# Analysis of Single-Chain Antibody Production in *Pichia pastoris* Using On-Line Methanol Control in Fed-Batch and Mixed-Feed Fermentations

**Stephan Hellwig,[1] Frank Emde,[2] Nicole P. G. Raven,[1] Mario Henke,[4] Paul van der Logt,[3] Rainer Fischer[1,4]**

[1]*Institut für Biologie I (Botanik/Molekulargenetik), RWTH Aachen, 52074 Aachen, Germany; telephone: +49 241 806631; fax: +49 241 871062; e-mail: fischer@bio1.rwth-aachen.de*
[2]*Heinrich Frings GmbH & Co. KG, Bio- und Chemietechnik, Jonas-Cahn-Straβe, 53115 Bonn, Germany*
[3]*Unilever Research Laboratories Colworth House, Sharnbrook, Bedford, MK441LQ UK*
[4]*Fraunhofer Department for Molecular Biotechnology, IUCT, Auf dem Aberg 1, 57392 Schmallenberg, Germany*

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**Abstract:** In the last few years the *Pichia pastoris* expression system has been gaining more and more interest for the expression of recombinant proteins. Many groups have employed fermentation technology in their investigations because the system is fairly easy to scale up and suitable for the production in the milligram to gram range. A large number of heterologous proteins from different sources has been expressed, but the fermentation process technology has been investigated to a lesser extent. A large number of fermentations are carried out in standard bioreactors that may be insufficiently equipped to meet the demands of high-cell-density fermentations of methylotrophic yeasts. In particular, the lack of on-line methanol analysis leads to fermentation protocols that may impair the optimal expression of the desired products. We have used a commercially available methanol sensor to investigate in detail the effects of supplementary glycerol feeding while maintaining a constant methanol concentration during the induction of a Mut$^+$ strain of *Pichia pastoris*. Specific glycerol feed rates in the range of 38–4.2 mg · g$^{-1}$ · h$^{-1}$ (mg glycerol per gram fresh weight per hour) were investigated. Expression of the recombinant scFv antibody fragment was only observed at specific feed rates below 6 mg · g$^{-1}$ · h$^{-1}$. At low specific feed rates, growth was even lower than with methanol as the sole carbon source and the harvest expression level of the scFv was only half of that found in the control fermentation. These results show that glycerol inhibits expression driven by the *AOX1* promoter even at extremely limited availability and demonstrate the benefits of on-line methanol control in *Pichia* fermentation research. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **74**: 344–352, 2001.

**Keywords:** single-chain antibody; methylotrophic yeast; *Pichia pastoris*; mixed-feed fermentation; methanol sensor; on-line methanol control

*Correspondence to:* Dr. Rainer Fischer
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## INTRODUCTION

The methylotrophic yeast *Pichia pastoris* has gained widespread attention as an expression system because of its potential to produce large quantities of heterologous proteins (Clare and Romanos, 1995; Sreekrishna et al., 1997). The highest recombinant protein yields reported are 22 g per L culture volume for an intracellularly expressed protein (Hasslacher et al., 1997) and ~11 g per L culture volume for a secreted protein (Werten et al., 1999). A commonly used approach for heterologous protein expression has been to express the gene of interest under the control of the *AOX1* promoter. In wild-type *Pichia,* this promoter controls expression of alcohol oxidase 1, the first enzyme in methanol metabolism (Cregg et al., 1989), which can account for up to 30% of the total soluble protein (Couderc and Baratti, 1980). For recombinant protein expression, genes of interest are integrated into the *Pichia* genome via homologous recombination, giving rise to two different integration types. Clones in which the expression cassette has been inserted at either the 5′ or 3′ *AOX1* UTRs or the *his4* gene via a single cross-over event retain their ability to metabolize methanol quickly (Mut$^+$), whereas clones in which the *AOX1* gene has been replaced by the expression cassette due to a double cross-over event show slow growth on methanol (Mut$^S$). The Mut phenotype of the expresser strain is important because expression from the *AOX1* promoter requires the presence of methanol. Special care must be taken to keep the methanol concentration below toxic levels while at the same time maintaining the induction. While it is not clear which Mut phenotype is more sensitive to methanol overdoses, as researchers have published different opinions (Stratton et al., 1998, and Chiruvolu et al., 1997, claim that Mut$^+$ is more sensitive to methanol overdoses, while Kim et al., 1997, Clare and Romanos, 1995, and Romanos, 1995, pro-

pose that the opposite is true), it is agreed that the $Mut^+$ strains can be advantageous for fermentation because they grow faster during the induction phase (Zhu et al., 1995).

