

Effect of Methanol Concentration on the Production of Human β_2 -Glycoprotein I Domain V by a Recombinant *Pichia pastoris*: A Simple System for the Control of Methanol Concentration Using a Semiconductor Gas Sensor

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The methylotrophic yeast *Pichia pastoris* is one of the best hosts for the production of foreign proteins because of the presence of the strong *AOX1* promoter induced by methanol. Methanol feeding during the production phase of the foreign proteins is important because methanol not only induces protein production but also provides energy source for the host cells. Excess methanol inhibits the growth of host cells, while an insufficient amount of energy source and/or methanol starvation lead to poor growth and production. We constructed a simple methanol control system consisting of a semiconductor gas sensor and a relay. Using this system, we studied the effect of methanol concentration on the production of a model foreign protein, human β_2 -glycoprotein I domain V. The methanol concentrations were kept constant at 1.5, 10, 17, or 31 g·l⁻¹ ($\pm 5\%$) during the production phase. Although the specific rates of growth and methanol consumption decreased with increase in the methanol concentration, the specific production rates increased, indicating that the energy for the production competed with that for cell growth. Accordingly, we provided glycerol as an extra energy source during the production phase, with the result that the specific production rate increased two times. Our simple and inexpensive system will help bioengineering studies on the production of recombinant proteins in *P. pastoris*, the growth and production of objective proteins in which are dependent on the methanol concentration.

[Key words: β_2 -glycoprotein I, *Pichia pastoris*, methanol control system, semiconductor sensor]

The methylotrophic yeast *Pichia pastoris* is one of the best hosts for the production of foreign proteins, especially of eukaryotic secretory proteins which require post-translational modifications. Many eukaryotic proteins have been produced using the *Pichia* expression system (1–4) because this system is more efficient, simpler and less expensive than other eukaryotic expression systems. This expression system is useful not only for the production of industrial proteins but also for biochemical researchers who are interested in tertiary structure analysis of proteins by X-ray crystallography or nuclear magnetic resonance (4). This yeast has two alcohol oxidase genes, *AOX1* and *AOX2*, which are induced strongly and weakly, respectively, by methanol (5, 6). The gene of the foreign protein is inserted between the *AOX1* promoter and the *AOX1* terminator of shuttle vectors between *P. pastoris* and *Escherichia coli*. When the vector is integrated into the chromosome of *P. pastoris*, the *AOX1* genotype of the yeast remains, which grows fast on methanol (integration-type). When the *AOX1* structural gene of the yeast genome is replaced by that of the foreign protein, the resultant yeast genotype is *aox1*, which grows slowly on methanol (replacement-type), because alcohol oxidase, a key enzyme for methanol utilization, is expressed only from *AOX2*. The transformed yeast is usually cultivated on glycerol, a non-inducible and non-repressive substrate, in the growth phase, and

thereafter, protein production is induced by methanol feeding. In both types of transformants, the methanol concentration in the medium affects not only the level of production of foreign proteins but also the growth of the host cell. However, no biochemical engineering studies have so far been conducted on the production of foreign proteins in the presence of constant methanol concentrations. Although some methanol feeding programs during the production phase were applied to the production of bovine lysozyme and human epidermal growth factor (7; Brerley, R. A. *et al.*, US patent 5,324,639, 1994; Siegel, R. S. *et al.*, International patent application, WO 90/10697, 1990; Buckholz, R. G. *et al.*, International patent application, WO 90/03431, 1989), it is difficult to keep the methanol concentration constant because the methanol consumption rate increases with cell growth. These programs are probably not suitable for being employed for the production of other proteins because the specific methanol consumption rate depends on the species of foreign proteins and the copy number of the gene.

It has been reported that a semiconductor sensor, which has been used as a gas-leakage sensor, can be used for monitoring the ethanol and methanol concentrations in microbial culture (8, 9), including recombinant *P. pastoris* culture (10). The sensor consists of a semiconductor and a heater unit which heats up the semiconductor. Since the heat increases the mobility of free electrons in the semiconductor, the density of free electrons at the surface of the semiconductor increases. When an oxygen molecule touches the free electrons, it detaches

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itself into atoms and traps the free electrons. As a result, since free electrons are the carriers of electric current, the resistance of the sensor increases. When flammable gas molecules are introduced into the sensor, the atomic oxygen reacts easily with them and releases the free electrons, the density of free electrons in the semiconductor then increases and the resistance of the sensor decreases according to the concentration of the flammable gas molecules (11, 12).

