioprocess Engineering, Institute of Biotechnology, Technische Universität Berlin, Ackerstr. 71–76, 13355 Berlin, Germany
E-mail: peter.neubauer@tu-berlin.de

Abbreviations: BSM, basal salt media; **DO**, dissolved oxygen; **scFv**, single chain antibody fragment; **UPR**, unfolded protein response

* Additional corresponding author: Dr. Frank Hoffmann
E-mail: frank.-.hoffmann@arcor.de
** Present address: Institute of Biotechnology 2, Forschungszentrum Jülich GmbH, Germany
*** Present address: Department of Plant Physiology, Technische Universität Kaiserslautern, Kaiserslautern, Germany

tions impairing production [6–8]. Therefore, dosage of pure oxygen is commonly employed [9]; nevertheless, the necessary oxygen transfer rates are difficult to scale-up [10]. Alternatively, the oxygen demand is reduced by growth-limiting feeding of methanol [11], but this may result in cell lysis and product degradation [12, 13]. Therefore, alternative strategies are continuously developed, such as the temperature-limited fed-batch technique [14] and two-stage pH-control strategy [15].

A phase of limiting glycerol feeding to derepress the *AOX1* promoter usually precedes the induction by methanol addition [11]. Continued glycerol co-feeding during a transition phase can improve the subsequent production [16], but during the actual production phase, already little co-feeding of glycerol represses the production of the recombinant protein [17]. An extended feeding phase before induction of recombinant gene expression is common for *Escherichia coli* and other organisms. Simple feed-forward control with a feeding rate increasing exponentially in time is often sufficient to ensure culture growth with a constant specific rate [18]. With *P. pastoris,* dissolved oxygen (DO)-spike control is recommended to control the glycerol feeding [11], although it burdens the cells with repeated short-term starvation. Exponential glycerol feeding strategies for derepression of the *AOX1* promoter are reported with *P. pastoris* [19, 20]. Moreover, duration of the feeding phase is usually short. However, long glycerol fed-batch phases have been used to adjust the specific methanol uptake rates by controlling the cell density at induction [21], and an optimum duration of the feeding phase for as long as 22 h has been calculated based on quantitative models [22].

Secretion of the recombinant protein enables disulfide bond formation, often resulting in proper protein folding, and simplifies product purification. However, slowly folding proteins may be retained in the endoplasmic reticulum (ER). This causes a 20-h delay in secretion of trypsinogen [23] and at the same time induces the unfolded protein response (UPR) [24]. With the yeast *Saccharomyces cerevisiae*, a single chain antibody accumulated for 20 h exclusively intracellularly, leading to growth arrest and UPR induction, until UPR proteases degraded the scFv completely, and in this case no product was secreted [25].

In the actual study the model protein expressed in *P. pastoris* is an scFv against fimbriae of enterotoxigenic *Escherichia coli* F4. Antibodies directed against the fimbriae of the enterotoxigenic *E. coli* strain F4 (formerly called K88) prevent adhesion of the pathogen to the porcine intestine, saving newborn piglets from lethal diarrhea [26]. We have shown previously that an scFv directed against the F4 fimbriae can be produced in *P. pastoris* and is secreted into the growth medium [12]. Application of this scFv as a preventive feed additive requires high doses and therefore very low production costs. Thus, further optimization of the production would be of advantage.

In this study, prolonged glycerol feeding before methanol addition was used to minimize the lag phase between induction and the onset of accumulation of anti-F4-fimbriae scFv in the culture supernatant, saving costly oxygen dosage under unlimited and methanol-limited conditions. To understand more about cellular responses in cultivations to protein production, mRNA analyses were performed for product-related genes and UPR-related genes *PDI* and *KAR2*.

2 Materials and methods

2.1 Strain and genetic constructs

An scFv against fimbriae of enterotoxigenic *E.coli* F4 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 *S. cerevisiae* in the plasmid pPic9 (Invitrogen, Carlsbad, CA) and a His_6 tag, and integrated in the genome of *P. pastoris* GS115 Mut^+ strain to give GS115:BA11 [12]. Successful transformants were selected for prototrophy after complementation of the GS115 *his4* strain by the *HIS4* gene of pPic9; a high producing strain was obtained by screening for scFv secretion [12].

2.2 Media

The growth media were prepared according to the recommendation of the Invitrogen Manual (EasySelect™ *Pichia* Expression Kit, cat. no. K1740-01): YPD (1% yeast extract, 2% peptone, 2% dextrose) and BMGY [1% yeast extract, 2% peptone, 100 mM KH_2PO_4 pH 6, 1.34% yeast nitrogen base with $(NH_4)_2SO_4$ without amino acids, 4×10^{-5}% biotin, 1% glycerol]. To the basal salt medium (BSM; 26.7 mL/L phosphoric acid, 14.9 g/L $MgSO_4 \cdot 7H_2O$, 0.93 g/L $CaSO_4 \cdot 2H_2O$, 18.2 g/L K_2SO_4, 4.13 g/L KOH, 40 g/L glycerol), 4 mL/L sterile-filtered trace element solution PTM1 (6 g/L $CuSO_4 \cdot 5\,H_2O$ 0.08 g/L NaI, 3 g/L $MnSO_4 \cdot H_2O$, 0.2 g/L $Na_2MoO_4 \cdot 2\,H_2O$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2$, 20 g/L $ZnCl_2$, 65 g/L $FeSO_4 \cdot 7\,H_2O$, 0.2 g/L biotin, 5 mL conc. H_2SO_4) was

Biotechnol. J. 2011, 6, 452–462

added after autoclaving [22]. The pH value was adjusted to pH 6 with 25% ammonium hydroxide solution.

2.3 Culture conditions

For preculture, 10 mL YPD were inoculated with a single colony of GS115:BA11 from an agar plate and incubated 24 h at 30°C and 160 rpm. Two times 200 mL BMGY in 1000-mL shake flasks were inoculated with 1% of the first preculture and incubated for 24 h as above. The reactor was inoculated with 5% of the second preculture.