In *Pichia* fermentation, cell densities of 150–400 $g \cdot l^{-1}$ (fresh weight) are routinely achieved on simple, defined media containing glycerol as a carbon source in batch and subsequent fed-batch fermentations. Heterologous protein expression is induced during fermentation by changing to methanol as the sole carbon source. The presence of glycerol has been stated to have either no effect on *AOX1*-driven expression (Katakura et al., 1998) or a repressive effect (Hollenberg and Gellissen, 1997; Lal et al., 1997; Sreekrishna et al., 1997; Waterham et al., 1997). Raschke et al. (1996) showed that there is a glycerol-dependent repression mechanism in *Pichia* (as opposed to *Hansenula polymorpha*) by comparing expression of a heterologous gene using the *AOX1* promoter in both systems. Stratton et al. (1998) and Gellissen et al. (1995) pointed out that an intermediate limiting glycerol fed-batch phase or a carbon starvation phase in *Pichia* fermentations may be helpful to derepress the *AOX1* promoter and aid the transition to methanol metabolism.

During methanol induction of gene expression the methanol feed rate must be constantly adjusted to the methanol consumption rate to ensure that methanol does not accumulate to toxic concentrations (Stratton et al., 1998). The methanol feed strategies used are either fixed feed rates (Clare and Romanos, 1995; Hellwig et al., 1999; Munshi et al., 1997; Nilsen et al., 1997; Rosenfeld et al., 1996; Van Heeke et al., 1996) or methanol feed rates controlled by the dissolved oxygen concentration (Stratton et al., 1998). The latter has the advantage of dependence on the actual methanol consumption; however, $dO_2$ response to methanol depletion is slower with $Mut^S$ strains and not all fermenters can be programmed to use this parameter in a feed control circuit. Furthermore, when this strategy is used methanol concentrations oscillate between 0% and a concentration determined by multiple factors such as feed pump rate, control circuit cycle times, methanol consumption and evaporation, and others. Maintaining methanol at a more constant and nonlimiting concentration of 0.4–3% would be more desirable (Stratton et al., 1998).

Various attempts have been made to improve this standard fermentation strategy such as feeding glycerol and methanol simultaneously during the induction phase (Cregg et al., 1993). Sreekrishna et al. (1989) produced tumor necrosis factor (TNF) using a $Mut^S$ strain in a continuous fermentation containing glycerol and methanol at a ratio of 5:1 (w:w) in the feed at a dilution rate of 0.05 $h^{-1}$. Although the expression levels in the outflow of the continuous fermentation were much lower than in the harvest broth of high-cell-density batch/fed-batch fermentations of the same clone, the volumetric productivity was much higher in the mixed-feed continuous fermentation. Loewen et al. (1997) increased expression levels of fish antifreeze protein in a $Mut^S$ strain from 2.5–35 $mg \cdot l^{-1}$ by using a mixed feed strategy containing glycerol and methanol in a 4:1 (w/w) ratio.

Fermentations of a $Mut^S$ strain secreting bovine lysozyme using mixed feeds with glycerol:methanol ratios ranging between 4:1 and 1:3 (w/w) were compared to standard fermentations of the same strain (Brierley et al., 1990). Expression levels were lower than standards when high glycerol:methanol ratios were fed and an improvement was only seen with ratios lower than 2:1. For a $Mut^+$ strain, McGrew et al. (1997) reported approximately doubled growth and CD40 ligand expression levels with mixed feeds at a glycerol:methanol ratio of 1:1 compared to feeding methanol only.

In the approaches described above the methanol concentration was not measured on-line during the fermentation. The only information on carbon source availability was obtained by observing $dO_2$ spikes upon stopping the feed, which does not provide information regarding which carbon source is present at which concentrations in the case of mixed-feeds. A methanol-controlled fermentation has been described (Katakura et al., 1998). Using a semiconductor gas sensor to measure methanol concentrations in the exhaust gas, methanol concentrations were maintained constant at different levels between 0.15 and 3.1% (v/v). At higher methanol concentrations, growth and productivity were lower. Feeding additional glycerol at a rate of 5 $ml \cdot l^{-1} \cdot h^{-1}$ at 0.55% (v/v) of methanol was reported to dramatically increase growth rate and product formation.

Sensor prototypes measuring methanol concentrations in the broth rather than in the exhaust gas were applied for shake-flask cultures of *Pichia pastoris* (Guarna et al., 1997), for fermentations of *Methylomonas mucosa* (Austin et al., 1992), and for the on-line determination of ethanol in fermentations of *Saccaromyces cerevisiae* (Vorlop et al., 1983).

In this article, we describe the production of a single-chain Fv antibody fragment in a *Pichia pastoris* $Mut^+$ strain in on-line-controlled methanol fed-batch fermentations and mixed-feed fed-batch fermentations in which glycerol was fed at a constant rate. Using a commercially available, non-invasive semiconductor sensor, methanol was measured directly in the fermentation broth and its concentration was controlled independently of the glycerol feed. The advantages of on-line methanol control in *Pichia pastoris* fermentations and the effects of different specific glycerol feed rates on methanol consumption and *AOX1*-driven expression are discussed as well as the new options that arise for *Pichia* fermentation research.

## MATERIALS AND METHODS

### Cloning

The gene coding for the VH and VL domains of mouse mAb4813 linked by a $(Gly_4Ser)_3$ linker peptide (Alfthan et al., 1995) was cloned into the *Pichia* expression vector pPIC9K (Invitrogen, San Diego, CA). The final construct (pPIC9K-4813His) contained an expression cassette encod-

ing scFv4813 fused to an N-terminal *S. cerevisiae* α-factor prepro leader peptide and a C-terminal His6 affinity purification tag under the control of the *AOX1* promoter.

