

On-Line Monitoring and Control of Methanol Concentration in Shake-Flask Cultures of *Pichia pastoris*

M. M. Guarna,¹ G. J. Lesnicki,¹ B. M. Tam,² J. Robinson,^{1,3}
C. Z. Radziminski,^{1,3} D. Hasenwinkle,¹ A. Boraston,^{1,3} E. Jervis,^{1,4}
R. T. A. MacGillivray,² R. F. B. Turner,^{1,5} D. G. Kilburn^{1,3}

¹The Biotechnology Laboratory, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada; telephone: 604-822-4182; fax: 604-822-2114; e-mail: Kilburn@unixg.ubc.ca

²Department of Biochemistry, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

³Department of Microbiology and Immunology, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

⁴Department of Chemical Engineering, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

⁵Department of Electrical Engineering, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada

Received 6 September 1996; accepted 13 March 1997

Abstract: The methylotrophic yeast *Pichia pastoris* can be used to express recombinant genes at high levels under the control of the methanol-inducible alcohol oxidase 1 (AOX1) promoter. Accurate regulation of the methanol concentration in *P. pastoris* cultures is necessary to maintain induction, while preventing accumulation of methanol to cytotoxic levels. We developed an inexpensive methanol sensor that uses a gas-permeable silicone rubber tube immersed in the culture medium and an organic solvent vapor detector. The sensor was used to monitor methanol concentration continuously throughout a fed-batch shake-flask culture of a *P. pastoris* clone producing the N-lobe of human transferrin. The sensor calibration was stable for the duration of the culture and the output signal accurately reflected the methanol concentration determined off-line by HPLC. A closed-loop control system utilizing this sensor was developed and used to maintain a 0.3% (v/v) methanol concentration in the culture. Use of this system resulted in a fivefold increase in volumetric protein productivity over levels obtained using the conventional fed-batch protocol. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* 56: 279–286, 1997.

Keywords: methanol sensor; methanol monitoring and control; methylotrophic yeast fermentation; *Pichia pastoris*; transferrin; shake-flask cultures

INTRODUCTION

Pichia pastoris has proven to be an effective host for the production of recombinant proteins (reviewed by: Buckholz and Gleeson, 1991; Cregg et al., 1993; Faber et al., 1995; Romanos, 1995; Romanos et al., 1992). This methylotrophic yeast combines several advantages of both prokaryotic

and animal cell expression systems. It is readily amenable to genetic manipulation, can be easily grown to high cell densities using minimal media, and is able to introduce some eukaryotic posttranslational modifications. *P. pastoris* is also capable of expressing heterologous genes to high levels under the control of the strong and tightly regulated alcohol oxidase 1 (AOX1) promoter (Tschopp et al., 1987). Methanol serves as the inducer and as the carbon source during the production phase of *P. pastoris* cultures. A challenge associated with methanol feeding is that the methanol concentration must be maintained within a relatively narrow range. We have observed that recombinant protein production decreases when the methanol concentration exceeds ca. 1.0% (v/v); this concentration is only ca. 0.5% (v/v) higher than that suggested in the *Pichia* Expression Kit (Invitrogen, San Diego, CA) to initiate induction.

While it is possible to monitor methanol concentration using HPLC or GC, these methods are expensive and not easily implemented on-line. Alternatively, methanol concentration can be estimated indirectly by interrupting the feed and measuring the time required for methanol to be exhausted, as indicated by a sharp increase in dissolved oxygen (i.e., “DO spike”). This approach, however, repeatedly exposes the cells to potentially noninducing levels of methanol and it is restricted to clones having a wild-type phenotype for methanol utilization (Mut⁺). Clones with a disrupted AOX1 gene, exhibiting a slow methanol utilization phenotype (Mut^S), consume methanol at a rate that is too slow for this technique to be practical. Furthermore, in shake-flask cultures, DO monitoring is difficult to implement using currently available probes.

The protocol for shake-flask cultures provided with the *Pichia* Expression Kit recommends 0.5% (v/v) methanol to

Correspondence to: D. G. Kilburn

Contract grant sponsor: The National Institutes of Health

Contract grant number: RO1 DK 21739

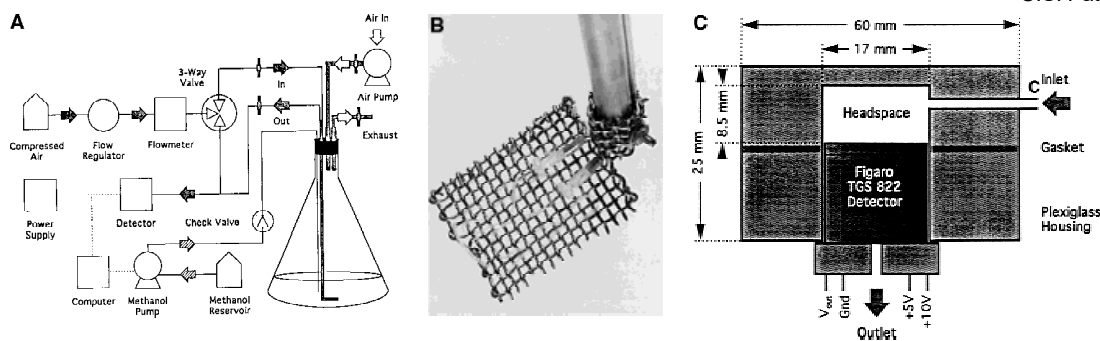


Figure 1. Methanol monitoring and control system for shake-flask cultivations of *Pichia pastoris*. (A) Schematic diagram of instrumentation and fluid/sample handling system components. (B) Photograph of silicone rubber tubing sensor probe showing stainless steel support fixture for shake-flask applications. (C) Drawing of in-house fabricated flow cell and detector housing.