By combining this sensor with a simple circuit, a relay and a pump, we constructed a feedback system for the control of methanol concentration. Recently Guarna *et al.* used this sensor to keep the methanol concentration constant in a flask-fed batch culture of recombinant *P. pastoris* (10). However, they emphasized the expedition of early selection and characterization of recombinant clones by their shake-flask system and did not discuss kinetic parameters such as specific rates. The purposes of this study are (i) to establish a simple methanol control system which is easy to use, and (ii) to investigate the effects of methanol concentration on the level of production of a foreign protein in a genetically engineered *P. pastoris* culture using the system for the control of methanol concentration.

MATERIALS AND METHODS

Strains and plasmid Human β_2 -glycoprotein I (β_2 GPI) domain V was used as the representative foreign protein. β_2 GPI is a glycosylated plasma protein and its fifth domain (β_2 GPI dV) was reported to interact with negatively charged phospholipids *in vivo* (13). β_2 GPI dV is a basic protein (isoelectric point 10) which consists of 86 amino acid residues with three disulfide bonds (molecular weight, 9.7 kDa). The plasmid pNPD5 (13), containing the β_2 GPI dV gene inserted in the vector pPIC9 (Invitrogen, San Diego, USA), was digested by *Sal*I and introduced into *P. pastoris* GS115 (*his*4, *AOX*1, *AOX*2) by electroporation. Most of the transformants grew fast on the minimum agar plate containing methanol as the sole carbon source (MM plate), because the *Sal*I digestion resulted in integration-type transformants (*HIS*4, *AOX*1, *AOX*2). The β_2 GPI dV expression level in the transformants on the MM plate was assayed by colony Western blotting using the anti-human β_2 GPI antibody from rabbit and alkaline phosphatase conjugated anti-rabbit immunoglobulins antibody from goat (Biosource, Camarillo, USA) as the primary and secondary antibodies, respectively. Ten clones which showed strong signals were selected and the production of β_2 GPI dV in a test tube culture was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the manufacturer's instructions (Invitrogen). A clone, designated SP216, which gave the highest level of β_2 GPI dV among the transformants was finally selected. SP216 had at least 3 copies of the β_2 GPI dV gene as judged from the results of Southern hybridization using the β_2 GPI dV gene as a probe (data not shown).

Analytical methods Dry cell weight was calculated based on the optical density at 660 nm and the β_2 GPI dV expression level was measured by cation-exchange chromatography. The culture supernatant was diluted three times with 20 mM acetic acid. The diluted sample was filtered (0.22 μ m filter) and loaded onto the SP-5PW column (4.6 mm $\times$ 75 mm, Tosoh Co., Tokyo) equilibrated with 20 mM acetic acid at a flow rate 1.0 ml $\cdot$ min⁻¹

at room temperature. β_2 GPI dV was eluted with a linear gradient, from 20 mM acetic acid to 50 mM sodium acetate containing 0.5 M sodium chloride (pH 5.5), and the concentration was calculated from the extinction coefficient of 10,185 at 276 nm (13). Glycerol concentrations were measured by F-kit glycerol (Boehringer Mannheim, Tokyo). Methanol concentrations in the culture broth were measured off-line by gas chromatography (G-3000, Hitachi Co., Tokyo) on the TC-1 column (GL Sciences Co., Tokyo) at a column temperature of 60°C, using 0.5 g $\cdot$ l⁻¹ *n*-propanol as the internal standard.

Methanol control system The semiconductor sensor model TGS822 was purchased from Figaro Engineering Inc. (Minoh, Osaka). As shown in Fig. 1, the heater of the sensor was connected to a 5 V stabilized power supply (the heater consumed 660 mW). The sensor, with a series resistance R_1 and variable resistance R_2 , was connected to a 10 V stabilized power supply. The sensor was connected in parallel to a conventional high-impedance recorder and to the detect relay model CR1 (Tsuruga Co. Ltd., Tokyo). Since the resistance of the sensor responds to the concentration of flammable gases and varies below about 10 k Ω , the output voltage range can be adjusted from 0 to 5 V by tuning R_2 . The methanol feeding pump is switched on or off by the detect relay corresponding to a desired voltage, which can be changed by rotating its dial. Methanol feeding was monitored by a conventional weight sensor. All of the exhaust gas from the jar fermentor was introduced into the sensor box. The sensor box and gas line from the fermentor were heated to 50–60°C by a ribbon heater to avoid the condensation of dew which delays the response and may damage the sensor. Methanol was introduced into the culture broth to avoid its direct evaporation to the gas phase. An automatic antifoam system was used to avoid overflow of the culture which could damage the sensor.