### Strain Maintenance

All yeast strains were kept as frozen stocks in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) glucose) supplemented with 15% (v/v) glycerol at –70°C. Working stocks of all strains were maintained as agar slants on YPD plates (YPD medium containing 2% (w/v) agar).

### Transformation

The expression vector pPIC9K-4813His was linearized with *Bgl*II prior to transformation. *Pichia pastoris* strain GS115 (*his4*) (Invitrogen) was transformed using the spheroplast transformation method as described in the *Pichia* expression kit manual (Invitrogen, San Diego). $His^+$ colonies appeared after 3–4 days of incubation at 28°C on RDB plates (1 M sorbitol, 1% (w/v) dextrose, 4 · 10-5% (w/v) biotin, 1.34% (w/v) yeast nitrogen base and 2% (w/v) agar).

### G418 Resistance Characterization

$His^+$ transformants were screened for G418 resistance to identify clones bearing multiple integrated copies of the vector, which contains the *kan* G418 resistance marker gene (Romanos et al., 1998; Scorer et al., 1994). Two hundred $His^+$ transformants were replica-plated on YPD plates (1% (w/v) yeast extract, 2% (w/v) peptone, 1% dextrose) containing 0.25, 0.5, and 1 mg · $ml^{-1}$ of G418. Colonies growing on elevated levels of G418 after 4 days of incubation at 30°C were analyzed by expression screening.

### Expression Screening

Individual $His^+$- and G418-resistant colonies were inoculated in 10 ml BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 · $10^{-5}$% biotin, 1% glycerol, 100 mM potassium phosphate, pH 6.0) in a 50-ml Falcon tube. After incubation at 30°C, 250 rpm for 24 h the cells were collected by centrifugation (2,000 *g*, 5 min, RT) and resuspended in 5 ml BMMY medium (BMGY with 4% methanol replacing glycerol). The cells were incubated at 30°C and 250 rpm for 24 h and the culture supernatant was separated from the cells by centrifugation (2,000 *g*, 5 min, RT). Expression levels were determined by surface plasmon resonance analysis.

### Determination of Methanol Utilization Genotype by PCR

Methanol utilization genotypes were determined by PCR using a modified version of the protocol described by Linder et al. (1996). Cells were grown in YPD for 16 h and cracked by incubation with the *Arthrobacter sp.* Yeast Lytic Enzyme (ICN, Eschwege, Germany) at 28°C for 40 min. Five μL of this lysate was subjected to PCR (50 μL total volume) using the primers 5′-AOX (5′-GAC TGG TTC CAA TTG ACA AGC) and 3′-AOX (5′-GGC AAA TGG CAT TCT GAC ATC). In $Mut^+$ clones these primers amplify both the native *AOX1* gene (2.2 kB) and the expression cassette. In the case of a replacement-type integration ($Mut^S$) these primers result only in the amplification of the expression cassette.

The amplified DNA fragments were separated by electrophoresis in 1.2% (w/v) agarose gels and visualized using ethidiumbromide staining.

### SDS-PAGE and Immunostaining

Clarified culture supernatant samples were separated on a 12% denaturing SDS-PAGE according to standard protocols. The gels were either directly stained with Coomassie brilliant blue or proteins were electrotransferred to a nitrocellulose membrane. In this case the membrane was blocked for 30 min at 24°C with 5% skim milk powder in PBS-T (137 mM NaCl, 27 mM KCl, 81 mM $Na_2HPO_4$, 15 mM $KH_2PO_4$, 0.05% (w/v) Tween 20, pH 7.4), then incubated for 1 h at 24°C with 100 ng/ml mouse monoclonal penta-his antibody (Qiagen, Hilden, Germany) in blocking solution. The membrane was then incubated with 100 ng/ml of polyclonal antibody $GAM\text{-}AP_{Fc}$ (Jackson ImmunoResearch, West Grove, PA) in PBS-T buffer for 1 h at 24°C. Bands were visualized by incubation with NBT-BCIP alcaline phosphatase substrate (Pierce, Rockford IL). Three washing steps with PBS-T were carried out between all incubation steps.

### Surface Plasmon Resonance

ScFv4813 was detected in culture supernatants using surface plasmon resonance technology via the C-terminal His6-tag on a BIAcore 2000 system using an Ni-NTA chip (BIAcore, Uppsala, Sweden). The flow rate was 5 μl · $min^{-1}$ with running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 0.005% (v/v) Tween20). Each sample cycle was composed of subsequent injections of 5 μl stripping buffer (running buffer containing 0.35 M EDTA at pH 8.3), 10 μl of loading buffer (running buffer containing 500 μM $NiCl_2$), and 20 μl of the clarified fermentation supernatant diluted 1:20-fold in running buffer.