start induction and the additions of methanol to 0.5% (v/v) every 24 h. However, the methanol consumption rate is strongly dependent on culture variables such as cell density, and hence the actual methanol concentration in a shake-flask culture after methanol adaptation is normally not known with any precision. A simple, inexpensive, and reliable on-line system for monitoring and controlling methanol concentration would be advantageous for optimizing and maintaining induction conditions in *P. pastoris* cultures both in shake-flask cultures and large-scale fermentations.

We designed and characterized a practical closed-loop methanol control system based on a commercially available organic solvent vapor detector, an in situ silicone rubber tubing probe, and a computer-actuated methanol feeding system. In this report we describe the components and operation of this control system and present data demonstrating its utility and performance in cell-free aqueous methanol solutions and in fed-batch shake-flask cultures of *P. pastoris* expressing recombinant human transferrin N-lobe (hTF/2N). Recombinant protein levels observed in the methanol-regulated cultures are compared to non-methanol-regulated cultures as a first step toward assessing the advantages of on-line control of methanol concentration in cell culture environments.

MATERIALS AND METHODS

Methanol Monitoring and Control System

The methanol sensor and methanol feeding system with associated instrumentation developed for use in this work are shown schematically in Figure 1A. The sensor itself comprises two components: a probe with a segment of silicone rubber tubing that remains immersed in the culture medium and facilitates conversion of the solution-phase methanol concentration to a partial pressure of methanol vapor in a carrier stream of air perfusing the lumen of the tubing (Fig. 1B); and a solid-state methanol vapor detector connected in-line with the outlet of the methanol:air flow stream (Fig. 1C). The voltage output signal from the sensor is digitized and fed to a personal computer where it is com-

pared to a manually entered set-point in order to generate an actuating signal to drive the methanol feed pump. The headspace of the shake flask is continuously flushed with sterile filtered air to maintain a constant gas-phase oxygen concentration.

The silicone rubber membrane probe was designed based on principles described earlier in work involving measurements of $p\text{CO}_2$ (Kilburn, 1967), $p\text{O}_2$ (Phillips and Johnson, 1961), and ethanol (Yamada et al., 1987; Yamane et al., 1981) using silicone rubber and polytetrafluoroethylene tubing probes. Physically, the methanol probe consists of a 20-cm length of silicone rubber tubing (Sani-Tech, Lafayette, NJ) with a lumen diameter of 0.31 mm and a wall thickness of 0.15 mm supported by a stainless steel wire mesh, as shown in the photograph of Figure 1B. The probe remains immersed in the liquid phase while the lumen of the probe is continuously perfused with filtered air. The flow rate is regulated by a needle valve and monitored using an Omega model FMA 1804 mass flow meter (Omega Engineering, Stamford, CT). Methanol dissolved in the culture medium diffuses across the gas-permeable silicone rubber "membrane" of the probe. Vapor-phase methanol in the lumen of the probe tubing, at a partial pressure determined by the concentration in the bulk liquid phase, is carried by the flowing stream of air to the downstream methanol detector element. The methanol-air sample mixture passes through a 0.2- μm pore size hydrophobic filter (Gelman Sciences, Ann Arbor, MI) inserted upstream of the detector to remove aerosol moisture from the flow stream, which can lead to instability in the detector signal. The carrier stream can be switched to bypass the methanol probe using a manually actuated three-way valve to provide a methanol-free sample to the detector at any time to check the baseline. For all media tested, the detector baseline voltage measured in methanol-free medium was within $\pm 1\%$ of the value measured in the bypass air stream.

The methanol vapor detector utilizes a Figaro model TGS 822 SnO₂ organic vapor sensor (Figaro Engineering, Wilmette, IL), which is sensitive to a broad range of alcohols and organic solvent vapors. The operating characteristics and instrumentation required for the detector element are

described in the manufacturer's products catalog (Figaro Engineering, Inc., 1990). The detector is mounted in a Plexiglas housing as shown in Figure 1C. The methanol-air sample mixture flows through an inlet port (1.7-mm diameter) on the side wall of the housing to an interior "head-space" (approximate volume 1.9 cm³) where it is exposed to the detector; the sample exhaust is vented through a port in the base of the detector element. The power supply for the detector, detector heater, and the conductance measuring circuit were designed and fabricated according to the manufacturer's specifications and application notes.

The sensor (probe plus detector) was calibrated on-line by adding prescribed aliquots of 100% methanol to a known volume of distilled water or culture medium in an agitated thermostated shake flask. Calibration was verified by withdrawing samples and determining the methanol concentration off-line by HPLC using a Shimadzu model SCL 6B auto-injector and LC 6A pump (Shimadzu Inc., Kyoto, Japan) with an HPX-87H column (BioRad, Hercules, CA).

