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EVALUATION OF THREE METHANOL FEED STRATEGIES FOR RECOMBINANT *PICHIA PASTORIS* MUT^S FERMENTATION

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Methylotrophic yeast *Pichia pastoris* is an ideal host organism for recombinant protein production. However, adequate methanol feed is still a critical point of successful product formation in *P. pastoris* Mut^S fermentations.

Three methanol feed strategies were tested: an organic vapor sensor, a dissolved oxygen controlled methanol addition and a pre-determined model, as well.

The organic vapor sensor proved to be unsuitable for methanol concentration measurements when samples were taken from the head space of the bioreactor, but may have the potential to substitute expensive gas analyzers in methanol fed-batch with a suitable selector submerged into the fermentation media.

Dissolved oxygen and substrate consumption did not show strict mathematical relation. However, drop of dissolved oxygen tension for periodic methanol addition may be applied for the determination of the substrate concentration in the media. The rate of methanol consumption shows peaks at 0.45 and 0.95% (v/v) substrate concentrations.

The rate of the specific methanol consumption of our model organism was determined. Based on the value of 0.023 h^{-1}, which is significantly less than suggested by earlier experiments, a successful pre-determined methanol feed strategy was set up.

Keywords: methanol, fermentation, control, *P. pastoris*

The methylotrophic yeast, *Pichia pastoris* has great potential in recombinant protein production (BUCKHOLZ & GLEESON, 1991). Recombinant *Pichia* has been reported to express several proteins from various host organisms at high level, such as HIV-1 envelope glycoprotein, hirudin or human tissue factor (SCORER et al., 1993; ROSENFELD et al., 1996; AUSTIN et al., 1998). Most of the proteins up to 90 kDa are

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expressed extracellularly and give the majority (>90%) of the protein content of the cell-free media, which simplifies the purification procedure (CREGG et al., 1993). Unlike *Saccharomyces cerevisiae*, *P. pastoris* lacks α1,3-glycosidase activity thus does not hyperglycolisate the asparagine residues of proteins (GRINNA & TSCHOPP, 1989; MIELE et al., 1997). Furthermore, recombinant products from *P. pastoris* appear to keep the wild-type disulfide forms (IKEGAYA et al., 1997). These features result in mammal-like glycoprotein structure with full enzyme functions and potentially no immune reaction in humans (BIEMANS et al., 1998; OHTANI et al., 1998).

P. pastoris has two alcohol-oxidase genes, AOX1 and AOX2, however the latter gives only the 10% of its alcohol-oxidase activity (CREGG et al., 1989). Both genes are under glucose and glycerol initiated catabolic repression and are activated by methanol. Recombinant protein genes are cloned into the AOX1 gene (type Mut^S) or into the HIS4 gene of *P. pastoris* under the control of an AOX1 promoter (type Mut^+). Mut^+ grows faster on methanol. However, in many cases Mut^S *Pichia* showed higher functional protein production than Mut^+ (LOEWEN et al., 1997). Since Mut^S consumes methanol with extremely slow rate and is sensitive to methanol accumulation in the media, the appropriate control of methanol feed is of stringent necessity.

So far two distinct methods have been applied for methanol feed control in recombinant *Pichia* Mut^S fermentations. The simpler method works with a pre-determined methanol-feed strategy (Easy Select Pichia Expression Kit; CHEN et al., 1996). The second one includes a gas analyzer connected to the headspace of the bio-reactor to measure the methanol concentration on-line. A major drawback of this kind of methanol concentration measurement is the high cost of the analytical instrument. Thus attempts have been made to substitute the gas analyzer with less expensive units such as organic vapor sensors (GUARNA et al., 1997).

A third method to control methanol feed can be measuring a parameter, which depends on the methanol concentration in the media. Simple on-line computer control strategy for methanol feed, based on dissolved oxygen level readings has been performed with other methylotrophic species (RENARD et al., 1985). Similar strategy seems to be applicable for *P. pastoris* Mut^S strains, although only a single report has been published to our knowledge (RODRÍGUEZ HIMÉNEZ et al., 1997). Even in this case, the dissolved oxygen control was combined with a pre-determined methanol-feed strategy.

