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Development of a responsive methanol sensor and its application in *Pichia pastoris* fermentation

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Abstract

A methanol sensor was developed with response time less than 2 min. It was unaffected by the dissolved O_2 concentration, agitation speed or pH value. When the sensor was used to monitor the methanol concentration on-line during hirudin production by recombinant *Pichia pastoris*, the cell dry weight was up to 155 g l^{-1}, and hirudin was 1.4 g l^{-1}.

Introduction

The methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha*, are outstanding eukaryotic hosts for heterologous gene expression (Sreekrishna *et al.* 1997, Cereghino & Cregg 2000).

Methanol is usually used as both inducer for the expression of heterologous protein and as carbon source for growth during the production phase of *Pichia pastoris* cultures. Methanol must kept below 10 g l^{-1}, otherwise growth of the yeast will be inhibited. However, it is difficult to feed methanol continuously and to maintain its concentration at a fixed level. In addition, *Pichia pastoris* Mut^+ strains are extremely sensitive to transient high methanol concentrations. Sudden changes in methanol concentrations often result in loss of alcohol oxidase activity and even cell death (Chiruvolu *et al.* 1997).

Several methods have been used to detect and control methanol concentration on-line in fermentation (Guarna *et al.* 1997, Wagner *et al.* 1997, Katakura *et al.* 1998). Among these methods, the silicone tubing sensor is simple and effective. The sensor is immersed in the liquid phase while the lumen of the sensor is continuously perfused with air. Methanol dissolved in the culture medium diffuses across the silicone tubing to the lumen of the sensor and the vapor phase of methanol in the lumen is carried by the flowing stream of air to a SnO_2 sensor. The SnO_2 sensor is sensitive to alcohols and other organic solvents by a resistance drop, which is measured by avometer. The response time of this method is usually more than 5 min. In this paper, an improved methanol sensor has been developed to reach a response time of less than 2 min and can be used for the rapid monitoring of the methanol concentration in the fermentation of *Pichia pastoris.*

Materials and methods

Organism

The synthetic gene of hirudin was designed according to the known amino acid sequence of hirudin variant HV2. The gene was then cloned into the *Bam*HI and *Sal*I sites of plasmid pPIC9K (Invitrogen, San Diego, CA). The 5′ terminus of the hirudin gene insert was designed such that the *Saccharomyces cerevisiae* pre-pro α-mating factor signal sequence cleavage site (Lys-Arg) was introduced in-frame with the hirudin coding sequence. The HV2 construct was transformed into *Pichia pastoris* GS115 by electroporation and high producers were selected as described by Sreekrishna & Kropp (1996). A strain with the phenotype GS115

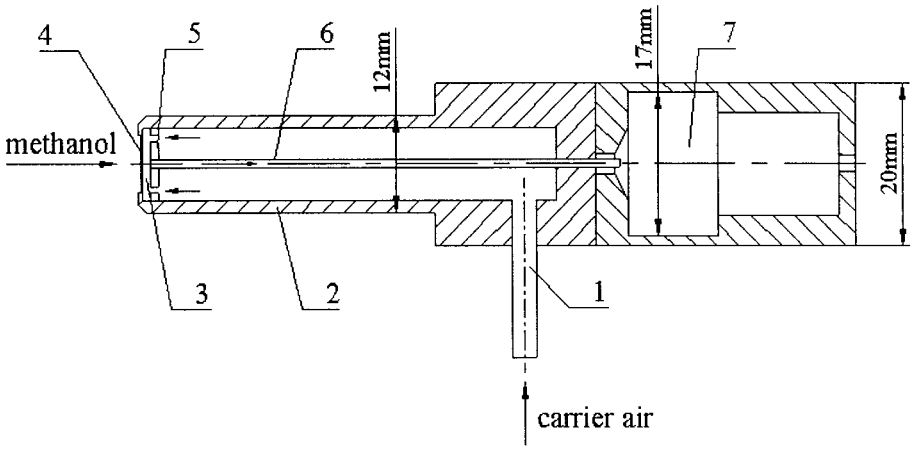

Fig. 1. Schematic diagram of methanol sensor. (1) Carrier air inlet; (2) shell; (3) mixing lumen; (4) semipermeable membrane; (5) sieve plate; (6) center airway; (7) SnO_2 sensor.