In fed-batch operation, the methanol feed stock (100% CH₃OH) was delivered using a Gilson Minipuls 3 peristaltic pump (Gilson, Villiers-Le-Bel, France) with an in-line check valve inserted between the pump and shake flask. Because the methanol consumption rate is slow compared to the slowest practical feeding rate, a simple on/off pulsed-feed control algorithm was used. When the methanol concentration, as determined by the sensor output signal, fell below the set-point value, the controller actuated the feed pump for a prescribed (user-defined) time interval to deliver a precise dose of methanol. The controller then entered a wait state for 5–7 min (a time slightly longer than the characteristic response time of the probe) before reevaluating the concentration. These feed-pulse + delay cycles were repeated until the methanol concentration exceeded the set-point value.

***Pichia pastoris* Cultures**

To evaluate the methanol control system in *P. pastoris* cultures, two parallel 2-L baffled shake flasks, each containing 500 mL of BMGY medium (buffered minimal glycerol complex medium, *Pichia* Expression Kit) and 0.1 mL of antifoam 289 (Sigma Chemical Co., St. Louis, MO), were inoculated. The inoculum consisted of 20 mL of an overnight culture (6.2 optical density units at 600 nm, OD₆₀₀) of a Mut⁺ *P. pastoris* GS115 clone producing hTF/2N (Mason et al., 1996). The cultures were grown in BMGY for 24 h to a cell density of 28 OD₆₀₀. The cells were then separated by centrifugation and resuspended in 500 mL of basal salts medium (26.7 mL L⁻¹ 85% o-phosphoric acid, 0.82 g L⁻¹ calcium sulfate anhydrous, 18.2 g L⁻¹ potassium sulfate, 14.9 g L⁻¹ magnesium sulfate-7H₂O, and 4.13 g L⁻¹ potassium hydroxide), containing 1 mL of PTM4 trace salts with biotin (2 g L⁻¹ cupric sulfate-5H₂O, 0.08 g L⁻¹ sodium iodide, 3.0 g L⁻¹ manganese sulfate-H₂O, 0.2 g L⁻¹ sodium molybdate-2H₂O, 0.02 g L⁻¹ boric acid, 0.5 g L⁻¹ cobalt

chloride, 7.0 g L⁻¹ zinc chloride, 22.0 g L⁻¹ ferrous sulfate-7H₂O, 1 mL L⁻¹ sulfuric acid, and 0.2 g L⁻¹ D-biotin).

Two different strategies were employed for feeding methanol to the cultures. For one culture, the standard (Invitrogen) protocol was followed: 2.5 mL of pure methanol was added daily. In the parallel culture, methanol was maintained at 0.3% (v/v) using the closed-loop control system based on the feedback signal provided by the methanol sensor. Culture samples were taken periodically to determine the OD₆₀₀. Additional samples were collected and filtered through a 0.2-μm pore size filter; the filtrate was used for off-line analysis of methanol and hTF/2N concentrations. Culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were visualized with Coomassie brilliant blue stain and quantified by scanning densitometry using a Fast-Scan Computing Densitometer (Molecular Dynamics).

Chemicals and Reagents

All chemicals used in the characterization of the sensor and in the preparation of culture media were obtained from Fisher Scientific (Nepean, ON), BDH Chemicals (Toronto, ON), Anachem Science (Montréal, PQ), and Sigma Chemical Co. and were used as received. All solutions were prepared using distilled water.

RESULTS AND DISCUSSION

Methanol Probe Characterization

Sensitivity to Methanol

A typical strip-chart record of the time-dependent response to methanol additions and the resulting methanol calibration curve are shown in Figure 2A and 2B, respectively. The baseline sensor voltage (i.e., the reading in a methanol blank solution) under the conditions used throughout this work was 1.39 ± 0.01 V; the baseline voltage periodically measured using the bypass stream of air remained within this range during all experiments. The sensor response time (to reach 90% of steady state) at 30°C in methanol-water solutions was ca. 4 min, using a carrier air-stream flow rate of 5 mL min⁻¹ with probe dimensions as indicated in the Materials and Methods section. In terms of the calibration characteristic and response time, the behavior of the probe was virtually the same whether measuring in methanol-water solutions or culture media, with or without cells. In the present configuration, the methanol concentration detection limit (defined as the concentration corresponding to a signal 3 standard deviations of the noise above the mean baseline response) at 30°C was approximately 1.2 × 10⁻⁴% (v/v).

The nonlinearity evident in the sensor response function results from a combination of two effects: one is an artifact of the electronic measuring circuit that employs a simple voltage divider configuration to measure conductance