Cultivations were carried out in a Biostat C bioreactor with a working volume of 10 L connected to a DCU3 digital control unit and the MFCSwin process supervisory system (B. Braun Biotech International, Melsungen, Germany). The initial culture volume was 6 L. The pH value was controlled at pH 6 by automatic addition of 25% ammonium hydroxide solution. DO saturation was determined by a Clark electrode (Ingold, Steinbach, Germany), and was maintained above 40% by increasing the stirrer speed from 300 to 1200 rpm, subsequently increasing the air flow rate from 2 to 16 L/min, and – where indicated – dosage of oxygen up to 10 L/min, until the maximum oxygen transfer capacity of the reactor was reached and the culture was harvested or the DO concentration declined. The cultivation temperature was 30°C. Antifoam was added automatically on demand.

When the process control system detected the DO-controlled decrease in stirrer speed and air flow rate upon depletion of glycerol from the BSM medium, glycerol feeding was activated with a rate r_{glycerol} given by Eq. (1).

$$r_{\mathrm{glycerol}} = \frac{\mu_{\mathrm{set}}}{Y_{X/\mathrm{S}}} \cdot X_{\mathrm{f}} \cdot \exp[\mu_{set} \cdot (t - t_{\mathrm{f}})] \quad (1)$$

The parameter values used were μ_{set} = 0.07/h (set growth rate), $Y_{X/\mathrm{S}}$ = 1 g/g (biomass yield coefficient on glycerol (experimental values are from 0.88 to 0.97 g/g) and X_{f} = 38.5 g/L (expected cell density at the time of feeding start t_{f}). The initial rate was hence 2.7 g L^{-1} h^{-1}.

For induction of scFv expression by methanol, the glycerol feeding was stopped and the methanol concentration was increased in three steps to final concentrations of 0.1%, 0.3% and 0.5%, while waiting for complete consumption of previously added methanol between the additions. The recorded resistance values were used to calibrate the methanol sensor (Alkosens electrode and Acetomat controller, Heinrich Frings GmbH, Bonn, Germany) as described earlier [17]. Afterwards, the methanol concentration was controlled to the concentration indicated in the results section. The methanol reservoir was placed on a balance to keep track of methanol consumption.

Alternatively, for limiting methanol feeding, the feeding rate was controlled to a profile given in Eq. (1) using μ_{set} = 0.04/h and X_{f} = 54 g/L for low cell densities. At high cell densities, the methanol feeding rate was kept constant at the maximum rate compatible with the oxygen transfer capacity of the reactor without dosage of pure oxygen.

To confirm repeatability, all bioreactor cultures were carried out at least twice. With regards to the discussed data the results were the same in the repeated experiments.

2.4 Purification of the scFv

After cell removal by centrifugation, the pH value of the supernatant was adjusted to 7.9 with 3 M K_2HPO_4. After overnight incubation with Ni-charged "His-Bind resin" (Novagen, Madison, WI) the His-Bind resin was transferred to plastic columns, washed with 5 vol 500 mM NaCl, 20 mM Tris-HCl pH 7.9, 5 mM imidazol, and the scFv was eluted with 500 mM NaCl, 20 mM Tris-HCl pH 7.9, 500 mM imidazol. The eluate was extensively dialyzed against solutions of decreasing NaCl concentrations (finally 1 mM NaCl) and lyophilized.

2.5 Cell density analysis

For cell density determination, 1.5 mL culture was centrifuged in pre-weighed Eppendorf tubes. Fresh weight was determined after removal of supernatant. The culture volume V was estimated from the initial volume V_0 and mass of added glycerol m_{glycerol} and methanol m_{MeOH}, neglecting density differences and volume contraction (Eq. 2).

$$V \approx V_0 + m_{\mathrm{glycerol}} + m_{\mathrm{methanol}} \quad (2)$$

2.6 scFv analyses

The concentration of scFv was quantified by ELISA in 96-well plates coated with F4 fimbriae using an anti-His_6 monoclonal antibody for detection as described earlier [12]. For Western blot, 20 µL culture supernatant was separated by SDS-PAGE, proteins were blotted onto a PVDF membrane and detected by an anti-His_6 monoclonal antibody [12].

2.7 RNA analysis

Quantitative analysis of the RNAs was performed as described earlier with a sandwich hybridization method [27–29]. Briefly, samples were collected by

fast inhibition of cellular activities immediately on ice-cold ethanol:phenol (95:5) solution and stored at –70°C after suspension in RNAlater (Ambion). Lysis was performed with total RNA extractions from an RNA extraction kit (Qiagen) and disrupted with a FastPrep cell homogenizer (ThermoSavant). Total extracted RNA was quantified using the RiboGreen RNA Quantification Kit (Molecular Probes). Ribosomal RNA (16S and 23S rRNA from *E. coli*; component C) was used as standard for total RNA quantification assays. The applied nucleotide probes are shown in Table 1; they were optimized compared to the previous publication [28] to obtain a higher sensitivity, especially in view of partially using a second helper probe. Primers for DNA amplification and oligonucletide probes for sandwich hybridization were designed using Clone Manager 5.0 (SE Central) and synthesized by Sigma Genosys. Capture probes were 5′ biotin labeled by Sigma Genosys and detection probes were labeled with digoxigenin at 3′ using the DIG Oligonucleotide Tailing kit (Roche) according to the manufacturer's instructions.

In vitro transcripts for the quantitative standard values were synthesized first by PCR with the primers shown in Table 1. The forward primer additionally always contained the sequence for T7-RNA polymerase 24-nucleotide T7 promoter sequence CTAATACGACTCACTATAGGGAGA at the 5′ end. *In vitro* transcription was then performed with the MAXIscript protocol (Ambion). The quality of the transcripts was controlled with the Agilent Bioanalyzer and they were quantified using the Ribogreen RNA quantification assay (Molecular Probes). The *in vitro* transcripts were diluted in

Table 1. Primers and probes used in this work[a)]