Preparation of inoculum The preculture medium was composed of 10 g yeast extract (Difco, Detroit, USA), 20 g tryptone (Difco), 100 mM potassium phosphate, 6.7 g yeast nitrogen base (without amino acids, Difco), 0.4 mg biotin and 18 g glycerol in 1 l of deionized water. The yeast grown in 50 ml of preculture medium was inoculated into a 5-l jar fermentor (MDL-

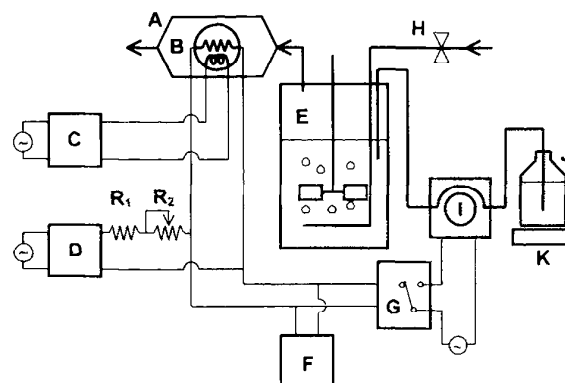


FIG. 1. Outline of the system for the control of methanol concentration. A, Sensor box; B, semiconductor sensor; C and D, stabilized direct current power supply (5 and 10 volts, respectively); E, jar fermentor; F, recorder; G, detect relay; H, flow control valve; I, peristaltic pump; J, methanol reservoir; K, digital weight sensor; R_1 , 10 k Ω resistance; R_2 , 10 k Ω variable resistance.

500S; Marubishi Bioeng. Co. Ltd., Tokyo) containing 2 l of the preculture medium and was cultivated to $OD_{600}=50$ ($11.7 \text{ g dry-cell} \cdot \text{l}^{-1}$) at 30°C , air flow rate of $3 \text{ l} \cdot \text{min}^{-1}$, dissolved oxygen (DO) $>20\%$ and pH 6.0 (with 28% ammonium hydroxide). The cells were centrifuged at $2000 \times g$ for 5 min and suspended in the fresh preculture medium containing $150 \text{ g} \cdot \text{l}^{-1}$ glycerol at a cell density of about $OD_{600}=200$. The suspension was dispensed in sterilized tubes of 15 ml volume and stored at -80°C as an inoculum stock.

Culture conditions

Growth phase Two liters of the Basal Salts Medium (BSM; containing $26.7 \text{ ml H}_3\text{PO}_4$ (85%), 0.93 g CaSO_4 , $18.2 \text{ g K}_2\text{SO}_4$, $14.9 \text{ g MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.13 g KOH and 40.0 g glycerol in 1 l of deionized water) was sterilized in the 5-l jar fermentor and its pH was adjusted to 5.0 by 28% ammonium hydroxide. After addition of 8.7 ml of the trace salts solution, PTM₁ (containing $6.0 \text{ g CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.08 g NaI , $3.0 \text{ g MnSO}_4 \cdot \text{H}_2\text{O}$, $0.2 \text{ g Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $0.02 \text{ g H}_3\text{BO}_3$, 0.5 g CoCl_2 , 20.0 g ZnCl_2 , $65.0 \text{ g FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g biotin and $5.0 \text{ ml H}_2\text{SO}_4$ in 1 l of deionized water), the cultivation was started with addition of the inoculum stock described above. The temperature, pH, air flow rate and DO concentration were 30°C , 5.0 (with 28% ammonium hydroxide), $3 \text{ l} \cdot \text{min}^{-1}$, $>20\%$, respectively.

Transition phase After 15–20 min of glycerol exhaustion (identified by a rapid increase in DO), a glycerol solution ($500 \text{ g} \cdot \text{l}^{-1}$, containing $12 \text{ ml} \cdot \text{l}^{-1}$ PTM₁) was fed at $20 \text{ ml} \cdot \text{h}^{-1} \cdot \text{l-medium}^{-1}$ for two hours. Glycerol feeding was continued for an additional 3 h at the flow rate 10, 7.5 and $5 \text{ ml} \cdot \text{h}^{-1} \cdot \text{l-medium}^{-1}$ in the first, second and third hour, respectively. Glycerol starvation is believed to allow the cells to consume metabolites which promote the toxicity of methanol, and glycerol feeding helps the cell to adapt to grow and adapt to the production on methanol. When the glycerol feed rate was reduced from 20 to $10 \text{ ml} \cdot \text{h}^{-1} \cdot \text{l-medium}^{-1}$, feeding of methanol containing $12 \text{ ml} \cdot \text{l}^{-1}$ PTM₁ was started, using a conventional peristaltic pump and the detect relay was set at a value corresponding to a methanol concentration $1.5 \text{ g} \cdot \text{l}^{-1}$. To avoid a rapid increase of methanol concentration which could damage the cells, the feed rate was set at $3.5 \text{ ml} \cdot \text{h}^{-1} \cdot \text{l-medium}^{-1}$. After 2 h of methanol feeding, the detect relay was set at a value corresponding to methanol concentrations of 1.5, 10, 17 or $31 \text{ g} \cdot \text{l}^{-1}$. When the methanol concentration was controlled at 10, 17, or $31 \text{ g} \cdot \text{l}^{-1}$, the feed rate was increased to 7.5, 7.5 or $15 \text{ ml} \cdot \text{h}^{-1} \cdot \text{l-medium}^{-1}$, respectively, so that the methanol concentration reached the desired value within corresponding periods.