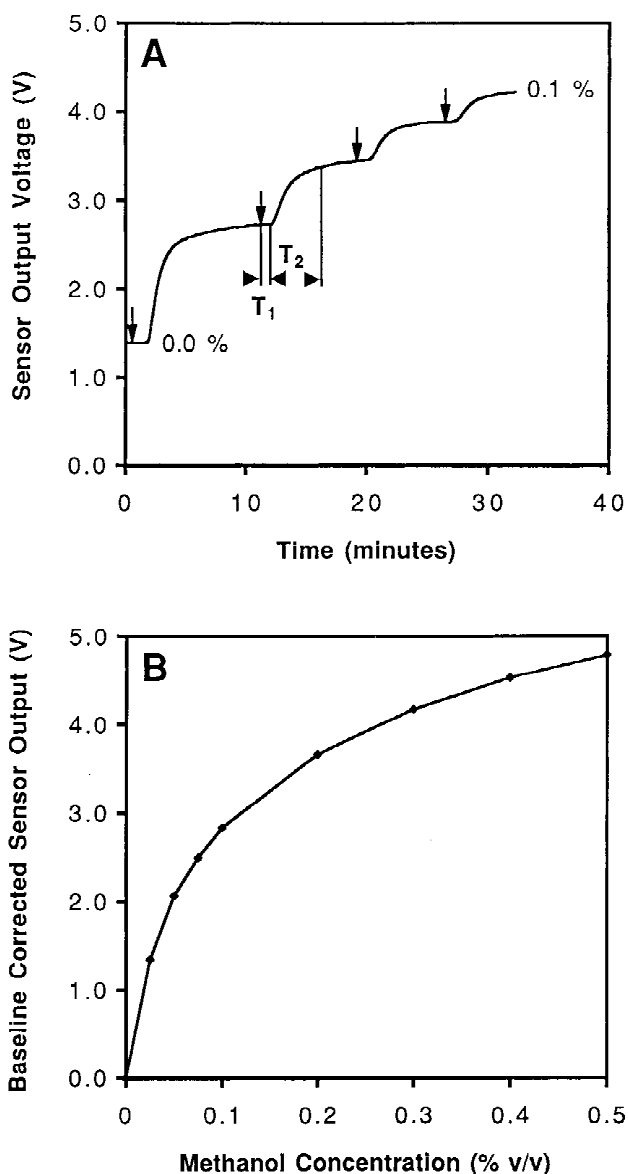


Figure 2. Methanol sensor response characteristics measured at 30°C with a carrier air flow rate of 5 mL min⁻¹. (A) Strip-chart record showing characteristic response time to 2.5 mL methanol injections (arrows); component T1 (0.71 ± 0.19 min) is the time for the sensor to initiate a response and component T2 (3.15 ± 0.63 min) is the additional time required to reach 90% of the steady-state signal. (B) Sensor response versus methanol concentration in methanol–water solution.

changes in the detector element, yielding a hyperbolic relationship between output voltage and conductance. The other is itself a composite effect that results from the fundamental physical chemistry and physical electronic principles of semiconducting metal-oxide gas sensors. These principles have been described in detail elsewhere (e.g., Heiland, 1982; Jones et al., 1985; Morrison, 1982; Watson, 1984); however, a summary of some of the salient points may be helpful here. Briefly, the conductivity of sintered metal oxides is mediated primarily by electrons populating surface states that arise due to a deficit of lattice oxygen at the grain

boundaries and lie energetically near the edge of the conduction band. The conductivity is modulated by any process that alters the availability of these surface states. In the case of the methanol probe described here, chemisorption of oxygen in the absence of methanol depletes the conduction electrons available at the surface and lowers the macroscopic conductivity. In the presence of methanol, which coadsorbs to the surface, the semiconductor catalyzes the reduction of surface-bound oxygen, forming the more weakly interacting formaldehyde that desorbs and frees surface conduction band electrons that, in turn, increases the observed conductivity. All of the processes involved are nonlinear over an appreciable range of methanol concentrations and hence so is the resulting response function, even though consideration of mass transfer limited by the diffusion of methanol across the silicone rubber membrane would lead to the expectation of a linear response.

Sensitivity to Other Organic Vapors

The detector element employed in this work is not intrinsically selective for methanol. Some extrinsic selectivity can be engineered in this family of sensors by the incorporation of appropriate dopant species in the sintered metal oxide pellet and by the choice of surface temperature (Gentry and Jones, 1986; Yamazoe et al., 1983). The particular model of detector element used here is specified for applications involving detection of a range of alcohols, as well as toluene, xylene, benzene, acetone, carbon monoxide, and several small hydrocarbons (Figaro Engineering, 1990). In the applications considered here under aerobic conditions, none of these potential interfering species exist in the culture medium in significant concentrations. However, if mix feeding of glycerol and methanol is used or if the culture becomes oxygen limited, low levels of ethanol can be produced by *P. pastoris*. Although the sensor is slightly more sensitive to ethanol than to methanol, the maximum ethanol concentration reported in mix-fed *P. pastoris* cultures was 0.02% (v/v) and only 0.003% in methanol-fed cultures (Brierley et al., 1990). Ethanol interference is thus of no practical concern when methanol is controlled at 0.3% (v/v), a level that seems adequate to maintain induction (see below).