### Fermentation

Fermentations were carried out in a 30-L working volume stirred tank reactor (Biopilot 40, Applikon, Schiedam, The Netherlands). Twenty-eight liters of basal salts medium (4.25 mL · $L^{-1}$ ortho-phosphoric acid, 9.4 mM $MgSO_4$, 1 mM $CaSO_4$, 16.4 mM $K_2SO_4$, 11.4 mM KOH, 50 mL · $L^{-1}$ glycerol) were sterilized inside the reactor; 4.0 mL · $L^{-1}$ PTM1 trace salts (24 mM $CuSO_4$, 0.53 mM NaI, 19.87 mM $MnSO_4$, 0.83 mM $Na_2MoO_4$, 0.32 mM boric acid, 2.10 mM

$CoCl_2$, 0.15 mM $ZnCl_2$, 0.23 M $FeSO_4$, 0.82 mM biotin) were added after sterilization.

Agitation was provided by three 6-bladed Rushton impellers of 1/3 the vessel diameter, operating at 500 rpm. The culture pH was adjusted to 6.0 and maintained by automatic addition of 28% (w/v) $NH_4OH$, which also served as the sole nitrogen source. The temperature was maintained constant at 30°C. Aeration was constant at a rate of 1 l $\cdot$ $l^{-1} \cdot min^{-1}$ of pressurized air. Fermentations were inoculated with 1.5 l of a preculture of the expresser strain (BMGY, 24 h, 30°C, 250 rpm).

For mixed-feed fermentations, glycerol was fed to the fermenter at fixed rates using a peristaltic pump operating in a pulsed mode with variable on-times in 2-min cycles. The FRINGS ALKOSENS alcohol sensor (Heinrich Frings, Bonn, Germany) was used for the on-line methanol determination. The probe was inserted into a standard 25 mm Ingold port and in-situ-sterilized. Highly purified pressurized air free of organic compounds was used as a carrier gas. Volatile alcoholic compounds that pervaporate from the fermentation medium through a selective, hydrophobic multilayer membrane are transported with the carrier gas to a semiconductor gas sensor. This sensor detects inflammable gases in air at low concentrations using a heated, dotted $SnO_2$ surface at which the reduction of adsorbed $O_2$, e.g., by organic acids or alcohols, causes a measurable decrease in the semiconductor's resistivity (for details on the sensor technology, see also Ebner et al., 1996; Maekawa et al., 1992).

The decrease in resistivity was measured by the FRINGS ACETOMAT display and control unit and correlated to a nonlinear calibration curve. According to the actual methanol concentration and the setpoint, methanol was fed to the fermenter via a peristaltic pump controlled by the ACETOMAT unit. The analog output from the ACETOMAT was linked to the process control software to monitor the methanol concentration on-line. Prior to the fermentation, the instrument was calibrated in the reactor using water at 30°C and agitation/aeration conditions identical to the conditions during the fermentation. The calibration curve was controlled at the beginning of the induction (1-point calibration) in fermentation broth and verified again after harvesting.

## RESULTS

### Transformation and Clone Selection

To generate recombinant *Pichia* clones secreting scFv4813, the host strain GS115 was transformed by the spheroplast method with the *Bgl*II-linearized vector pPIC9K-4813His. Linearization of the plasmid with *Bgl*II prior to electroporation results in a mixture of integration-type and replacement-type recombinant clones showing either $Mut^+$ or $Mut^S$ phenotypes (Romanos, 1995; Sreekrishna et al., 1997). Recombinant clones were selected on RDB minimal medium for a $His^+$ phenotype and screened for their tolerance towards G418. The highest G418 tolerance found among 200 screened clones was 0.5 mg $\cdot$ $l^{-1}$. In this group of clones, expression levels were determined by small-scale expression and subsequent surface plasmon resonance analysis on a BIAcore 2000 system. The clone with the highest expression level (N55) was used for further experiments.

The methanol utilization phenotype of clone N55 was determined by PCR. Using 5′AOX and 3′AOX primers, two bands of approximately 2.2 and 1.25 kB were amplified, which correlates with the expected sizes of the intact *AOX1* structural gene and the scFv4813 expression cassette, respectively (Fig. 1). This result revealed the $Mut^+$ phenotype of clone N55. All other clones tested for their Mut-phenotype by PCR also possessed $Mut^+$ phenotypes.

### scFv Production by Mixed-Feed Fermentation Using On-Line Methanol Control

scFv4813 was produced in three mixed-feed fermentations using different fixed glycerol feed rates and a constant methanol concentration of 0.5% (v/v), which was maintained using the on-line methanol control system. A salt-reduced medium containing approximately one-eighth the salt concentrations used for *P. pastoris* fermentations by other groups (Brankamp et al., 1995; Katakura et al., 1998; Kim et al., 1997; Rosenfeld et al., 1996) was used. This medium had previously been used for *Pichia* fermentations in our group without any effects on cell growth or final cell densities (Hellwig et al., 1999). The initial glycerol concentration was 5% (v/v).