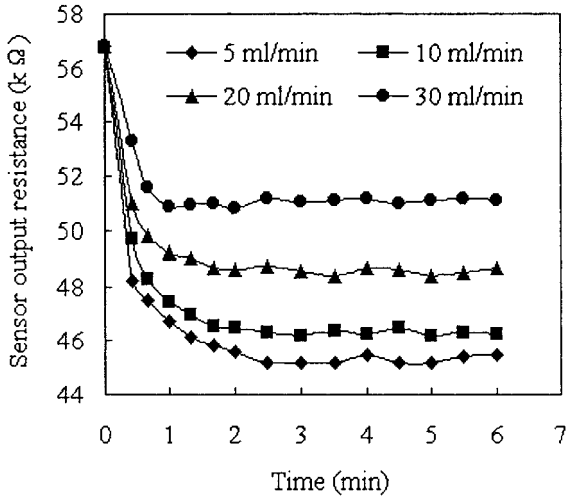

Fig. 2. Time course of sensor output resistance with different carry air flow rate when the methanol concentration was 1 g l^{-1} in a 5-l fermenter.

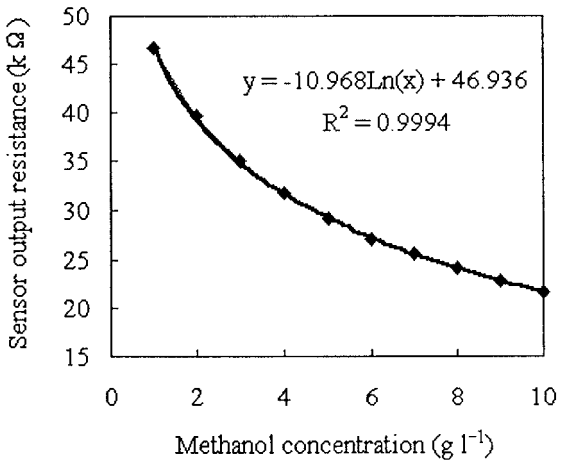

Fig. 3. Sensor output resistance in different methanol concentrations with a carrier air flow rate of 10 ml min^{-1}.

His^+ Mut^+ showing the highest expression level was chosen.

Fermentation system

Fermentation medium and preparation protocol were as those described by Sreekrishna & Kropp (1996). *Pichia pastoris* was inoculated into a 5 l fermenter containing 2.5 l basal salts medium (BSM), which, per liter, consisted of 26.7 ml 85% (w/v) H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, 40 g glycerol and 4.35 ml PTM1 trace salts (containing, per liter, 6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 5 ml concentrated H_2SO_4). The temperature, pH, and dissolved O_2 (DO) concentration were controlled at 30 °C, 5 (with 15 M NH_4OH), and $\geqslant$30% respectively. After glycerol was exhausted (identified by a rapid increase in DO), a 50% (w/v) glycerol solution (containing 12 ml of PTM1 per liter) was fed at a rate of 15 ml l^{-1} h^{-1} for 5 h, and then methanol (containing 12 ml of PTM1 per liter) was added.

Methanol sensor

Figure 1 shows the schematic diagram of the novel methanol sensor. The semipermeable membrane with a diameter of 8 mm and a thickness of 0.1 mm was

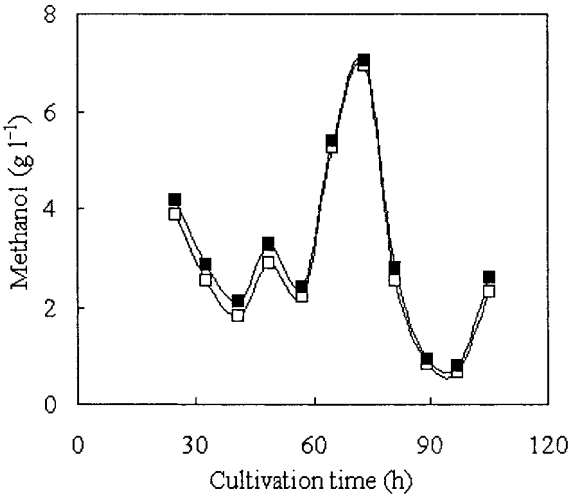

Fig. 4. Comparison of methanol concentration determined on-line by sensor (■) with those determined off-line by gas chromatography (□) in *Pichia pastoris* fermentation in a 5-l fermenter.

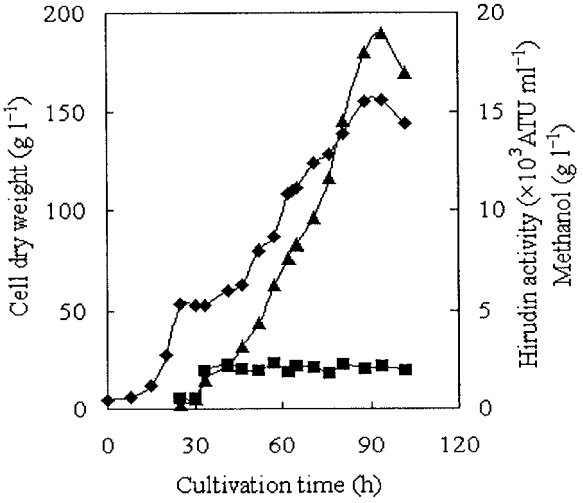

Fig. 5. Profiles of methanol concentration determined on-line by sensor (■), cell dry weight (♦) and hirudin activity (▲) during *Pichia pastoris* fermentation in a 5-l fermenter.