Probe Dimensions and Air Flow Rate

The main physical parameters of the probe that dictate both the kinetics and the sensitivity of the response are the silicone rubber tubing length, tubing diameter and wall thickness, and the flow rate of the carrier stream of air. In the present work, the tubing diameter and wall thickness as specified in the Materials and Methods section were chosen to yield reasonable durability with a minimal compromise of sensitivity and response time. The tubing length and air flow rate were then optimized based on the performance needed for the intended application. The dependence of tubing length and air flow on the sensor response is shown in Figure 3A and 3B, respectively. Longer tubing lengths

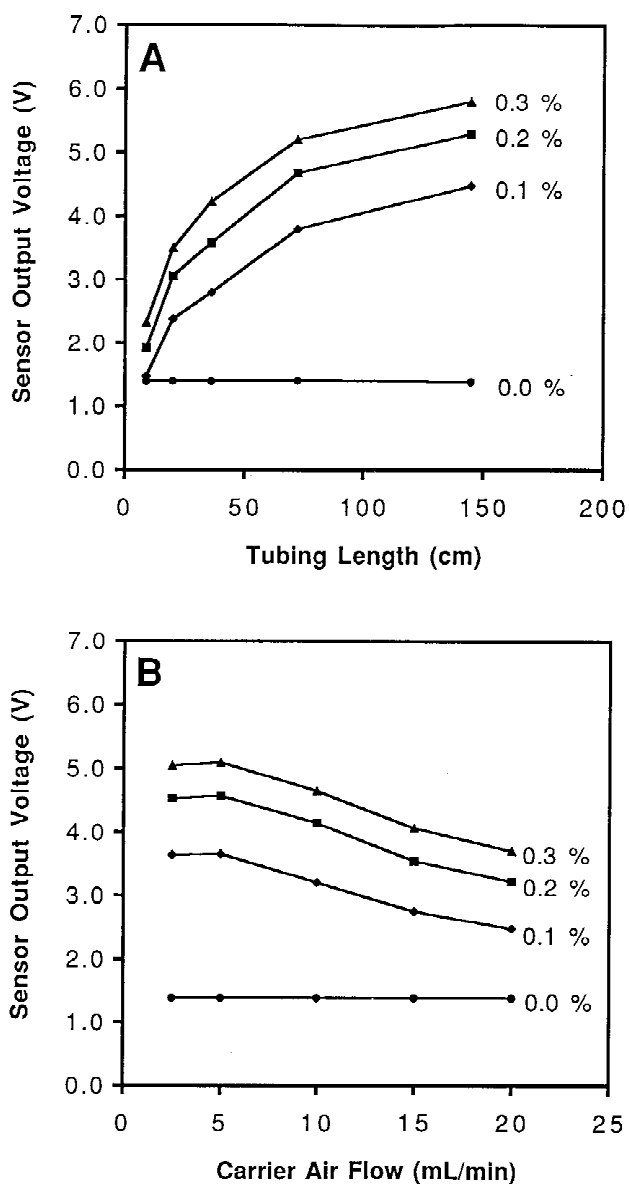


Figure 3. Tubing length and carrier air flow-rate dependence determined at 30°C. (a) Sensor response versus silicone rubber sensor tubing length for a methanol blank solution and three nonzero methanol concentrations. (b) Sensor response versus carrier air flow rate for the same methanol concentrations as in (a).

clearly yield higher sensitivity and larger output signals, albeit with rapidly diminishing returns beyond ca. 50 cm; however, the response time beyond 40–50 cm increases (data not shown) to a point where the system becomes impractical for use in *P. pastoris* fermentations. A length of 20 cm was chosen as the optimal compromise between sensitivity and response time. Response time is also (inversely) dependent on the carrier air flow rate. However, as shown in Figure 3B, the sensitivity drops as a function of air flow above 5 mL min⁻¹; hence, a rate of 5 mL min⁻¹ was again chosen as the optimal compromise between sensitivity and response time.

Temperature and pH Dependence

The temperature and pH dependence of the sensor, measured in a methanol–water solution, is shown in Figure 4A and 4B, respectively. Essentially all of the physical and chemical processes involved in the operation of the sensor are inherently temperature dependent. However, the detector element is remotely located and thereby thermally isolated from the culture environment; the detector also incorporates an integrated heating coil that maintains the surface temperature at ca. 400°C. No significant cooling of the detector is effected by the methanol–air gas flow through the