After the initial amount of glycerol was depleted a limiting glycerol feed was started and maintained at a constant rate for the rest of the fermentation. Thus, the specific glycerol feed rate decreased with the increase in biomass, as shown in Figure 2 for the three different glycerol feed rates. During the first 4–6 h of glycerol feeding no methanol was

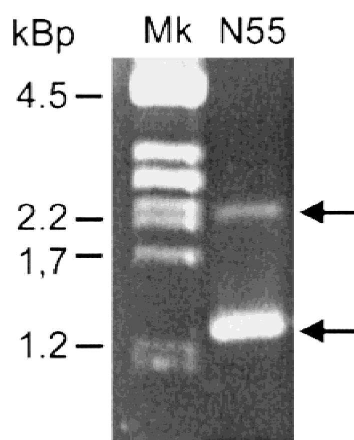


**Figure 1.** PCR-analysis of *Pichia* clone N55 using 5′AOX and 3′AOX primers. *PstI*-digested λ-phage DNA was used as a size-marker. N55: PCR products obtained from direct PCR of clone N55. Arrows indicate the PCR-amplified wild-type *AOX1*-gene (2.2 kB) and the scFv expression cassette (1.25 kB).

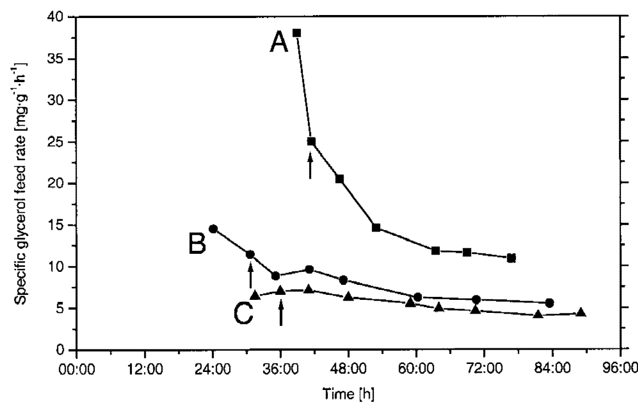


**Figure 2.** Specific glycerol feed rates in three mixed-feed fermentations of *Pichia pastoris* clone N55. The absolute feed rates were (**A**) 4.9 $g \cdot L^{-1} \cdot h^{-1}$, (**B**) 2.46 $g \cdot L^{-1} \cdot h^{-1}$, and (**C**) 1.23 $g \cdot L^{-1} \cdot h^{-1}$. Arrows indicate the beginning of induction by adjusting the methanol concentration to 0.5% (v/v).

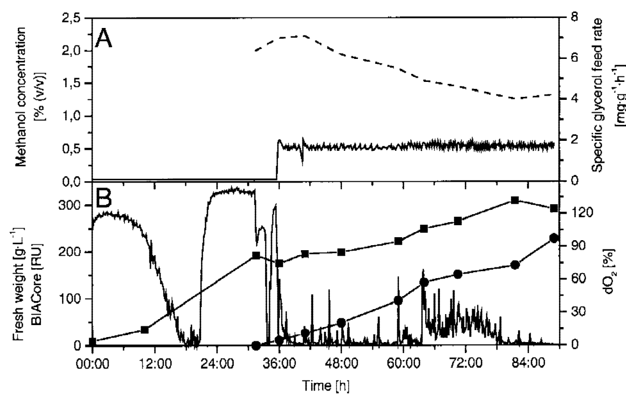


**Figure 3.** Mixed-feed fermentation of *Pichia pastoris* clone N55 using on-line methanol control and a fixed-rate glycerol feed of 1.23 $g \cdot l^{-1} \cdot h^{-1}$. **A**: Methanol concentration (solid line) was maintained constant at 0.5% (v/v) using the FRINGS ALKOSENS methanol sensor. The specific glycerol feed rate (dashed line) results from the glycerol feed correlated to the fresh weight. **B**: Fresh weight (squares) was determined gravimetrically, expression levels are shown as BIAcore response units (circles) detected in supernatant samples. The dissolved oxygen concentration ($dO_2$, solid line) was measured on-line using a Clark-type oxygen sensor electrode.

added. From the dissolved oxygen signal oscillations and from "$dO_2$-spiking" experiments (see Introduction) it was evident that glycerol was fed at limiting rates. After 4–6 h of glycerol feeding the methanol concentration was adjusted to 0.5% and maintained at this value for the rest of the fermentation. The basic parameters of all fermentations discussed in this article are summarized in Table I.

In the first two fermentations with specific glycerol feed rates ranging from 38–11 and 14.5–5.5 $mg \cdot g^{-1} \cdot h^{-1}$, respectively, (Fig. 2A,B), biomass was built up quickly to high final cell densities. The overall growth rates observed during the mixed-feed induction phase were 0.026 $h^{-1}$ and 0.016 $h^{-1}$, respectively. Both fermentations were terminated when the broth became too thick to be effectively mixed. Expression levels of scFv4813 were poor. Only one sample taken at a specific glycerol feed rate of 5.5 $mg \cdot g^{-1} \cdot h^{-1}$ gave an elevated signal in BIAcore analysis of the clarified supernatant. A third mixed-feed fermentation was carried out, in which the specific glycerol feed rate was further reduced (Fig. 2C).