Production phase Following the transition phase, the control of methanol concentration was continued at 1.5, 10, 17 or $31 \text{ g} \cdot \text{l}^{-1}$. The feed rate of the pump was set two or three times higher than the methanol consumption rate, to avoid fluctuations of the methanol concentration in the culture.

Calculation of the specific rates During the production phase, the following equations were applied.

$$\frac{dVX}{dt} = \mu VX \quad (1)$$

$$\frac{dV_s P}{dt} = \rho VX \quad (2)$$

$$\frac{dV_s S}{dt} = FS_r + \nu VX = 0 \quad (3)$$

Since the cell concentrations in the production phase were more than $50 \text{ g dry-cell} \cdot \text{l}^{-1}$, the total cell volume was not negligible compared with the culture volume. Therefore, the amounts of the product were calculated using the volume of the culture supernatant.

$$V_s = V - aVX \quad (4)$$

where V and V_s are culture volume and the volume of the culture supernatant, and a is the cell volume per dry-cell. We provided $a=0.003 \text{ l} \cdot \text{g dry-cell}^{-1}$ in this study. Since equations 1–3 can be rewritten as follows under constant specific rates:

$$\ln(VX) = \mu t + \ln(V_0 X_0) \quad (5)$$

$$V_s P = \rho \int_0^t VX dt + V_{s0} P_0 \quad (6)$$

$$\int_0^t FS_r dt = -\nu \int_0^t VX dt \quad (7)$$

the specific rates μ , ρ and ν were calculated from t vs $\ln(VX)$, $\int_0^t VX dt$ vs $V_s P$, and $\int_0^t VX dt$ vs $\int_0^t FS_r dt$ plot, respectively (14), where $\int_0^t FS_r dt$ and $\int_0^t VX dt$ were obtained from the weight change of the methanol reservoir and the area of t vs VX plot, respectively.

RESULTS AND DISCUSSION

Response of the sensor The sensor was calibrated using various dilutions of methanol in distilled water under the same conditions as for the yeast culture (at $30.0 \pm 0.1^\circ\text{C}$, air flow rate at $3 \text{ l} \cdot \text{min}^{-1}$ using the 5-l jar fermentor). A good linearity between the output voltage of the sensor and methanol concentration was observed (Fig. 2, circles). It was reported that flammable gases as well as ethanol in the exhaust gas affected the response of the sensor in the case of baker's yeast production (8). In our experiments, however, the methanol concentrations in the culture, as determined by gas chromatography, agreed well with those determined by the sensor (triangles in Fig. 2). Accordingly, we concluded that the effect of other flammable gases on the on-line monitoring of methanol concentration, if any, was negligible in our experiments. It was reported that oxygen concentration in the exhaust gas also affects the

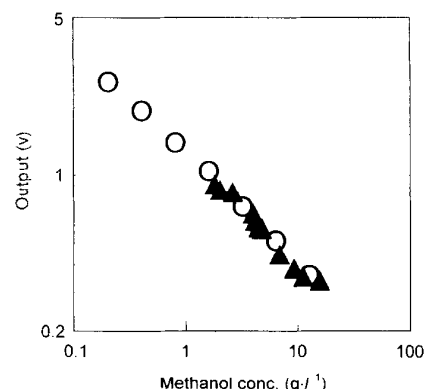


FIG. 2. Relationship between the output voltage of the sensor and methanol concentration. Symbols: circles, in distilled water; triangles, in the culture.