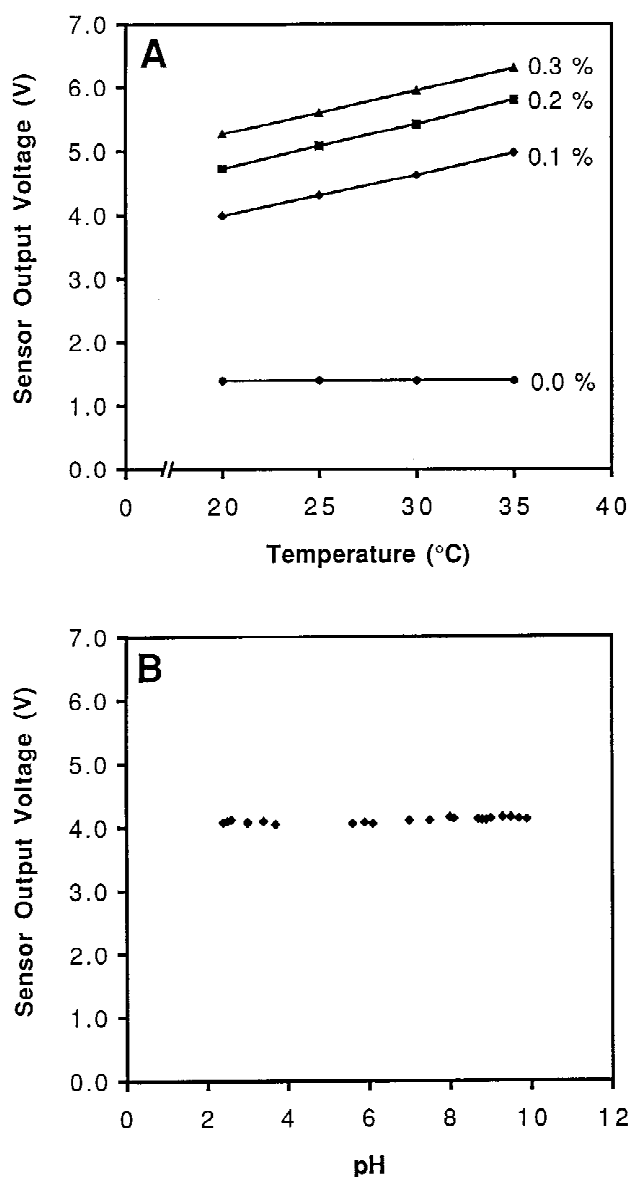


Figure 4. Effect of variations in temperature and pH determined at 30°C using a carrier air flow rate of 5 mL min⁻¹ and a tubing length of 20 cm. (a) Sensor response versus temperature of the methanol–water analyte matrix. (b) Sensor response versus pH of the methanol–water analyte matrix with a methanol concentration of 0.1%.

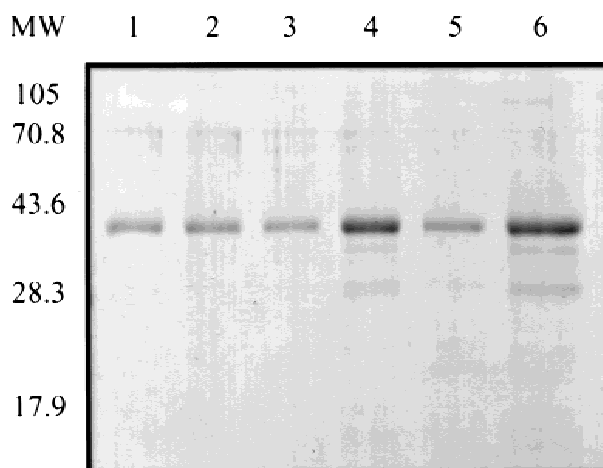


Figure 5. SDS-PAGE analysis of hTF/2N produced by *P. pastoris*. Culture medium after (lanes 1, 2) 10 h, (lanes 3, 4) 33 h, and (lanes 5, 6) 56 h of production was sampled from (lanes 1, 3, 5) the flask receiving daily additions of methanol and (lanes 2, 4, 6) the flask with constant (ca. 0.3%) methanol concentration.

housing as evidenced by the flat response as a function of flow rate for a methanol blank solution shown in Figure 3B. Furthermore, no temperature dependence is observed in the absence of methanol. The observed temperature dependence of the sensor is therefore dominated by effects relating to the mass transfer of methanol across the silicone rubber membrane of the probe. With regard to pH dependence, it was important to establish that the sensor response is independent of fluctuations in culture pH, as demonstrated by the data in Figure 4B, because pH is normally not controlled in shake-flask cultures.

Increased Protein Productivity in *P. pastoris* Cultures

The results obtained using the two different methanol feeding protocols in parallel shake-flask cultures are shown in Figures 5, 6, and 7. SDS-PAGE showed that recombinant hTF/2N comprises the majority of the total protein in the medium under both conditions (Fig. 5). In the production phase of one 500-mL culture, the standard Invitrogen protocol was followed with daily additions of 2.5 mL of methanol. After each dose, methanol was rapidly consumed such that its concentration was lower than 0.3% (v/v) for more than half of the production phase and the methanol was consistently exhausted by the culture before the next scheduled feeding time was reached. In response to the first methanol dose, recombinant protein concentration in the culture medium increased to 32 mg L⁻¹. However, as the run progressed, hTF/2N not only ceased to accumulate, but its concentration actually declined, presumably due to protein degradation in the medium. After the third dose of methanol, however, recombinant protein accumulation resumed but only to 82% of the hTF/2N concentration obtained after the first methanol dose. In the parallel culture,

the methanol control system was used to maintain a methanol concentration of 0.3% (v/v) in the production phase. In contrast to the uncontrolled culture, recombinant protein accumulated continuously for 2 days, reaching a maximum concentration of 120 mg L⁻¹.

Under the experimental conditions used, the standard protocol of daily methanol additions was insufficient to maintain induction. The concentration of hTF/2N in the medium reached a maximum 10 h after the first methanol addition. Maintaining the culture for longer times with daily additions did not result in any further increase in volumetric protein yield. In contrast, control of methanol concentration