	Sequence 5'→3'	Position	Tm (°C)	Th (°C)
AOX1				
Forward primer	AGC AGG TGA GAA CAA CCT CAA C	583	66	
Reverse primer	AAG GTC CAC CGT AGG CAT TAG A	1988	66	
Detection	CTT CGA TAC CGT TAG TG	1541	50	45
Capture	GTC TGA TCT TGA CAC CA	1559	50	45
Helper 1	AAG AGG ACC AGT ACC AT	1522	50	45
Helper 2	GAG TTC TTC TGG TGT TG	1576	50	45
KAR2				
Forward primer	GCC ATG CTA TTG GTC GTA GT	49	60	
Reverse primer	GGT AGG CTC GTT CAC AAT TC	633	60	
Detection	TTG AAG TAG GCT GGA ACG GT	554	60	50
Capture	GTG GCT TGA CGT TGA GCG T	575	60	50
Helper	CGA TGA GAC CGG CAT CCT T	595	60	50
PDI				
Forward primer	TGT CCG CTC TCA CAC TAG CA	726	62	
Reverse primer	TTC CTC GAC TGG TCT GAG TG	2182	62	
Detection	CCG ACT AGC TTG AAG ACT	1827	54	50
Capture	CCT CTC CTG CGA TGA ATT	1760	54	50
Helper	TCT CAA TCA GCT CGG TCA	1742	54	50
26S rRNA				
Forward primer	AAT GAG TGG TGA CAC GAG AC	8	60	
Reverse primer	GGA TGT CGG AGA TGC GTA G	168	62	
Detection	CGC CCT TAA GAA GAA GAG A	82	56	50
Capture	CTA AAC CAT CCA GGC ACA A	101	56	50
Helper 1	AAC TCC TCC TAC GCC TT C	62	56	50
Helper 2	TTT ACT GCC CTT CGC CG	119	54	50
scFv (BA11)				
Forward primer	TTC GCA GCA TCC TCC GCA TT	82	62	
Reverse primer	GAA CGT CCA CGG AAG CGT AT	1029	62	
Detection	TAG GCT GTG CTG GAG GAT	563	56	50
Capture	TCA GAG GTC AGG CTG CT	590	56	50
Helper 1	TCA GTG TGG CCT TGC CTT	535	56	50
Helper 2	CAG TAA TAG ACC GCA GAG T	611	56	50

a) Tm, melting temperature; Th, hybridization temperature. Position: First nucleotide localization within the corresponding gene sequence at GenBank or from the known gene sequence (scFv).

DEPC water and used as standards in sandwich hybridization assays.

3 Results

3.1 Production and secretion of scFv under methanol and oxygen sufficiency

Recombinant *P. pastoris* was cultivated to produce an scFv. In the construct used, the expression was under the control of the methanol inducible *AOX1* promoter [12]. After a batch phase on glycerol, additional glycerol was fed at a growth-limiting rate for 2 h to derepress the *AOX1* promoter (Fig. 1). For induction of scFv expression, glycerol feeding was stopped and three pulses of methanol were added to adapt the cells and to calibrate the methanol sensor. Afterwards, the methanol concentration was controlled above 0.3%. The methanol consumption rate increased fivefold during the course of the cultivation (Fig. 1B). The maximum methanol consumption rate was 235 g/h. It took, however, 20 h to reach this maximum rate. During the production phase, a specific growth rate of 0.07/h was maintained; the cell density also increased fivefold and approached 500 g/L (fresh weight) 25 h after induction (Fig. 1A). By supplying pure oxygen, a DO saturation of 40% was maintained for 25 h after induction. When the DO decreased thereafter (Fig. 1C), the culture was harvested.

After 28 h of production, scFv (1.2 g, lyophilized) was purified from 4 L culture supernatant by immobilized metal affinity chromatography (IMAC) using the C-terminal His-tag (Table 2, run 1). The average specific production rate of this cultivation was thus 8.7 mg kg^{-1} h^{-1} (scFv per final cell fresh weight at per induction time).

The kinetics of product accumulation showed that the product started to accumulate in the culture supernatant only after a lag phase of 14 h after induction (Fig. 2B).

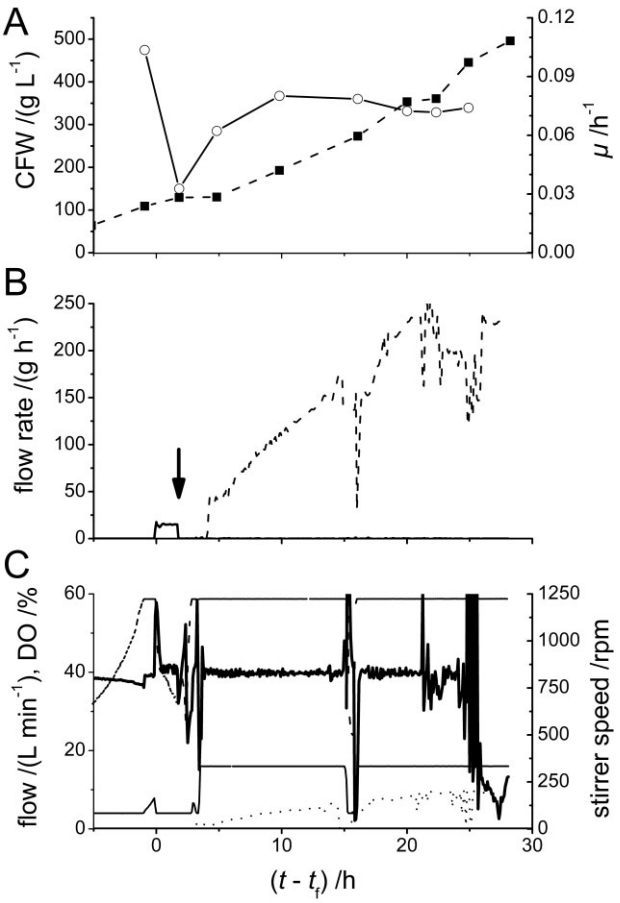

Figure 1. Production of a single-chain antibody (scFv) with recombinant *P. pastoris* under methanol and oxygen sufficient conditions. After growth in batch and fed-batch mode on glycerol, recombinant protein production was induced by addition of methanol (arrow). (A) Cell density (squares) was determined from cell fresh weight (CFW) measurements. The specific growth rate (μ, circles) was calculated from CFW and culture volume V (Eq. 2). (B) Feeding rates of glycerol (solid line) and methanol (dashed line) were determined as changes of the reservoir weights. (C) Dissolved oxygen (DO) saturation (dotted line) was maintained at 40% by increasing the stirrer speed (dashed line) and air flow rate (solid line) and flow rate of pure oxygen (short dotted line). Time is given relative to the start time of glycerol feeding, t_f. The disturbance at 15 h was connected to a refilling of the methanol feed bottle.

