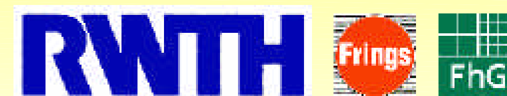
Production of a single-chain Fv antibody fragment in 30-L fermentations of *P. pastoris* using on-line methanol control

Stephan Hellwig[1], Frank Emde[2], Nicole P. G. Raven[1], Mario Henke[3], Rainer Fischer[1,3]

[1] Institut für Biologie I, RWTH Aachen, Worringer Weg 1, D-52074 Aachen
[2] Heinrich Frings GmbH u. Co. KG, Jonas-Cahn-Straße 9, D-53115 Bonn
[3] Fraunhofer IUCT, Abt. Molekulare Biotechnologie, Auf dem Aberg 1, D-57392 Schmallenberg



Introduction

In the past years, the methylotrophic yeast *Pichia pastoris* has gained widespread attention as an expression system because of its ability to produce large quantities of heterologous proteins. In most cases the genes of interest are integrated into the Pichia chromosome under control of the strong AOX1 promoter. Expression driven by this promoter is tightly regulated and induced only when methanol is the sole carbon source.

Fermentations are usually carried out in stirred tank reactors, in which biomass is built up quickly using glycerol as a carbon source. Sometimes an intermediate glycerol fed-batch phase is used to achieve high cell densities prior to induction and to de-repress expression from the AOX1-promoter. Subsequently, the culture is adapted to methanol and the expression of the recombinant protein is carried out in a methanol fed-batch phase, in which the methanol feed rate is set to a fixed rate based on the estimation of methanol consumption and evaporation.

Information on the actual methanol concentration is only obtained through „dO2 spikes", sharp rises in the dissolved oxygen concentration, occurring when the carbon source is entirely depleted. Therefore, adjusting the methanol feed rate to maintain inducing methanol concentrations while at the same time not allowing toxic concentrations of methanol to accumulate, becomes a task requiring much experience and more or less constant supervision by the operator. Even then, in cases where the dO2 spike method does not work, fermentations may give unsatisfactory results in terms of expression levels due to poor induction or methanol accumulation.

The importance of the constant presence of inducing levels of methanol in Pichia expression was shown by Guarna *et al.* (1997), who could improve the shake-flask expression yields by a factor of five by maintaining a constant concentration of 0.3 % (v/v) methanol in shake flasks instead of feeding 0.5 % (v/v) methanol at 24 h intervals. Stratton *et al.* (1998) have also pointed out that the maintenance of a constant above-limiting concentration of methanol is important.

Here, we present the use of the commercially available alcohol sensor ALKOSENS to on-line monitor and control methanol concentrations during the methanol fed-batch phase of *Pichia pastoris* fermentations.

Methanol sensor technology:

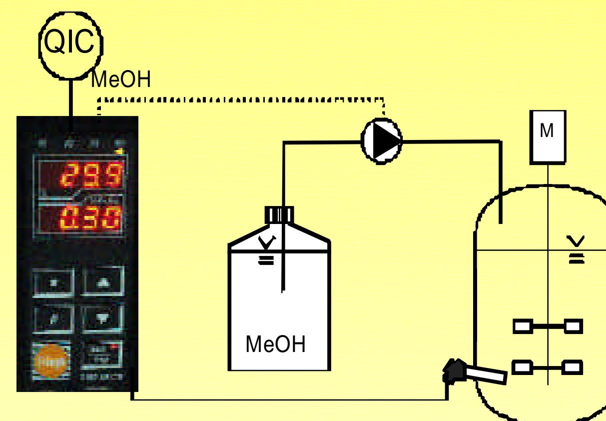


Fig. 1: Schematic function diagram of the ALKOSENS methanol sensor and control unit

The ALKOSENS methanol sensor and control unit is commercially available from Heinrich Frings, Bonn, Germany. The in-situ sterilizable probe fits a standard 27 mm Ingold port. Pressurized air passes through a channel separated from the fermentation broth by a silicone membrane, through which methanol can diffuse. The methanol-enriched carrier gas is then forced through a gas sensor, in which methanol is oxidized, thus lowering the resistance of the semiconductor gas sensor. The decrease in resistance is proportional to the concentration of methanol in the fermentation broth. The methanol feed pump is activated by the control unit when the methanol concentration falls below the setpoint.