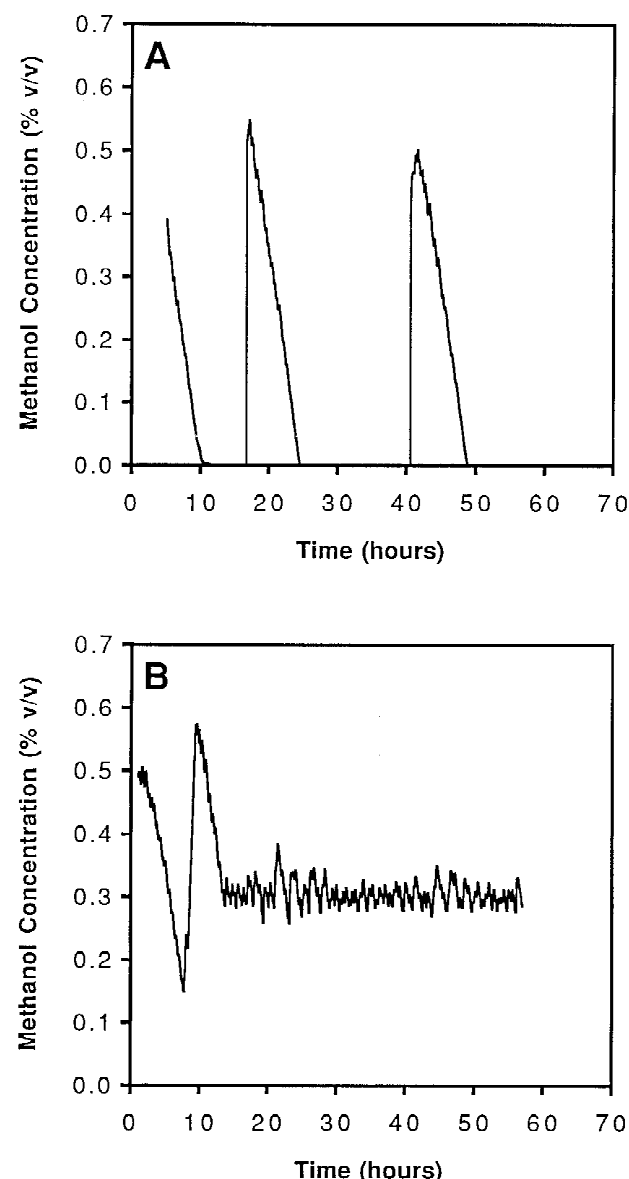


Figure 6. Methanol concentration versus time in two parallel *P. pastoris* shake-flask cultures. (a) the 500-mL culture was fed daily with 2.5 mL methanol. (b) The closed-loop control system was set to maintain the methanol concentration at 0.3% (v/v), as determined by in situ calibration of the sensor.

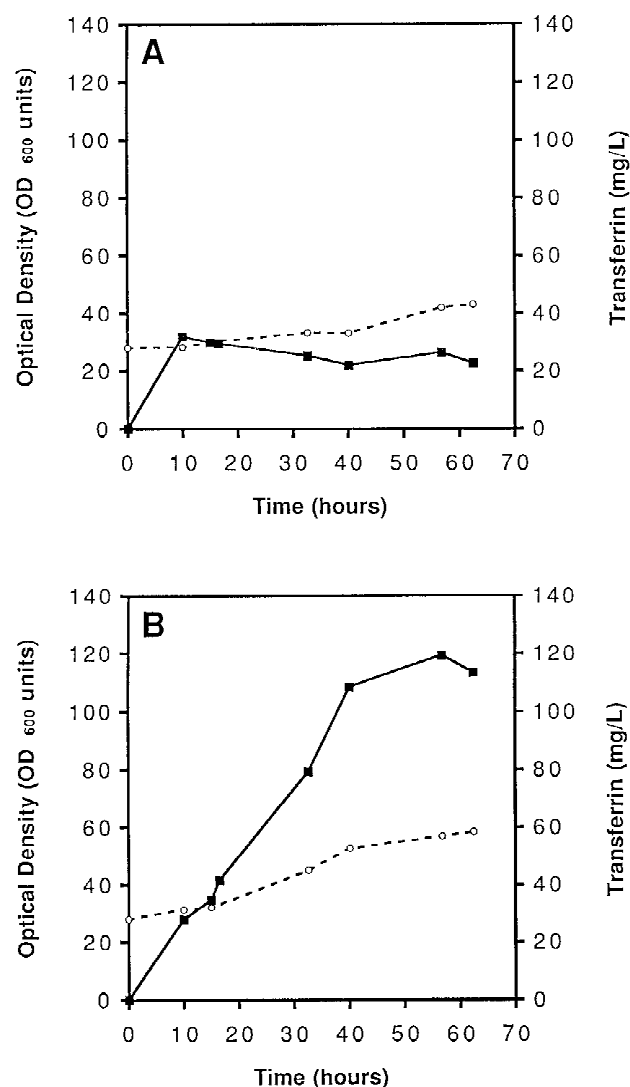


Figure 7. Effect of on-line control on protein productivity. (○-○) Cell density and (■-■) recombinant protein production were analyzed in (a) a culture receiving daily doses of methanol and (b) in a controlled culture in which the methanol concentration was maintained at 0.3% (v/v).

at 0.3% (v/v) resulted in continuous culture growth and hTF/2N production, reaching levels fivefold higher than in the flask that received daily methanol feedings.

CONCLUSIONS

A practical and effective system for on-line monitoring and control of methanol concentrations in *P. pastoris* shake-flask cultures has been presented. The sensor element, based on a commercially available organic vapor detector and a custom designed gas-permeable methanol probe, has been optimized with respect to performance-determining physical parameters (e.g., carrier stream flow rate, tubing length) and characterized as a function of the key fermentation environmental variables (e.g., methanol concentration, tem-

perature, pH) over their typical respective ranges. The probe is autoclavable, can be calibrated in situ, and is stable for several days under routine operating conditions for *P. pastoris* fermentations.