The purpose of our work was to establish the potentials of three different methanol-feed control strategies in the case of a human serum albumin producing Mut^S type *P. pastoris* as a model organism: a pre-determined, a dissolved oxygen controlled as well as an organic vapor sensor controlled methanol addition.

1. Materials and methods

1.1. Microorganism

Pichia pastoris GS115 Mut^S $His4^+$ with a single copy of human serum albumin V fraction (HSA) gene downstream of its AOX1 promoter (Invitrogen, USA) was maintained on YEPD agar slant (2% D-glucose, 2% peptone, 1%yeast extract {all media recipes are from Easy Select Pichia Expression Kit, (CHEN et al., 1996)}).

1.2. Media and cultivation parameters

One point two and 1.5 l stirred tank-reactors (Biostat Q and M, respectively, B. Braun, Germany) with B. Braun CSFV2 acquisition and control program was used in the experiments. In the 1.2 l reactor, the agitation speed was 1000 min^{-1} (magnetic stirrer of triangle-shaped cross-section), whereas it was 800 min^{-1} in the 1.5 l fermentor (flat blade turbine impeller). The 1.5 l bio-reactor was used for methanol-sensor experiments, while 1.2 l reactors were used for all other fermentations. Constant 1 l min^{-1} airflow was applied. pH control was applied in the methanol-fed phases. The temperature of the fermentations was 30 °C.

Seventy ml of a 24 h-old inoculum (BMGY media: 1% glycerol, 2% peptone, 1% yeast extract, 1.34% yeast nitrogen base, 0.00004% biotin, 0.1 mol l^{-1} KH_2PO_4/K_2HPO_4 at pH 6.0; 28 °C, 280 r.p.m.) was used to inoculate the 800 ml final volume fermentors resulting in a 1 g l^{-1} dry weight initial cell concentration (SIEGEL & BRIERLEY, 1989). The pH of the fermentation media (4% glycerol, 2.67% cc. H_3PO_4, 1.82 g l^{-1} K_2SO_4, 1.49 g l^{-1} $MgSO_4.7H_2O$, 0.413 g l^{-1} KOH, 0.093 g l^{-1} $CaSO_4$) was set to 5.0 prior to inoculation. The glycerol batch phase lasted for 16–24 h and its end was indicated by the raise of dissolved oxygen tension (DO). As the DO reached 85%, the methanol feed was started (100% methanol with 12 ml l^{-1} PTM_1 solution (g l^{-1}): 65.0 $FeSO_4.7H_2O$, 20.0 $ZnCl_2$, 6.0 $CuSO_4$. $5H_2O$, 3.0 $MnSO_4$. H_2O, 0.5 $CoCl_2$. $6H_2O$, 0.2 $NaMoO_4$. $2H_2O$, 0.2 biotin, 0.08 NaI, 0.02 H_3BO_4, 5 ml l^{-1} H_2SO_4). The methanol was added periodically by sterile syringes through a silicone membrane or continuously by infusion pumps (MTA KUTESZ, Hungary). In the methanol-phase, the pH was maintained at pH 3.0 or 5.2.

1.3. Assays

Cell concentration was determined by optical density (at 600 nm, 1 cm light path, in appropriate dilution; Ultrospec Plus, Pharmacia LKB, Sweden) and by dry weight measurements (filtration on Millipore 0.42 μm membrane, dried at 105 °C for 5 h). Glycerol concentration was determined by iodometry (HORWITZ et al., 1975), methanol was measured by gas chromatography (Laboratorni Pristoje, Czech R.). Cell-free

filtrated supernatant from fermentation media was used for total protein measurements and electrophoresis. The samples were stored at –20 °C in a buffer with 0.37 g l^{-1} Na_2EDTA, 1.21 g l^{-1} TrisHCl final concentration, at pH 8.0. Total protein concentration was determined by bicinchoninic acid reagent (Pierce, USA) or by the coumassie blue method. SDS electrophoresis was used to analyze the protein content of cell-free samples with gradient pre-fabricated poly-acrylamide gels (Pharmacia Phast System, Sweden, Phast Gel Gradient 8–25, Phast Gel SDS Buffer Strips). The gels were developed by silver staining, which has the capability to detect 5 μg protein per band as a minimal concentration. Crystallized HSA V (Sigma, USA) was used as positive standard for electrophoresis in 200 μg ml^{-1} solution. The scanned pictures of the gels were evaluated by Kodak Digital Science 1D Image Analysis Software. The set-up of the semiconductor on-line methanol sensor experiments with SnO_2 organic vapor sensor (Figaro Engineering Inc., Japan), air pump (KNF Neuberger, Germany), heating ribbon (Barnstead/Thermoline, USA) and recorder (Radelkis, Hungary) is described in the experimental part.