Table 2. Growth and production parameter of cultivations with *Pichia pastoris*

Cultivation run	1	2	3
At induction			
$t_{ind} - t_f$ (h)	2	12	18
glycerol consumed (g)	22.5	320	572
spec. growth rate (h^{-1})[a]	0.09	0.06	0.04
CFW[c] (g/L)	130	216	270
30 h after glycerol feeding			
$r_{MeOH,max}$ (g/h)	235[b]	170	115
spec. growth rate (h^{-1})	0.07[b]	0.05	0.03
methanol consumed (g)	3547[b]	1824	907
Total oxygen used (L)	8270[b]	5970	2226
At harvest			
$\Delta t = t_{end} - t_f$ (h)	28	35	40
CFW[c] (g/L)	491	440	379
scFv (mg)	1190	1048	1166
scFv (mg/kg)	243	271	376
scFv (mg kg^{-1} h^{-1})	8.7	8.0	9.6
scFv/ methanol (mg/g)	0.34	0.42	0.72
scFv/total subs[d] (mg/g)	0.33	0.37	0.53

a) Initial rate after resumption of growth.
b) Data at the end of the cultivation, 28 h after start of glycerol feeding.
c) CFW, cell fresh weight.
d) Total substrate (glycerol + methanol) consumed in g.

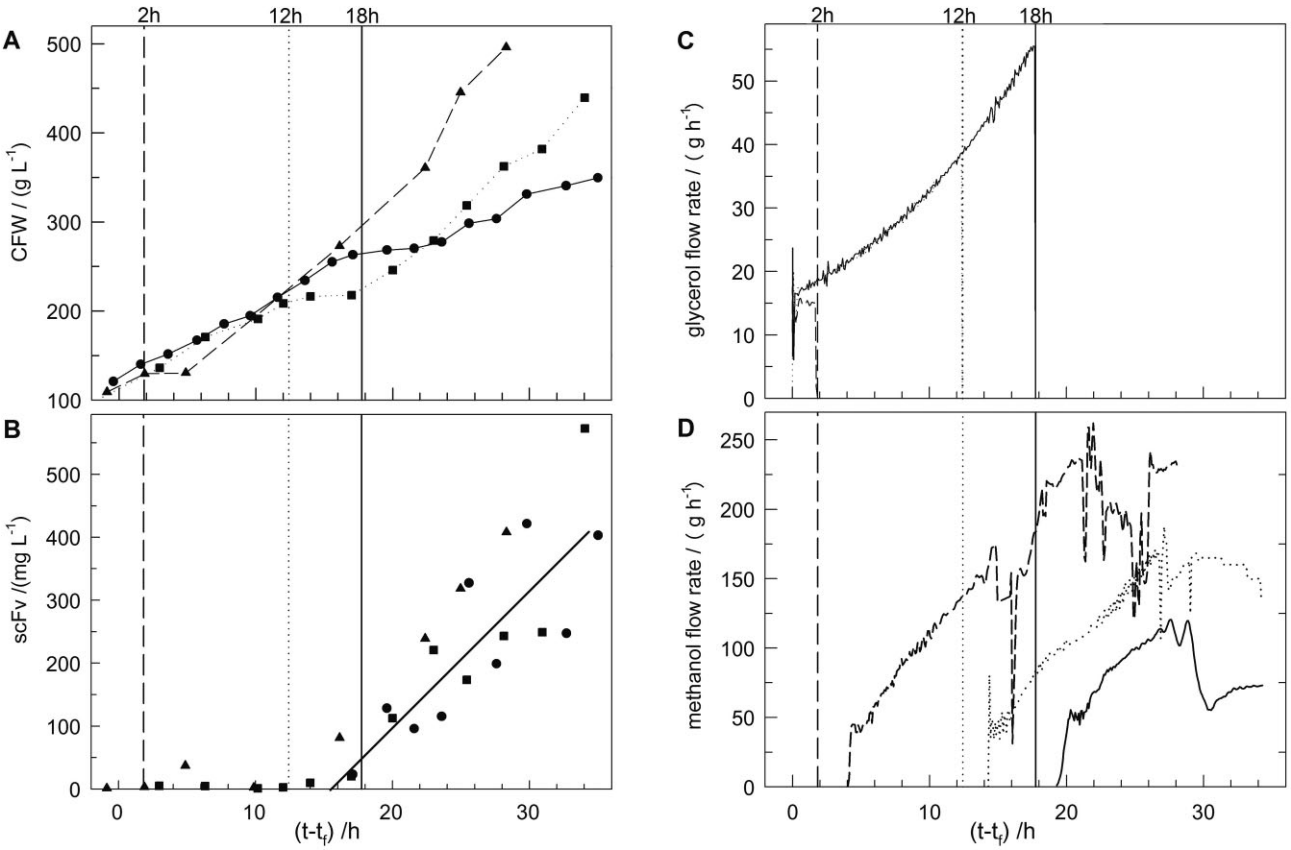

Figure 2. Impact of an extended glycerol feeding phase on the scFv production profiles. After the batch phase, glycerol was fed for 2 h (triangles, dashed lines), 12 h (squares, dotted lines), or 18 h (circles, solid lines) before induction of scFv production (methanol induction points indicated by vertical lines). (A) Cell fresh weight (CFW); (B) scFv concentrations (functional product) as determined by quantitative ELISA; (C) glycerol flow rates; (D) methanol flow rates. Time is given relative to the start time of glycerol feeding, t_f. The line indicates the trend of the experimental data.

3.2 Impact of prolonged glycerol feeding on the lag phase of product secretion

The impact of the length of the glycerol-feeding phase on subsequent scFv production was examined. To avoid starvation during the feeding phase, while maintaining a limiting glycerol feed, the feeding rate was increased exponentially with time. Based on the initial growth rate of the previous cultivation, a set growth rate μ_{set} =0.07/h was employed.