This fermentation is shown in detail in Figure 3. The initial amount of 5% (w/v) of glycerol was used up 20 h after inoculation. After a glycerol starvation phase of 10 h the glycerol feed was initiated at a rate of 1.23 $g \cdot l^{-1} \cdot h^{-1}$, corresponding to an initial specific feed rate of 6.4 $mg \cdot g^{-1} \cdot h^{-1}$. At 36 h the methanol concentration was adjusted to 0.5% (v/v) and maintained at this level for the rest of the induction period, which lasted for another 53 h, in which the fresh weight increased to over 300 $g \cdot l^{-1}$. The specific glycerol feed rate dropped to 4.22 $mg \cdot g^{-1} \cdot h^{-1}$ accordingly. During the induction phase the methanol consumption was in the range of 4.7–7 $mg \cdot g^{-1} \cdot h^{-1}$ as calculated using a balance to monitor the methanol feed. The overall growth rate during the induction was 0.007 $h^{-1}$.

The accumulation of secreted scFv4813 in the culture supernatant was monitored by BIAcore analysis and showed a continuous increase until the end of the fermentation. The final scFv concentration in the fermentation supernatant was estimated as 25–30 $mg \cdot l^{-1}$ from the BIAcore analysis. This estimation was consistent with Coomassie-stained SDS-PAGE (data not shown).

To determine if the low specific glycerol feed rates used in the fermentation described above exerted an inhibitory effect on *AOX1*-driven expression of scFv4813, a fourth fermentation in which methanol was the sole carbon source during induction was carried out.

## scFv Production by Methanol Fed-Batch Fermentation Using On-Line Methanol Control

Cells were grown in a batch fermentation mode until glycerol was depleted. To adapt the cells to methanol as sole

**Table I.** Summary of basic parameters of *Pichia pastoris* clone N55 fermentations

| Glycerol feed rate [$g \cdot L^{-1} \cdot h^{-1}$] | Specific glycerol feed rates [$mg \cdot g^{-1} \cdot h^{-1}$] | Total fermentation time [h] | Harvest fresh weight [$g \cdot L^{-1}$] | Overall growth rate during induction [$h^{-1}$] | Harvest expression level [RU] |
|---|---|---|---|---|---|
| 4.9 | 38–11 | 77 | 449 | 0.023 | 23 |
| 2.46 | 14.5–5.5 | 85 | 450 | 0.016 | 81 |
| 1.23 | 7.0–4.0 | 89 | 310 | 0.007 | 228 |
| 0 | 0 | 86 | 324 | 0.012 | 436 |

carbon source, methanol was fed at a fixed rate of 1 $mL \cdot L^{-1} \cdot h^{-1}$. For the first 6 h of the adaptation time, methanol accumulated in the fermenter to a maximum concentration of 0.26% (v/v) because the combined consumption and evaporation rates of methanol were lower than the methanol feed rate. As adaptation proceeded and more methanol was consumed, methanol decreased again. After a total adaptation time of 12 h the adaptation was considered complete and 0.5% (v/v) methanol was maintained constant for the rest of the fermentation. Figure 4 shows this fermentation in detail. The induction phase in which methanol was kept constant lasted for 47 h. During the methanol fed-batch phase, the biomass increased almost linearly with an overall growth rate of 0.012 $h^{-1}$ to a final cell density of 324 $g \cdot l^{-1}$. ScFv4813 accumulated steadily in the fermentation supernatant to a harvest expression level of 40–45 $mg \cdot l^{-1}$ as estimated from BIAcore analysis. This was consistent with the estimation of expression levels from Coomassie-stained SDS-PAGE and Western blot (Fig. 5).

## DISCUSSION

We investigated growth and production characteristics in fermentations of a recombinant strain of *Pichia pastoris*. Using an on-line methanol sensor and control unit, a constant methanol concentration was maintained throughout the induction phases, allowing independently controlled glycerol feeding. With methanol being nonlimiting, we investigated the effects of several limiting glycerol feed rates on growth and expression. The strain used in these fermentations showed a $Mut^+$ phenotype, as verified by PCR. Interestingly, no $Mut^S$-phenotypes were detected among 40 $His^+$-transformants tested.

The feed rates used by other authors in similar limited glycerol fed-batch fermentations were 10.4 $g \cdot L^{-1} \cdot h^{-1}$

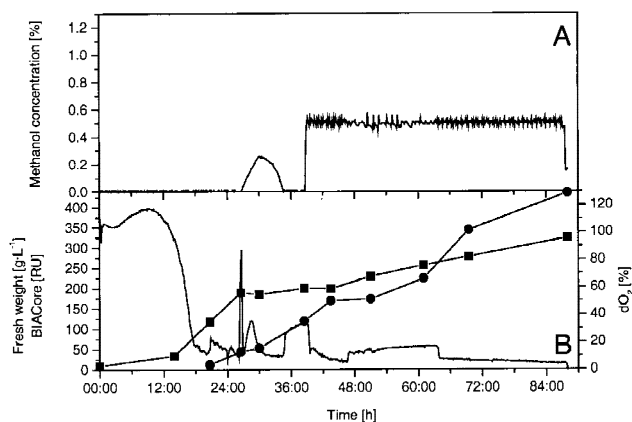


**Figure 4.** Fermentation of *Pichia pastoris* clone N55 using on-line methanol control without additional glycerol feeding. **A**: Methanol concentration (solid line) was maintained constant at 0.5% using the FRINGS ALKOSENS methanol sensor after the adaptation phase. **B**: Fresh weight (squares) was determined gravimetrically, expression levels are shown as BIAcore response units (circles) found in supernatant samples. Dissolved oxygen ($dO_2$, solid line) levels were measured on-line.