2. Results and discussion

2.1. Test of semi-conductor organic vapor sensor for P. pastoris *fermentation*

To improve recombinant *P. pastoris* AOX1 fermentation, three strategies for methanol feed control were tested. As a first approach, we used a semi-conductor methanol-sensor to measure the methanol concentration on-line during the fermentation (Fig. 1). The exhaust-air was connected to the methanol sensor through an oil-free air pump. The air-pump forwarded a constant air-flow through the methanol-sensor.

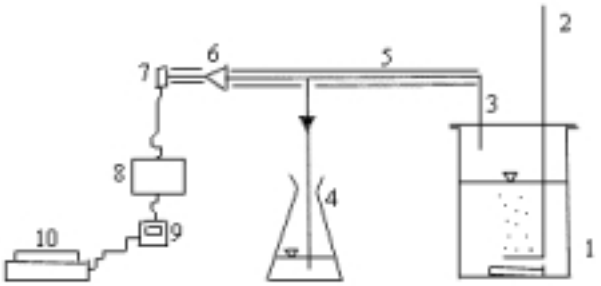

Fig. 1. Experimental set-up with semi-conductor methanol sensor. 1: fermentor; 2: air supply; 3: exhaust air; 4: extra air outlet; 5: heating ribbon; 6: air pump; 7: methanol sensor; 8: amplifier; 9: digital multimeter; 10: recorder

The extra air was led into a water bath. An adjustable heating ribbon ensured that the exhaust-air would be at 160 °C. The tubing between the fermentor and the sensor was as short as possible and composed of glass. An amplifier received the signal from the sensor. The amplifier had a built-in voltamater to set the zero level at the beginning of the fermentation. The amplifier was connected to a recorder and a multimeter, which showed the signal in mV.

To establish the capabilities of the sensor, its characteristics were determined in a water/methanol model system. We added the methanol to the water stepwise, increasing the concentration from 0 to 1.1% (v/v). We always waited with the following methanol addition until stable electrode-signal was given, which took 10–20 min. The semi-conductor methanol sensor gave similar results in our model system than in earlier experiments (AUSTIN et al., 1992). Figure 2 shows both the measured and the calculated values from a hyperbolic fit.

The sensor was used during a 1.5 l fermentation (Fig. 3). The measured values do not show accordance with the points calculated from the hyperbolic equation gained in the model system. Moreover, there could be no significant mathematical connection set up between the methanol concentration and the signal of the sensor. Also, the signal/noise ratio was large at higher measured voltages probably due to the low specificity of the sensor (data not shown). An additional problem was that the volume of the fermentation media decreased almost to its half during the 6-day-long fermentation, since the exhaust cooler had to be turned off.

To decrease disturbances and volume loss, a submerged unit was applied. The stainless steel unit had a 2 cm^2 silicon membrane surface submerged into the fermentation media. A stable oil-free air-flow washed the membrane's inner surface axially, then the air was led to the sensor. This set-up could not ensure sufficiently high methanol concentrations for the sensor to detect. The two different experimental set-ups showed that this kind of sensor can not be used for on-line methanol measurements during fermentation without a suitable selector for methanol.

2.2. Dissolved oxygen as a possible factor to control methanol feed in recombinant P. pastoris *fermentation*

A second way to control the methanol feed during recombinant *Pichia* fermentation can be measuring a parameter, which depends on the methanol concentration of the fermentation media and/or the activity of the yeast's alcohol-oxidase system. The most common parameters of this kind are the acid releasing rate of the microbe and the dissolved oxygen level in the fermentation media.