Glycerol was fed for 12 and 18 h, respectively (Fig. 2C). During this phase, 320 and 572 g glycerol were consumed, and cell densities of 216 and 270 g/L were reached (Fig. 2A, Table 2). After induction by switching to methanol, a constant volumetric methanol addition rate needed to be maintained (0.3%) (Fig. 2D). Methanol concentration followed a profile, as a function of time, similar to that of the standard short glycerol-feeding curve. This was not influenced by higher cell densities at induction. Therefore, 30 h after the start of glycerol feeding, only the cultivation that was induced earliest had reached the maximum volumetric methanol uptake rate of r_{MeOH} =235 g/h, whereas the cultivation with the longest glycerol-feeding phase reached only half as much, r_{MeOH} =115 g/h. Correspondingly, the amount of methanol consumed at this time was in the range of 900 g (run 3) to 3500 g (run 1). While more than 8000 L (0.5 vvm) oxygen was necessary to maintain a constant DO in the early induced culture, only 2200 L (0.2 vvm) were consumed with late induction (Table 2).

Consequently, the specific methanol uptake rates were 0.09, 0.06 and 0.04 g g^{-1} h^{-1} after 2, 12 and 18 h of glycerol feeding (data not shown), and growth during the production phase was slower after prolonged feeding (Fig. 2A, Table 2). Product accumulation, however, started sooner. It took 14 h after induction before the product could be detected in the culture supernatant with 2-h glycerol feeding; with 12-h glycerol feeding, the product was found already 5 h after induction, and immediately with 18-h glycerol feeding (Fig. 2B). Thus, product

Biotechnol. J. 2011, 6, 452–462

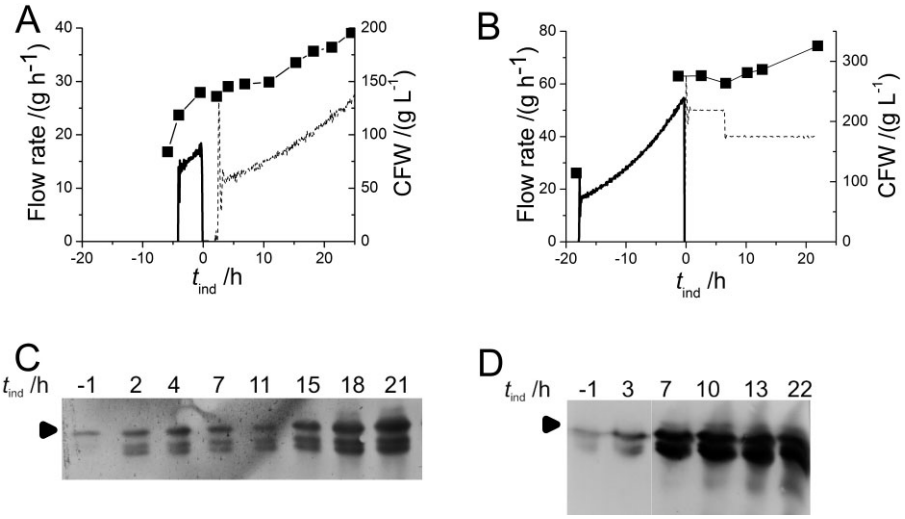

Figure 3. Production of scFv with limiting methanol feeding. After the batch phase as before, glycerol was fed for 4 h (A) or 18 h (B) before induction of scFv production by addition of methanol. Methanol was added pulsewise as described in the Materials and methods section, followed by limiting feeding with exponentially increasing rate (A), or – at high cell density – fed at constant rate (B). Feeding rates for glycerol (solid lines) and methanol (dashed lines), and cell fresh weight (squares) are shown. Time is given relative to the time of induction, t_{ind}. (C, D) Western blots of culture supernatant (constant volume) taken at different times in relation to induction from cultivations with 4 h (C) or 18 h (D) of glycerol feeding. Full-length scFv is indicated by a mark on the left. Lower bands may result from partial degradation of the product.

accumulation started about 16–18 h after feeding start independent of the time of induction by switching to methanol feeding. The scFv concentrations showed similar profiles in all three cultivation runs (Fig. 2B). The amounts of products purified and the average specific production rates were identical in all cases (Table 2, runs 1–3).

Although the final cell density was considerably higher with early induction compared to cultivations with longer glycerol feeding, similar product yields were obtained after purification and lyophilization; the specific product concentration was 1.5 times higher with a glycerol-feeding phase of 18 h, compared to cultivations with short glycerol feeding (Table 2). Lower cell densities simplify downstream processing. Thus, a prolonged glycerol feeding increased the selectivity of methanol conversion to scFv by reducing gratuitous biomass formation due to lower post-induction specific growth rate.

3.3 Impact of prolonged glycerol feeding in a methanol-limited process

Another common cultivation strategy to reduce the toxic effects of formaldehyde on cell lysis in case of faster methanol consumption is limited feeding of methanol. While this strategy is, in the long run, not appropriate for this scFv due to degradation of the product [12] (see also Figs. 3C and D), the impact of the glycerol-feeding phase on the lag phase can also be examined in this process. After 4-h glycerol feeding and pulse addition of methanol, methanol was fed at an exponentially increasing rate (Fig. 3A). To ensure a similar mean specific methanol feeding rate of 130 mg g^{-1} h^{-1} (methanol per cell fresh weight per time) at the higher cell density obtained after 18 h of glycerol feeding, methanol was fed at the maximum rate that did not result in methanol accumulation (Fig. 3B). This process did not include pure oxygen supply.

Accumulation of the scFv in the culture supernatant was analyzed by Western blot. While a slight product band was detectable soon after induction in both cultivations, substantial product formation initiated only after a delay. With short glycerol feeding, the lag phase lasted for 15–18 h after induction (Fig. 3C), while with prolonged glycerol feeding high product levels were detected already 7 h after induction (Fig. 3D). In all cases significant bands for product degradation appeared.