Results and discussion

A *Pichia pastoris* strain secreting a single-chain Fv antibody fragment (generated by transforming the host strain GS115 with the expression vector pPIC9K (Invitrogen, San Diego, CA, USA), mut+) was subjected to fermentation in a 30-L working volume stirred tank reactor (Biopilot 40, Applikon Biotek, Remsfeld, Germany).

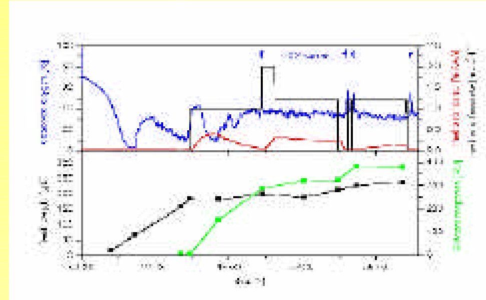


Fig. 2: Standard fermentation of *P. pastoris*.
A: The methanol concentration (red) was monitored on-line, but the methanol feed rate (black) was adjusted according only to the information obtained from the dissolved oxygen level (blue). B: Fresh weight (black) and product formation (green) were determined off-line.

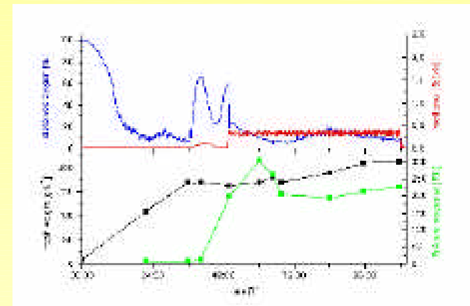


Fig. 3: Fermentation using on-line methanol control.
A: The end of the glycerol batch phase and the duration of the adaption phase, in which methanol was fed at a fixed rate, was determined according to the dissolved oxygen level (blue). During the induction the methanol concentration was maintained constant at 0.3 % (v/v). B: Fresh weight (black) and product formation (green) were determined off-line.

Fig. 2 shows a fermentation carried out according to the strategy employed, when on-line information on the methanol concentration is not available: 28.5 L of a basal salts medium containing 5 % (v/v) of glycerol were inoculated with 5 % (v/v) of an overnight culture of *Pichia pastoris* in buffered glycerol complex medium (BMMY). 25 % (v/v) ammonium hydroxide was used to control the pH at 6.0 and as nitrogen source. Fresh weight was determined gravimetrically. The accumulation of the product scFv was monitored by surface plasmon resonance analysis using a BIAcore 2000 system in combination with a Ni-NTA sensor chip (BIAcore, Uppsala, Sweden). The aeration rate was 0.25 vvm for the first 17 hours and 1 vvm for the remaining fermentation time.

At the end of the glycerol batch phase, methanol was fed at a fixed feed rate of 1 $mL \cdot L^{-1} \cdot h^{-1}$ in order to allow the cells to adapt to methanol. The end of the adaption phase was indicated by a dO2 spike.

During the induction phase, the feed rate was adjusted according only to experience from previous fermentations and information obtained through dO2 spiking. The actual methanol concentrations resulting from this strategy were monitored, but not controlled using the ALKOSENS and are shown in fig. 2: Although great attention was paid to adjusting the feed rate, the concentrations still varied between 0 and 0.5 % (v/v) in the course of the induction phase.

The fermentation shown in fig. 3 was conducted identically to the one described before until the end of the adaption phase. From this point, the methanol level was controlled automatically: As soon as the concentration fell below the setpoint at 0.3 % (v/v), a fixed volume of 30 mL of methanol (0.1 % (v/v)) was added to the fermenter. As a consequence, the methanol concentration was maintained constant in the range of 0.3 to 0.4 % over a period of 60 hours. The methanol consumption during the period of automatically controlled feed was 1.65 to 1.85 $mL \cdot L^{-1} \cdot h^{-1}$.