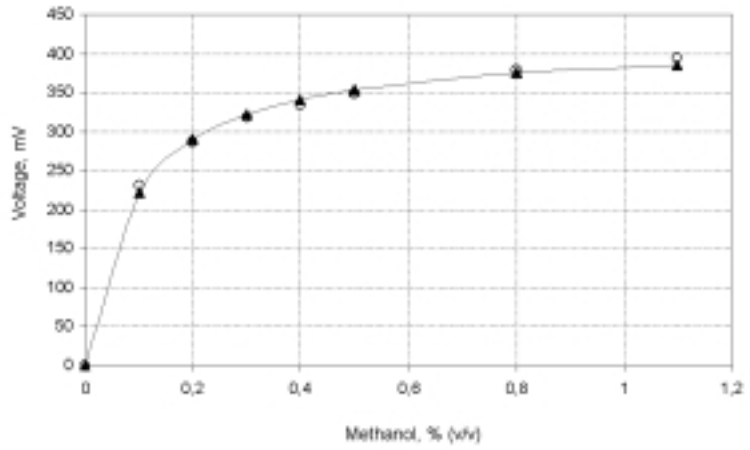

Fig. 2. Test of methanol sensor in methanol/water model-system. O: measured output signal from amplifier (mV); ▲: 415.43c/(0.09+c) R=0.9976; c: methanol concentration (%, v/v)

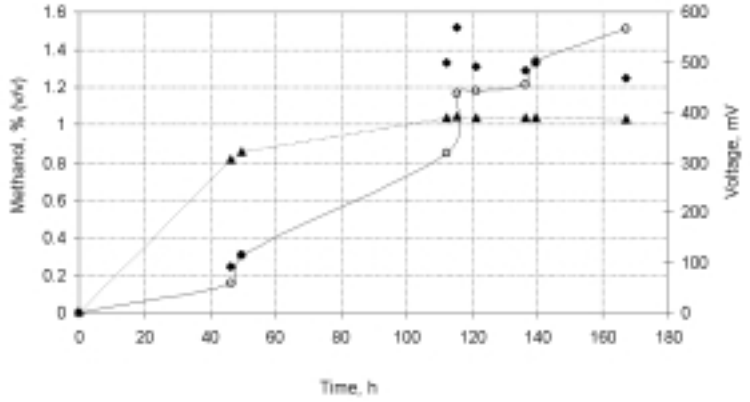

Fig. 3. Test of methanol sensor during 1.5 l fermentation. ◆: methanol concentration in media (%, v/v); O: measured output signal from amplifier (mV); ▲: 415.43c/(0.09+c); c: methanol concentration (%, v/v)

However, the pH does not change during the methanol-fed phase of *P. pastoris* fermentation, in contrast to the glycerol-phase, in which the pH decreases from 6.0 to 2.8. For this reason the dissolved oxygen level seemed to be the sole measured parameter other than the methanol, suitable to control the substrate feed during the product formation phase.

The oxygen utilization is more directly connected to the activity of the alcohol-oxydase system, since methanol is the sole carbon source during product formation. The activity of the alcohol-oxydase system depends on the methanol concentration in the media. Also, the cell mass determines the amount of catabolized oxygen. The dissolved oxygen level of the media divided by the dry cell mass can show the activity of the alcohol-oxidase 2 system, if constant aeration is applied. Nevertheless a major problem is that the *P. pastoris* Mut^S can utilize methanol only at an extremely slow rate and changes during the slow substrate consumption may not be noticed on the bases of dissolved oxygen level. To test, if dissolved oxygen is suitable to control methanol feed, first we measured how the alcohol utilization of the recombinant yeast depends on the methanol level in the media. We also determined if the dissolved oxygen level reflects the substrate utilization by the recombinant yeast. Finally, the influence of the methanol concentration on the dissolved oxygen level was measured.