3.4 Quantitative analysis of RNA markers indicates higher expression of product (scFv) and UPR-related stress genes

Various mRNAs were analyzed to obtain a better view about the cellular responses in the cultivations for production of scFv with short (2 h) and long (18 h) glycerol feeding (Fig. 4). The mRNA encoding for scFv showed a higher expression level before methanol induction in the cultivation with

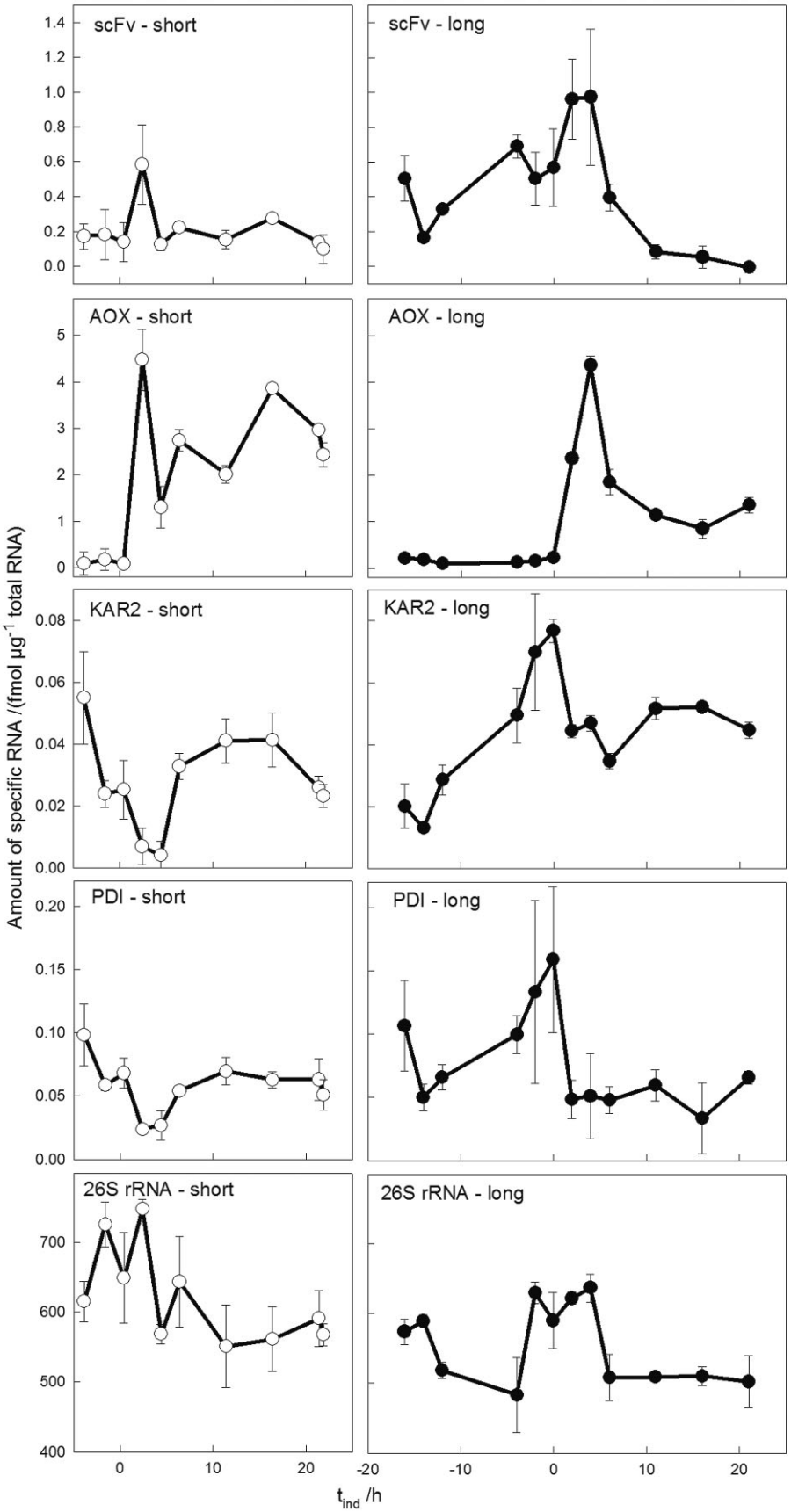

Figure 4. Amount of selected mRNAs in analyses during production of a single-chain antibody (scFv) with recombinant *P. pastoris* under methanol and oxygen sufficient conditions with short and with extended feeding of glycerol. Culture samples for mRNAs were analyzed from cultivations with short term (2 h, left column, open symbols) and extended (18 h, right column, filled symbols) glycerol feeding by sandwich hybridization as described in Materials and methods. The SDs were analyzed from fourfold analysis. Time t_{ind} refers to time of methanol induction.

extended glycerol feeding. The scFv mRNA level was also higher after methanol induction. However, the concentration of the scFv mRNA only increased for a very short time after induction in the cultivation with a short glycerol-feeding phase. Interestingly, the profiles for scFv are not directly reflecting the level of *AOX1* mRNA, which was very low in both cases before induction and showed a higher prolonged induction in the cultivation with the short glycerol-feeding phase. In order to check the UPR, we analyzed UPR-related genes *KAR2* and *PDI*. The levels of *KAR2* and *PDI* showed a continuous increase in the cultivation with prolonged glycerol feeding, in all cultivations; however, their levels decreased significantly after methanol induction. In the cultivation with short glycerol feeding, they nearly disappeared and recovered then over 5–10 h, whereas in the cultivation with prolonged glycerol feeding the decrease was less severe. Interestingly, in the last phases of all fermentations the *KAR2* and *PDI* mRNA levels were very similar. No significant differences were observed in the *26S rRNA* levels.

The results of mRNA analysis indicate that there is a derepression of scFv mRNA synthesis before methanol induction in the cultivation with long glycerol feeding, and during this period the levels of *KAR2* and *PDI* seems to be growth rate related.

4 Discussion

Derepression of the *AOX1* gene by glycerol-limited fed-batch cultivation before methanol induction has been described earlier as beneficial for an efficient uptake of methanol and induction of the *AOX1* promoter [19]. Therefore, a short glycerol-feeding phase is mostly applied in *P. pastoris* fed-batch cultivations. Recently, we showed for production of an antibody scFv fragment that, if methanol induction is performed under oxygen limitation, a prolongation of the glycerol-feeding phase was beneficial to maximize the amount of product [30]. To further understand this observation, we investigated whether the production of the same protein was affected if prolonged glycerol feeding was applied under purely aerobic conditions.

Here we show that a prolonged glycerol-limited fed-batch feeding phase really is beneficial also in purely aerobic cultivations. The same final product yield and similar high quality are achieved using both short and prolonged glycerol-feeding processes.