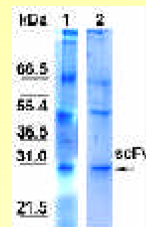


Fig. 4: Coomassie-stained SDS-PAGE of the harvest samples.
1: standard feed strategy
2: on-line methanol control
30 l of clarified supernatant were loaded to each slot.

In this fermentation, the product concentration decreased considerably between 60 and 68 h. This was not observed in other fermentations in the same reactor using an identical methanol control setup and may be due to the fact that the dissolved oxygen concentration was below 20 % in this fermentation. In general, we have observed the same or higher final product levels in on-line controlled fermentations.

Comparison of the coomassie stained SDS-PAGE of the harvest samples showed that in both fermentations the product scFv was one of the major bands in the culture supernatant. The concentration was estimated to be in the range of 35 to 45 mg/L or a total of approximately 850 mg of scFv per fermentation.

Conclusions

The production of recombinant proteins expressed in *Pichia pastoris* under the control of the AOX1 promotor by fermentation involves a methanol fed-batch induction phase, in which the methanol concentration must be maintained at levels high enough to induce expression of the recombinant protein, but low enough to avoid toxic effects. The standard fermentation strategy relies on the "dO2 spike" method to adjust the methanol feed rate. As a result, it is impossible to maintain a constant concentration of methanol which is not only the sole carbon source, but also the inducing agent. Consequently, this fermentation strategy requires considerable experience and intense supervision to avoid either suboptimal growth and induction or methanol accumulation. Additionally, the nonavailability of on-line information on this key substrate is unsatisfactory regarding the fermentation documentation, which is essential in the production of proteins under cGMP conditions.

We have used a commercially available on-line methanol sensor to monitor the methanol concentration in a fermentation conducted according to the standard fermentation strategy. In a second fermentation run the methanol concentration was kept constant within a window of 0.3 - 0.4 % (v/v) throughout the entire induction phase.

The comparison of accumulated product concentrations in the fermentation supernatant show that the on-line methanol control may not be indispensable to achieve satisfactory productivities. However, it is clearly demonstrated that it facilitates better control and documentation of the fermentation.

Moreover, with on-line methanol control available, we will now be able to investigate more sophisticated fermentation strategies like advanced continuous or mixed glyerol/methanol feed fermentations.

References

Guarna, M. M., Lesnicki, G J., Tam, B. M., Robinson, J., Radziminski, C. Z., Hasenwinkle, D., Boraston, A., Jervis, E., MacGillivray, R. T. A., Turner, R. F. B. Kilburn, D. G (1997): On-Line Monitoring and Control of Methanol Concentration in Shake-Flask Cultures of *Pichia pastoris*. Biotechnol. Bioeng. **56**: 279-286

Stratton, J., Chiruvolu, V., and Meagher, M (1998): High-cell-density fermentation. *In: Higgins, D. R. and Cregg, J. M. (eds.): Methods of Molecular Biology: Pichia protocols.* Humana press, Totowa, NJ.: 107-121

Business contacts

ALKOSENS distributors:
Heinrich Frings GmbH und Co. KG, Bio- und Chemietechnik, Jonas-Cahn-Straße 9, D-53115 Bonn, Phone: 0228-9833-0, Fax: 0228-9833-195, email: frings-biotec@t-online.de
Biotechnologie Kempe GmbH, Lehrter Str. 16-17, 10557 Berlin, Phone: 030-3949744 Fax: 030-394-9825

5 or 30-L fermentations of *Pichia pastoris*: Dr. Rainer Fischer, Institut für Biologie I (Botanik, Molekulargenetik), Worringerweg 1, 52074 Aachen, Phone: 0241-80-6631, Fax: 0241-871062, email: fischer@bio1-rwth-aachen.de