The methanol concentration in the media was measured by offline gas-chromatography. The methanol consumption was counted as the difference of the methanol content of two following samples corrected with the amount of substrate supplied in this time interval. Unfortunately the rate of methanol consumption was commensurable to the methanol taken by the exhaust air flow, thus we had to estimate the methanol concentration decrease caused by the aeration. In our model, the methanol, leaving through the surface of the media in the bio-reactor was neglected. Only the mass transfer from the liquid phase into the air-bubbles was taken into consideration. A simple equilibrium between the two phases was supposed:

$$c_{liquid} \xleftrightarrow{K_I} c_{gas} \qquad K_I = \frac{c_{gas}}{c_{liquid}}$$

$$c_{gas} = K_I \cdot c_{liquid} \qquad \frac{dc_{liquid}}{dt} = -f \cdot c_{gas} = -f \cdot K_I \cdot c_{liquid}$$

$$\int_{c_{liquid,1}}^{c_{liquid,2}} \frac{1}{c_{liquid}} dc_{liquid} = -\int_0^t f \cdot K_I dt$$

$$c_{liquid,2} = c_{liquid,1} \cdot \exp(-f \cdot K_I \cdot t) = a \cdot \exp(-b \cdot t) \qquad \text{(I)}$$

where

K_I – equlibrium constant between gas and liquid phase
c_{gas}, c_{liquid} – methanol concentration in gas and liquid phase
t – time
f – air flow (constant)
a, b – constants

Based on equation I, the methanol concentration in the media can be defined as an exponential function of the time. This description shows a good correspondence with the values which we gained in a model experiment using a fermentation media without cells and glycerol (Fig. 4). Thus a correction can be taken into account on the basis of this model when calculating methanol consumption rates of *P. pastoris*.

Examining the methanol consumption by unit amount of biomass and the dissolved oxygen as the function of methanol concentration in the media at different fermentations, no strict correspondence was found between them (Fig. 5). However, the alcohol-oxydase system is virtually activated from 0.3% (v/v) of methanol, with a putative local methanol consumption maximum around 0.45% (v/v) substrate and a peak between 0.8–1.1% (v/v). This assumption is supported by earlier data.

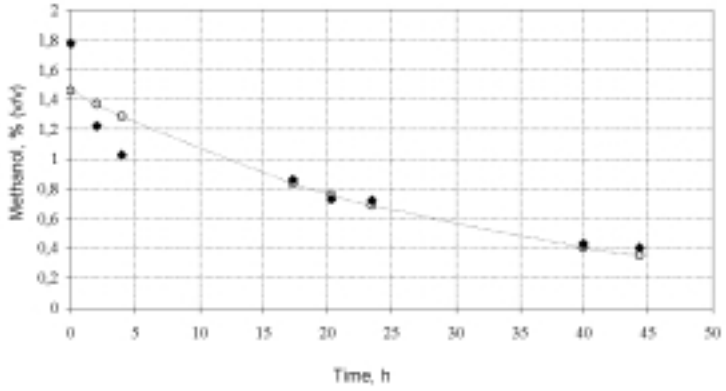

Fig. 4. Model experiment to determine the methanol concentration change in the media caused by constant air-flow. ◆: measured methanol concentration in media (%, v/v); O: 1.457 exp (–0.0321t), R=0.9322, t: time