While the appearance of scFv in the culture supernatant was delayed for 10–20 h with the standard glycerol-feeding procedure (see Fig. 2 and [31]), immediate product accumulation was achieved after limiting feeding of glycerol for 18 h. The sum of duration of the glycerol-feeding phase and the product lag phase after induction hence was about constant. Similar product concentrations were obtained at 30 h after feeding start.

This observation is interesting as the prolonged glycerol-feeding strategy resulted in very significant savings of methanol and pure oxygen of about 75%. Furthermore, the same amount of product was obtained with a 23% lower cell density, which provides a clear advantage to the downstream purification process.

The same behavior (faster appearance of the product in the growth medium after methanol induction) was observed not only for the optimal feeding strategy of methanol (non-limiting, 0.3%), but also when methanol limitation was applied during the induction phase.

Within this work we followed the mRNA levels of two genes involved in protein folding and secretion, *KAR2/BiP* and *PDI*. Both of these genes have been related previously to improved secretion of human trypsinogen [32]. Here we saw strongly increased levels of these mRNAs – but only in the cultivation with long glycerol feeding. Interestingly, both genes were derepressed before induction during the glycerol-limitation phase, leading to two (*PDI*) or three times higher expression levels at the time of induction for *PDI* or *KAR2/BiP*, respectively. Surprisingly, in the cultivation with a short glycerol-feeding phase the mRNA levels of both genes strongly decrease at the time of induction and are only recovering slowly. The clearly different behavior of *PDI* and *KAR2/BiP* indicates that the folding/secretion system is different under the two culture conditions. Although still to be confirmed by measurements at the proteomic level, the mRNA analysis suggests a higher level of the chaperones *PDI* and *KAR2/BiP* at the time of induction in the cultivation with prolonged glycerol feeding, which would enhance the folding process. The strong decrease of these mRNAs, especially of *PDI* after methanol induction would be especially detrimental for folding if the actual *PDI* level is low, as can be assumed for the cultivation with short glycerol feeding.

A further interesting observation at the mRNA level is that the expression of the scFv mRNA does not reflect the expression of the *AOX1* mRNA. Whereas the *AOX1* mRNA level was very low in the glycerol-feeding phase in both cultivation types, the scFv mRNA showed a strong derepression over the time in the process with prolonged glycerol feeding. This derepression of the *AOX1* promoter is well known from the literature, and thus in many

fermentation processes an initial glycerol-limited growth phase is applied before methanol induction ([33] and comprehensively reviewed in [34]). Furthermore, in both cases methanol induction caused a only transient increase of scFv gene expression, and this induction was stronger under conditions of prolonged glycerol feeding compared to a shorter glycerol-feed phase. Therefore, in addition to the better conditions in view of the folding/secretion apparatus, the mRNA data of the scFv product also suggest a lower product synthesis with short glycerol feeding.

It is remarkable that, without external limitation, the cell density did not influence the methanol uptake profiles. Internal limitation determines the specific methanol uptake rate; it is likely that the consumption of methanol by alcohol oxidase limits the methanol uptake [35]. The induction of alcohol oxidase depends on the methanol concentration, which is limited in part by the toxic effect of methanol, but also by the high oxygen consumption. Therefore, the level of alcohol oxidase is autocatalytically controlled and reaches a volumetric maximum rather than being dependent on the cell density. Furthermore, the expression of alcohol oxidase also is effected by the growth status of the cells. Our investigations of *AOX1* mRNA showed that the maximum level of *AOX1* mRNA was similar in the cultivations with short- and long-term glycerol feeding after methanol pulsing. However, the maximum level is reached much faster in the cultivation with short-term glycerol feeding and the *AOX1* mRNA level stays higher for a long time. This is in agreement with the lower specific methanol uptake rates after prolonged glycerol feeding.

In conclusion, a prolonged glycerol-feeding phase followed by a short production phase under methanol and oxygen sufficiency, and also under methanol or oxygen limitation, maximized the efficiency of secreted recombinant protein production. A clear benefit of the longer glycerol-feeding phase was a 75% reduction in the consumption of pure oxygen and methanol, as well as a higher specific production rate of functional scFv product. The same amount of product was obtained in the same total process time, with only 320 g/L of cells compared to 500 g/L with the short glycerol-feeding phase, which is a clear advantage for downstream processing. Additional benefits of long glycerol-feeding process may be related to a better adaptation of the folding/secretion apparatus. Indication for this is a higher expression of *KAR2* and a higher level of *PDI* at the time of induction. Additionally, the higher level of the scFv mRNA at the time of induction, possibly accumulating by derepression, may have contributed to the faster product accumulation. This is an initial study with a single secreted protein with *P. pastoris*. To better understand, the positive benefits of long glycerol feeding on protein expression and secretion, further investigations are necessary.

N.K. received a grant from CIMO and Tauno Tönningin Foundation and D.G. was recipient of a "Postgraduate Education" ESF scholarship.

The authors have declared no conflict of interest.

5 References

[1] Werten, M. W. T., van den Bosch, T. J., Wind, R. D., Mooibroek, H., de Wolf, F. A., High-yield secretion of recombinant gelatins by *Pichia pastoris*. *Yeast* 1999, *15,* 1087–1096.

[2] Damasceno, L. M., Pla, I., Chang, H.-J., Cohen, L. *et al*., An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. *Protein Expr. Purif.* 2004, *37,* 18–26.

[3] Freyre, F. M., Vázquez, J. E., Ayala, M., Canaán-Haden, L. *et al*., Very high expression of an anti-carcinoembryonic antigen single chain Fv antibody fragment in the yeast *Pichia pastoris*. *J. Biotechnol.* 2000, *76,* 157–163.

[4] Lin Cereghino, G. P., Lin Cereghino, J., Ilgen, C., Cregg, J. M., Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Curr. Opin. Biotechnol.* 2002, *13,* 329–332.

[5] Yurimoto, H., Sakai, Y., Kato, N., Methanol metabolism, in: Gellisen G. (Ed.), *Hansenula polymorpha. Biology and Applications,* Wiley-VCH, Weinheim 2002, pp. 61–75.