made from silicone. Methanol in liquid phase diffuses across the semipermeable membrane to the mixing lumen. Carrier air flows across a 8 mm-diameter sieve plate from the inlet. The pore size of the sieve mesh is 1 mm. The methanol/air mixture passes through the center airway, with a 2 mm diam lumen into the SnO_2 sensor (TGS822, Figaro Electronic Co. Ltd., Tianjin, China). The resistance output signal of sensor is detected by an avometer. As the partial pressure of methanol in the vapor phase is dependent on the methanol concentration in liquid phase, the resistance of the sensor reflects the methanol concentration in liquid phase. All experiment was performed at 30 °C.

Assays

Methanol concentration was measured off-line by gas chromatography. The cell dry weight was determined gravimetrically on cells that had been washed and then dried at 80 °C for 24 h. The hirudin activity in culture supernatant was determined using thrombin and chromogenic substrate, chromozym TH (Rao *et al.* 1999). Hirudin activity was measured in antithrombin units (ATU) with 1 ATU unit hirudin neutralizing 1 NIH unit thrombin.

Results and discussion

Effect of carrier air flow rate on the response time of methanol sensor

When methanol was added into the fermenter at 1 g l^{-1}, the output of sensor responsed in 12 s (Figure 2). A lower carrier air flow rate yielded a larger output signal while the response time increased. When sensitivity and response time were measured simultaneously, a flow rate of 10 ml min^{-1} with response time of less than 2 min was chosen as the optimum, while the response time of the silicone tubing sensor reported earlier was more than 5 min (Guarna *et al.* 1997, Wagner *et al.* 1997).

At carrier air flow rate of 10 ml min^{-1}, the relationship between output resistance (R, kΩ) and the methanol concentration (C, g l^{-1}) can be summarized as follows:

$$R = -11 \ln(C) + 46.9,$$

where $C \geqslant 1$ (Figure 3).

Influence of DO concentration, agitation speed and pH value on the response of methanol sensor

Pure O_2 is usually aerated into a fermenter to keep the DO at its setpoint in *Pichia pastoris* fermentations. The DO concentration, however, affects the response of the SnO_2 sensor (Puhar *et al.* 1980). With our sensor, when aeration was shifted from air to pure O_2

and the DO concentration in the fermenter rose from 0.16 mM to 1.14 mM in 1.5 min, the output resistance of the sensor almost remained constant. The result indicates that the influence of DO concentration on the sensor response was negligible.

The agitation rate, varying from 200 rpm to 1000 rpm, did not affect the response of methanol sensor and the sensor response was independent of variant pH value (data not shown).

On-line monitoring of methanol concentration by sensor in hirudin fermentation by Pichia pastoris

Figure 4 shows a comparison between methanol concentration determined on-line by the sensor and those determined off-line by gas chromatography in *Pichia pastoris* fermentation. At high methanol concentration ($\geqslant$5 g l^{-1}), the results obtained by the two methods agreed well with each other. When methanol was below 5 g l^{-1}, the off-line data were 10% less than the on-line data. If sodium azide, which inhibits respiratory chain and arrests metabolism of the yeast, was added to the off-line samples, the deviation between on-line and off-line determination decreased. This demonstrated that the deviation was mainly caused by the continuous utilization of methanol by *Pichia pastoris* during sample preparation. At high methanol concentrations, the metabolism of the yeast was inhibited and the deviation was negligible.

Figure 5 shows the application of methanol sensor in hirudin fermentation by *Pichia pastoris.* Methanol was added to the medium at 25 h to induce hirudin expression. In the first 5 h of inducing, methanol concentration maintained at 0.5 g l^{-1} for the adaptation of *Pichia pastoris* to methanol. Then methanol concentration was raised and maintained at 2 g l^{-1}. The cell dry weight reached 155 g l^{-1} and hirudin activity was 1.9×10^4 ATU ml^{-1} at 94 h. The maximum of hirudin concentration in the culture supernatant was 1.4 g l^{-1} on the basis of specific activity of 1.3×10^4 ATU mg^{-1} protein (Sohn *et al.* 1995). These results suggest that methanol sensor could be used as an effective instrument to monitor methanol on-line in *Pichia pastoris* fermentation.

Conclusions

To shorten the response time of the silicone tubing sensor, a large diffusing area and a thin wall are necessary. This type of silicone tubing, though, is difficult to make. In our work, a semipermeable silicone membrane meets these requirements. With a short response time and satisfactory stability, the performance of the semipermeable silicone membrane sensor was preferable. On the other hand, the shape of the sensor was designed as those standard electrodes and was more acceptable than the silicone tubing sensor.

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