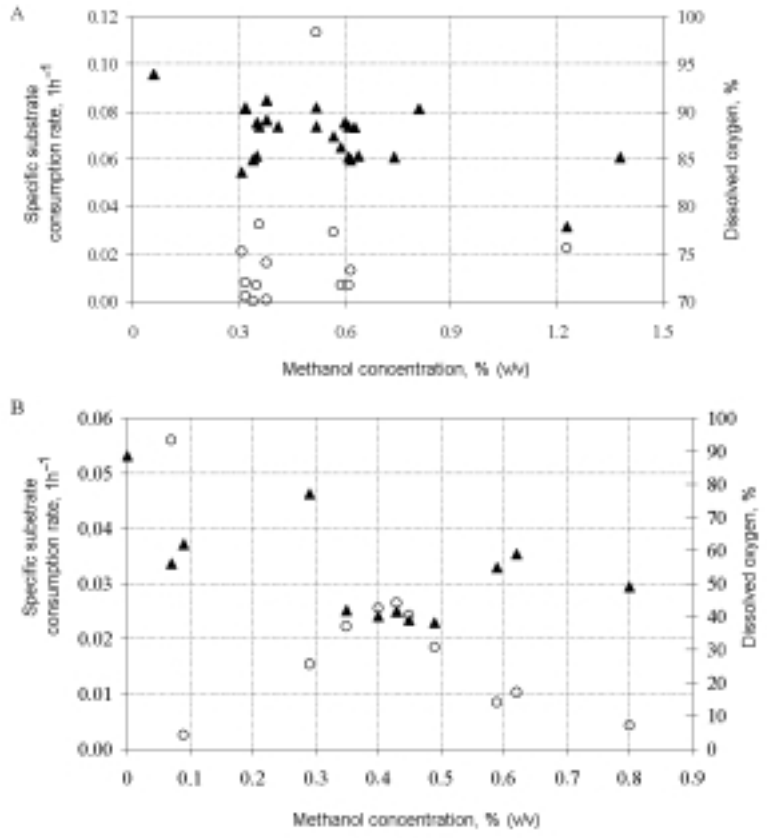

Fig. 5. Specific substrate consumption and dissolved oxigen levels at two different fermentations (A and B) as the functions of methanol concentration in the media. ○: specific substrate consumption rate [g methanol (g cell dry weight)$^{-1}$ h^{-1} = h^{-1}]; ▲: dissolved oxygen level in the media (%)

We also examined, wheather the drop of dissolved oxygen level for periodic methanol addition reflects the methanol concentration in the fermentation media. It was supposed that the dissolved oxygen level alters the methanol concentration and the methanol concentration change in the media, since both of these influence the metabolic activity of the cells. Indeed, a maximum seemed to describe the dissolved oxygen drop as the function of the methanol concentration change/methanol concentration in the media (Fig. 6). The change of substrate concentration for periodic methanol addition can be counted from the amount of methanol added to the fermentor divided by the fermentor volume. This way, the actual methanol concentration can be determined from

the oxygen level drop, using the polynomial fit from Fig. 6. The problem with the equation is that it describes only dissolved oxygen changes lower than 9% and applies only to the 0.4–0.85% (v/v) methanol concentration range. Also, the changes of dissolved oxygen for the same methanol concentration change/methanol values showed differences with the age of the microbes in the product forming phase.

2.3. A pre-determined model based on methanol consumption measurements

Pre-determined methanol feed is widely used in recombinant *P. pastoris* fermentations. However, the most accepted substrate feed strategy, suggested by the developer of the Pichia Expression Systems proved to be unproper in case of our Mut^S HSA strain. Gradually increasing the continuous methanol feed rate from 1 to 3 ml l^{-1} h^{-1} and keeping this value during the rest of the fermentation proccess at around 200 g l^{-1} wet weight of cells resulted in undesired methanol accumulation in the media (data not shown). Furthermore HSA production was not observed in this fermentation. The calculated specific methanol consumption in our fermentations varied between 0.016 and 0.032 h^{-1} compared to the 0.056 h^{-1} dry weight suggested by Invitrogen manuals. Since limiting substrate concentration should be applied, when the alcohol consumption is relatively low, we suggest to count with 0.023 h^{-1} specific methanol consumption. To test if this methanol feed was adequate for recombinant protein production, we applied higher and lower continuous feed as well.

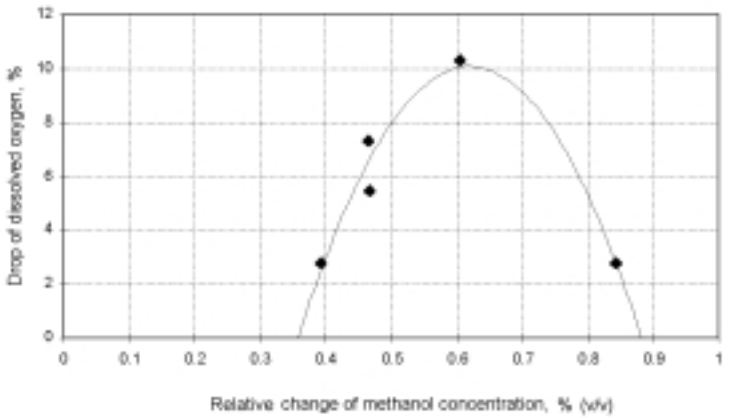

Fig. 6. Correlation between the drop of dissolved oxygen for periodic substrate addition and the relative change of methanol concentration (change of methanol concentration for substrate addition/methanol concentration after the addition). ◆: drop of dissolved oxygen; –: $y=-149x^2+181.65x-46.20$, $R^2=0.9451$ where y: dissolved oxygen, x: relative change of methanol concentration