[6] Bushell, M. E., Rowe, M., Avignone-Rossa, C. A., Wardell, J. N., Cyclic fed-batch culture for production of human serum albumin in *Pichia pastoris*. *Biotechnol. Bioeng.* 2003, *82,* 678–683.

[7] Lee, C. Y., Lee, S. J., Jung, K. H., Katoh, S., Lee, E. K., High dissolved oxygen tension enhances heterologous protein expression by recombinant *Pichia pastoris*. *Proc. Biochem.* 2003, *38,* 1147–1154.

[8] Lin Cereghino, J., Cregg, J. M., Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol. Rev.* 2000, *24,* 45–66.

[9] Thiry, M., Cingolani, D., Optimizing scale-up fermentation processes. *Trends Biotechnol.* 2002, *20,* 103–105.

[10] Curvers, S., Brixius, P., Klauser, T., Thömmes, J. *et al*., Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing. Part 1. Fermentation. *Biotechnol. Prog.* 2001, *17,* 495–502.

[11] Stratton, J., Chiruvolu, V., Meagher, M., High cell-density fermentation, in: Higgins D.R.., Cregg J.M. (Eds.), *Pichia Protocols,* Humana Press, Totowa 1998, pp. 107–120.

[12] Trentmann, O., Khatri, N. K., Hoffmann, F., Reduced oxygen supply increases process stability and product yield with recombinant *Pichia pastoris*. *Biotechnol. Prog.* 2004, *20,* 1766–1775.

[13] Zhou, X.-S., Zhang, Y.-X., Decrease of proteolytic degradation of recombinant hirudin produced by *Pichia pastoris* by

controlling the specific growth rate. *Biotechnol. Lett.* 2002, *24,* 1449–1453.

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

[15] Qureshi, M. S., Zhang, D., Du, G., Chen, J., Improved production of polygalacturonate lyase by combining a pH and on-line methanol control strategy in a two-stage induction phase with a shift in the transition phase. *J. Ind. Microbiol. Biotechnol.* 2010, *37,* 323–333.

[16] Minning, S., Serrano, A., Ferrer, P., Solá, C., Schmid, R. D., Valero, F., Optimization of high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. *J. Biotechnol.* 2001, *86,* 59–70.

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

[18] Korz, D. J., Rinas, U., Hellmuth, K., Sanders, E. A., Deckwer, W. D., Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J Biotechnol.* 1995, *39,* 59–65.

[19] Jahic, M., Rotticci-Mulder, J. C., Martinelle, M., Hult, K., Enfors, S.-O., Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. *Bioprocess Biosyst. Eng.* 2002, *24,* 385–393.

[20] Xie, J., Zhang, L., Ye, Q., Zhou, Q. *et al.*, Angiostation production in cultivation of recombinant *Pichia pastoris* fed with mixed carbon sources. *Biotechnol. Lett.* 2003, *25,* 173–177.

[21] Cunha, A. E., Clemente, J. J., Gomes, R., Pinto, F. *et al.*, Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. *Biotechnol. Bioeng.* 2004, *86,* 458–467.

[22] Zhang, W., Bevins, M. A., Plantz, B. A., Smith, L. A., Meagher, M. M., 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.* 2000, *70,* 1–8.

[23] Hohenblum, H., Borth, N., Mattanovich, D., Assessing viability and cell-associated product of recombinant protein producing *Pichia pastoris* with flow cytometry. *J. Biotechnol.* 2003, *102,* 281–290.

[24] Hohenblum, H., Gasser, B., Maurer, M., Borth, N., Mattanovich, D., Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. *Biotechnol. Bioeng.* 2004, *85,* 367–375.

[25] Kauffman, K. J., Pridgen, E. M., Doyle, F. J. I., Dhurjati, P. S., Robinson, A. S., Decreased protein expression and intermittent recoveries in BiP levels result from cellular stress during heterologous protein expression in *Saccharomyces cerevisiae*. *Biotechnol. Prog.* 2002, *18,* 942–950.

[26] Sun, R., Anderson, T. J., Erickson, A. K., Nelson, E. A., Francis, D. H., Inhibition of adhesion of *Escherichia coli* k88ac fimbria to its receptor, intestinal mucin-type glycoproteins, by a monoclonal antibody directed against a variable domain of the fimbria. *Infect. Immun.* 2000, *68,* 3509–3515.

[27] Rautio, J., Barken, K. B., Lahdenpera, J., Breitenstein, A., Molin, S., Neubauer, P., Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates. *Microb. Cell Fact.* 2003, *2,* 4.

[28] Resina, D., Bollok, M., Khatri, N. K., Valero, F., Neubauer, P., Ferrer, P., Transcriptional response of *P. pastoris* in fed-batch cultivations to *Rhizopus oryzae* lipase production reveals UPR induction. *Microb. Cell Fact.* 2007, *6,* 21.

[29] Ruottinen, M., Bollok, M., Kogler, M., Neubauer, A. *et al.*, Improved production of human type II procollagen in the yeast *Pichia pastoris* in shake flasks by a wireless-controlled fed-batch system. *BMC. Biotechnol.* 2008, *8,* 33.

[30] Khatri, N. K., Hoffmann, F., Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with recombinant *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* 2006, *72,* 492–498.

[31] Khatri, N. K., Hoffmann, F., Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant *Pichia pastoris*. *Biotechnol. Bioeng.* 2006, *93,* 871–879.

[32] Gasser, B., Sauer, M., Maurer, M., Stadlmayr, G., Mattanovich, D., Transcriptomics-based identification of novel factors enhancing heterologous protein secretion in yeasts. *Appl. Environ. Microbiol.* 2007, *73,* 6499–6507.

[33] Brierley, R. A., Siegel, R. S., Bussineau, C. M., Craig, W. S. *et al.*, Mixed feed recombinant yeast fermentation. In *The Patent Cooperation Treaty* 1990, C12N, 5/00, C12 21/00, US.

[34] Jahic, M., Veide, A., Charoenrat, T., Teeri, T., Enfors, S.-O., Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. *Biotechnol.Prog* 2006, 22, *6*, 1465–1473.

[35] Brinkmann, U., Mueller, R. H., Babel, W., The growth rate-limiting reaction in methanol-assimilating yeasts. *FEMS Microbiol. Rev.* 1990, *7,* 261–265.