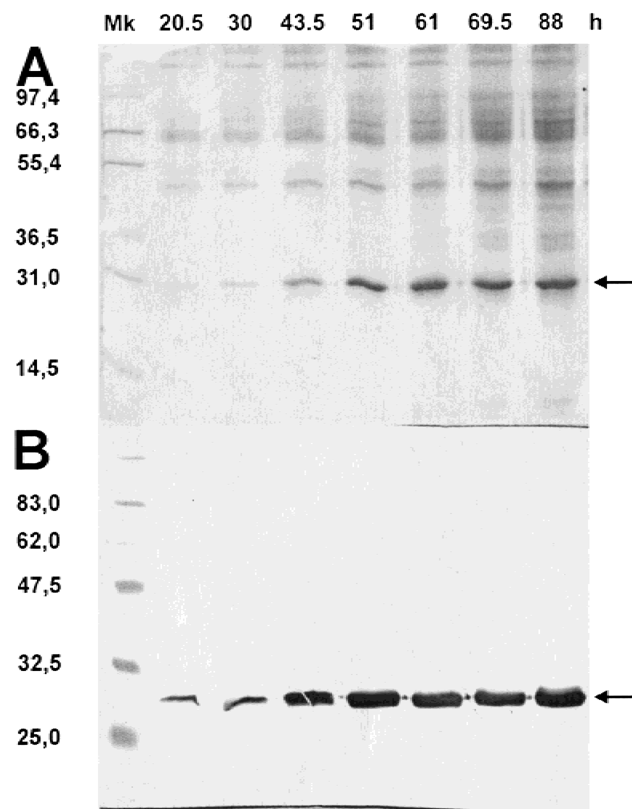


**Figure 5.** SDS-PAGE of *Pichia pastoris* clone N55 methanol-fed-batch fermentation samples. Twenty-seven μL of clarified supernatant taken at the times indicated above were loaded to each slot. Arrows indicate bands corresponding to the expected size of scFv4813. **A**: Coomassie staining. **B**: Western blot of the same samples after immunostaining using anti-penta-his antibodies and goat-anti-mouse antibodies conjugated to alcaline phosphatase. Bands were visualized using NBT-BCIP.

(Loewen et al., 1997), ~5.3 $g \cdot l^{-1} \cdot h^{-1}$ (McGrew et al., 1997), or 6.3 $g \cdot l^{-1} \cdot h^{-1}$ (Katakura et al., 1998), corresponding to fresh weight-specific feedrates at least 26 $mg \cdot g^{-1} \cdot h^{-1}$ or higher. In the first two fermentations described, glycerol was fed at 4.9–2.46 $g \cdot L^{-1} \cdot h^{-1}$, corresponding to fresh-weight specific feed rates decreasing from 38 to 11 $mg \cdot g^{-1} \cdot h^{-1}$ and 14.5 to 5.5 $mg \cdot g^{-1} \cdot h^{-1}$, respectively (Fig. 2A,B). These feed rates were evidently limiting, as shown by the $dO_2$ signal oscillations and $dO_2$ spiking experiments. Expression levels were much lower than in the expression level screening. Only one sample, taken at the lowest specific glycerol feed rate, showed an elevated expression level in surface plasmon resonance analysis. This indicated that the expression of scFv4813 was inhibited by the glycerol availability and a third fermentation was carried out with further reduced glycerol feed rate (Fig. 2C). In this fermentation the specific glycerol feed rate decreased from 7.0 $mg \cdot g^{-1} \cdot h^{-1}$ at the beginning of induction to 4.0 $mg \cdot g^{-1} \cdot h^{-1}$ at the end of the fermentation (Table I). Surface plasmon resonance revealed the expression of His6-tagged scFv4813 shortly after the beginning of induction (Fig. 3).

To compare the mixed-feed fermentations with a methanol fed-batch, a fourth fermentation was carried out using

the same conditions but without glycerol feed (Fig. 4). In this fermentation the growth rate during the induction was calculated to be 0.012 $h^{-1}$. Similar growth rates have been reported for $Mut^{+}$ strains previously (Hellwig et al., 1999; Katakura et al., 1998). Surprisingly, the overall growth rate found during the induction phase as well as the final cell density were higher than in the mixed-feed fermentation with the lowest glycerol feed rate (Table I). ScFv4813 accumulated in the supernatant to a final concentration of ~45 $mg \cdot l^{-1}$ as determined by surface plasmon resonance analysis and Coomassie-stained SDS-PAGE (Fig. 5). This was almost twice as high as in the best mixed-feed fermentation. The expression level found for scFv4813 is comparable to the expression levels of other scFv antibody fragments produced in *P. pastoris* (Eldin et al., 1997; Luo et al., 1995, 1997; Ridder et al., 1995). Immunostaining of the fermentation supernatant samples using anti-penta-his antibody (Qiagen) showed that only the scFv4813 band of the expected size was recognized in the Western blot, indicating that the product was homogenous and no proteolytic fragmentation or glycosylation occurred (Fig. 5).