Shake-flask cultures in which the sensor was used to control the methanol concentration exhibited higher recombinant protein productivity than simultaneously run cultures carried out using the conventional protocol. These experiments also provided the first quantitative data demonstrating the drastic variability in the methanol concentration profile that can result from the conventional protocol. The higher productivity observed in the controlled cultures was attributed to elimination of this variability.

On-line monitoring of substrate concentration is seldom, if ever, used in simple shake-flask cultures and we know of no similar example of controlled fed-batch operation in this type of system. These features have previously been limited to much more expensive and complex bioreactor systems not routinely available to most investigators. The ability to monitor and control methanol concentration in shake-flask cultures will expedite the early selection and characterization of recombinant clones of methanol utilizing organisms (e.g., *P. pastoris*, *Hansenula polymorpha*).

Although beyond the scope of this work, the methanol sensor described here provides a valuable tool for further optimization of *P. pastoris* expression systems, particularly with respect to target methanol concentrations during the induction phase of the culture. This sensor system can easily be adapted for use in applications involving larger scale cultures in stirred-tank bioreactors of methylotrophic organisms or for monitoring production in biomass to ethanol processes. Continuing work in our laboratories includes application of this system to Mut⁺ and Mut^S mixed-feed fermentations of *P. pastoris* for enhanced productivity of recombinant proteins.

The authors wish to thank Mr. Randy Deane for assisting with the fabrication of electronic components used in this work. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada, The Medical Research Council of Canada, and Ciba-Geigy Corporation.

References

- Brierley, R. A., Bussineau, C., Kosson, R., Melton, A., Siegel, R. S. 1990. Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: Bovine lysozyme. *Ann. NY Acad. Sci.* **589**: 350-362.
- Buckholz, R. G., Gleeson, M. A. G. 1991. Yeast systems for the commercial production of heterologous proteins. *Bio/Technology* **9**: 1067-1072.
- Cregg, J. M., Vadick, T. S., Raschke, W. C. 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Bio/Technology* **11**: 905-910.
- Faber, K. N., Harder, W., Ab, G., Veenhuis, M. 1995. Review: Methylotrophic yeasts as factories for the production of foreign proteins. *Yeast* **11**: 1331-1344.
- Figaro Engineering Inc. 1990. Figaro gas sensors. Figaro Products Catalog pp. 2-12.

- Gentry, S. J., Jones, T. A. 1986. The role of catalysis in solid-state gas sensors. *Sensors Actuators* **10**: 141–163.
- Heiland, G. 1982. Homogeneous semiconducting gas sensors. *Sensors Actuators* **2**: 343–361.
- Jones, T. A., Firth, J. G., Mann, B. 1985. The effect of oxygen on the electrical conductivity of some metal oxides in inert and reducing atmospheres at high temperature. *Sensors Actuators* **8**: 281–306.
- Kilburn, D. G. 1967. Some factors affecting growth of mammalian cells in suspension cultures. Ph.D. thesis. Department of Chemical Engineering, University College, London, Chap. 2.
- Mason, A. B., Woodworth, R. C., Oliver, R. W. A., Green, B. N., Lin, L.-N., Brandts, J. F., Tam, B. M., Maxwell, A., MacGillivray, R. T. A. 1996. Production and isolation of the recombinant N-lobe of human serum transferrin from the methylotrophic yeast *Pichia pastoris*. *Protein Expr. Purif.*, to appear.
- Morrison, S. R. 1982. Semiconductor gas sensors. *Sensors Actuators* **2**: 329–341.
- Phillips, D. H., Johnson, M. J. 1961. Measurement of dissolved oxygen in fermentations. *J. Biochem. Microb. Technol. Eng.* **111**: 261–275.
- Romanos, M. A. 1995. Advances in the use of *Pichia pastoris* for high-level gene expression. *Curr. Opin. Biotechnol.* **6**: 527–533.
- Romanos, M. A., Scorer, C. A., Clare, J. J. 1992. Foreign gene expression in yeast: A review. *Yeast* **8**: 423–488.
- Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A., Gingeras, T. R. 1987. Expression of the *lacZ* gene from two methanol regulated promoters in *Pichia pastoris*. *Nucl. Acids Res.* **15**: 3859–3876.
- Watson, J. 1984. The tin oxide gas sensor and its applications. *Sensors Actuators* **5**: 29–42.
- Yamada, M., Mizuno, M., Tsukamoto, Y., Yamada, K. 1987. Method for a measurement of alcohol concentration in acetic acid fermenting broth. U.S. Pat. 4,656,140.
- Yamane, T., Matsuda, M., Sada, E. 1981. Application of porous Teflon tubing method to automatic fed-batch culture of microorganisms. II. Automatic constant-value control of fed substrate (ethanol) concentration in semibatch culture of yeast. *Biotechnol. Bioeng.* **13**: 2509–2524.
- Yamazoe, N., Kurokawa, Y., Seiyama, T. 1983. Effects of additives on semiconductor gas sensors. *Sensors Actuators* **4**: 283–289.