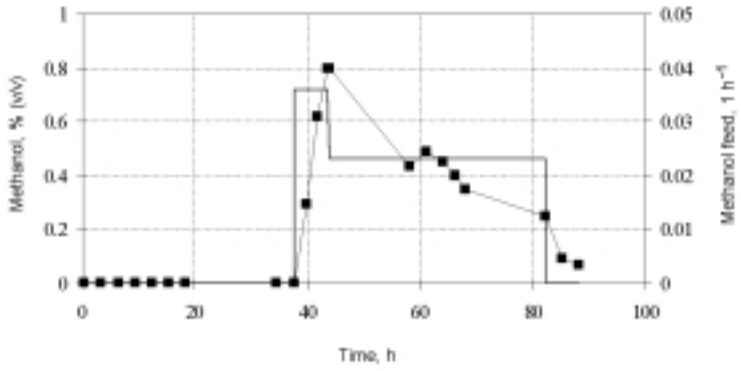

Fig. 7. Pre-determined methanol feed in *P. pastroris* Mut^S fermentation, based on substrate consumption measurements. ■: methanol concentration (%, v/v); –: specific methanol addition (h^{-1})

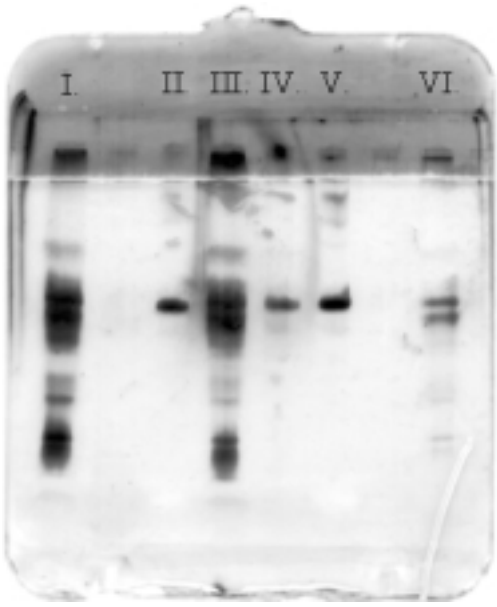

Fig. 8. Electrophoresis of samples from the fermentation with 0.023 h^{-1} pre-determined specific methanol addition. IV: 68th h of fermentation (149 mg l^{-1} HSA); VI: 90th h of fermentation (89 mg l^{-1} intact HSA); II, V: HSA standards (200 mg l^{-1}); I, III: samples condensed by ultrafiltration

After the adaptation period, the methanol feed was set to 0.036 h^{-1} which resulted in a substrate concentration increase to 0.8% in 6 h (Fig. 7). Then the methanol feed was reduced to 0.023 h^{-1}. Keeping this substrate feed, the methanol concentration stayed in the substrate limiting range in a 30-h-long period. Finally, turning off the methanol supply caused the rapid decrease of the alcohol concentration. The produced HSA concentration in the supernatant of the media peaked at the end of the 0.023 h^{-1} substrate feed period (70th h) with 149 mg l^{-1} HSA (Fig. 8). After the substrate supply was terminated, proteolytic degradation occured, decreasing the HSA concentration, showed by a second band on the gel. When 50% lower methanol supply was applied, after an initiative methanol concentration peak of 0.37%, the substrate concentration decreased below 0.1% and no HSA production was observed.

3. Conclusion

The organic vapor sensor applied has the potential to provide methanol concentration data during the fermentation of recombinant *P. pastoris*. However, a suitable selector for methanol with large contact surface submerged into the media is still required.

Dissolved oxygen level is not proper to control methanol feed, but drops in dissolved oxygen level for periodic methanol addition seem to correlate with methanol concentration in the media. This correlation is not strict enough to be used succesfully in *P. pastoris* Mut^S fermentations, but may be useful for *P. pastoris* Mut^+ cultivations.

A 0.023 h^{-1} specific methanol consumption rate was determined, which greatly differs from the 0.056 h^{-1} value suggested by manuals. Based on this specific substrate consumption value, a pre-determined methanol-feed strategy was tested and proved to be applicable for HSA producing *P. pastoris* Mut^S.

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