Comparing the basic parameters of the four fermentations (Table I), it becomes obvious that glycerol supplementation during induction resulted in increased growth rates and biomass accumulation. At the same time, glycerol represses expression of scFv4813.

The fact that specific and volumetric productivity found at the lowest glycerol feed rate are even lower than those found with methanol as the sole carbon source clearly indicates that inhibition of *AOX1*-driven expression occurs even at extremely low glycerol concentrations. This conclusion is supported by the observation that at the lowest specific glycerol feed rate in mixed-feed fermentations the growth rate is even slower than on methanol alone. This effect could be interpreted as a lower expression of the alcohol oxidase 1 enzyme itself. At higher specific glycerol feed rates, the increased growth rate resulting from the availability of supplemented glycerol could mask the inhibition of *AOX1*-driven expression.

The results obtained by other researchers show that upon additional glycerol feeding the specific productivity of *AOX1*-controlled protein expression decreases (Chiruvolu et al., 1997; McGrew et al., 1997). Glycerol supplementation is always reported to result in increased growth rates, but the specific glycerol feed rates are usually higher than 20 $mg \cdot g^{-1} \cdot h^{-1}$ (Katakura et al., 1998; Loewen et al., 1997; McGrew et al., 1997; Sreekrishna et al., 1989), so that the effect of impaired methanol metabolism is not visible in the growth rate. Besides, especially in $Mut^{S}$ strains (Loewen et al., 1997; Sreekrishna et al., 1989), the increased energy availability may more than make up for the inhibition. Other factors, such as oxygen limitation or the inherent properties of a heterologously expressed protein, may also make it difficult to clearly distinguish the influence of key factors in *Pichia* fermentations.

In fermentations in which methanol is used as the sole carbon source at limiting concentrations, as is the case in oxygen-controlled or limited feed control strategies, carbon source availability may be the limiting factor for expression and the increase in energy supply by glycerol feeding could also mask the effects of *AOX1* inhibition. Therefore, the effects of glycerol supplementation may be different with strains other than $Mut^{+}$ or other methanol control strategies. This work strongly emphasizes the necessity to obtain a maximum of information on the process parameters and to maintain strict process control in order to optimize the high-cell-density fermentation of *P. pastoris*. It also shows that the use of a methanol sensor can be a powerful tool in the research and optimization of *P. pastoris* fermentation strategies.

## CONCLUSIONS

This work demonstrates the advantages of on-line methanol analysis and control in *Pichia* fermentations. Using a commercially available alcohol sensor measuring the methanol concentration in the fermentation broth, we were able to maintain the methanol concentration between 0.48 and 0.53% (v/v) during the entire induction phase, thus allowing uncoupled glycerol/methanol feeding and detailed analysis of the effects of changes in the specific glycerol feed rate. At extremely low specific glycerol feed rates, expression of the recombinant protein as well as growth rate were lower than in fermentations in which no glycerol was fed. The results show that with on-line methanol control it is possible to obtain more reproducible fermentation results with methylotrophic yeasts and to employ more sophisticated fermentation modes in order to methodically approach research in this area. Our current work includes investigation of the methanol concentration optimum as well as continuous fermentations of *P. pastoris* with independent control of dilution rate and methanol concentration. On-line methanol analysis can help avoid safety hazards during large-scale fermentations. Finally, the availability of on-line data on the concentration of the toxic substrate methanol during the production of therapeutically relevant proteins must be of vital interest to process documentation according to GLP and GMP.

## NOMENCLATURE

| | |
|---|---|
| BMGY | Buffered complex medium containing glycerol as the sole carbon source |
| BMMY | Buffered complex medium containing methanol as the sole carbon source |
| $dO_2$ | Dissolved oxygen |
| EDTA | Ethylen-diamine-tetra-acetate |
| $GAM\text{-}AP^{Fc}$ | Goat-anti-mouse antibody, Fc-specific, alcaline-phosphatase conjugated |
| mAb | Monoclonal antibody |
| NBT-BCIP | Nitro-Blue tetrazolium chloride/5-bromo-chloroindol-3-yl phosphate |
| NTA | Nitrilo-tri-acetic acid |

| | |
|---|---|
| PBS-T | Phosphate buffered saline containing 0.005% Tween 20 |
| PCR | Polymerase chain reaction |
| RDB | Regeneration dextrose medium (selection agar) |
| RT | Room temperature |
| SDS-PAGE | Sodium-dodecyl-sulfate poly-acrylamide gel electrophoresis |
| UTR | Untranslated region |

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