response of the sensor (9). When the oxygen concentration in the exhaust gas decreases, the resistance of the sensor decreases because the density of free electrons increase. In our case, even at the highest cell concentration ($80 \text{ g} \cdot \text{l}^{-1}$), the relationship between methanol concentration, as determined by gas chromatography, and the output voltage of the sensor agreed well with that determined from the calibration curve shown in Fig. 2 (see also Fig. 3). This result indicates that the effect of decrease in the oxygen concentration in the exhaust gas due to respiration of the host cell, on the sensor response was negligible for our purposes. The temperature drift of the culture significantly influenced the sensor response, because the drift directly affects the methanol equilibrium between liquid and gas phase. We controlled the culture temperature at $30.0 \pm 0.1^\circ\text{C}$ to minimize the effect of the drift. The agitation rate, which was increased during the fermentation from 300 rpm to 1000 rpm to keep the DO from exceeding 20%, did not affect the on-line monitoring of methanol concentration. Since the sensor resistance decreased with increasing air flow, the air flow rate was fixed at $3 \text{ l} \cdot \text{min}^{-1}$; all of the exhaust gas was introduced into the sensor box in our study to avoid the effect of air flow rate on the sensor response.

Yamane *et al.* (15) and Guarna *et al.* (10) controlled the ethanol and methanol concentrations using a Teflon and a silicon tubing system, respectively, in which the ethanol and methanol permeating across the tubes are introduced into the detector by a carrier gas. Although this system can prevent the effect of foaming in aerobic processes and be used for anaerobic processes, parameters such as tube length and flow rate of the carrier gas must be optimized to obtain a suitable response and sensitivity of the sensor, even when a tube with suitable properties is selected. We introduced the exhaust gas from the fermentor directly into the sensor to simplify the system. In conclusion, when the temperature of the fermentor is kept at $30.0 \pm 0.1^\circ\text{C}$, the exhaust line is heated up by the ribbon heater, and the air flow rate is constant, the methanol concentration in the culture could be maintained within $\pm 5\%$ of the set point even when the agitation rate was increased with cell growth.

Time course of the fermentation The time-courses of fermentation in which methanol concentrations were kept at 10 and $31 \text{ g} \cdot \text{l}^{-1}$ are shown in Fig. 3. The growth of the cells in the production phase at a methanol concentration of $31 \text{ g} \cdot \text{l}^{-1}$ was slower than that at $10 \text{ g} \cdot \text{l}^{-1}$. After 60 h, the specific production rate decreased. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the culture supernatants showed that the decrease was due to proteolytic degradation of the protein (data not shown). Although it might be possible to avoid degradation by the addition of casamino acid to the medium (16) or by changing the pH of the culture (Brerley, R. A. *et al.*, US patent 5,324,639, 1994; Siegel, R. S. *et al.*, International patent application W/O90/10697), we evaluated the effects of methanol concentration on the production of $\beta_2\text{GPIdV}$ by the specific rates before the proteolysis.

Trends of the specific rates The specific rates of growth, methanol consumption and production of $\beta_2\text{GPIdV}$ were calculated from the experimental results under the methanol concentrations of 1.5, 10, 17, and $31 \text{ g} \cdot \text{l}^{-1}$ based on equations (5–7). The tendency for transition of the specific rates was almost similar at all methanol concentrations. The specific growth rates dur-

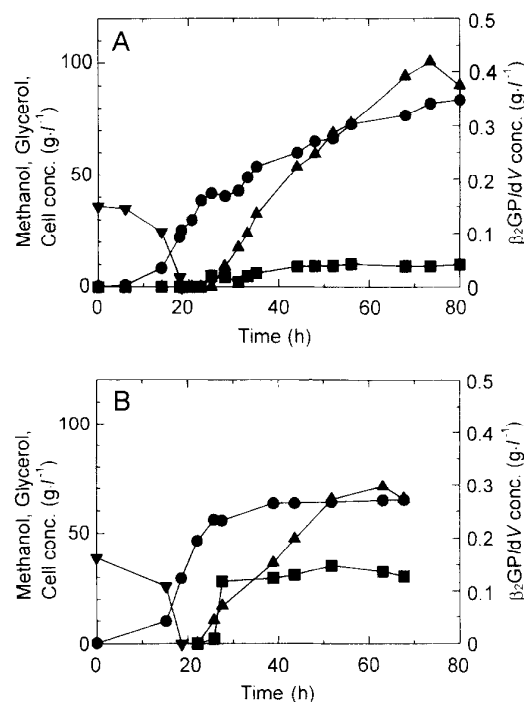


FIG. 3. Time-course of the culture. Methanol concentration in the culture during the production phase was controlled at 10 (A) and $31 \text{ g} \cdot \text{l}^{-1}$ (B), respectively. Symbols: $\bullet$, cell; $\blacktriangle$, $\beta_2\text{GPIdV}$; $\blacksquare$, methanol; $\blacktriangledown$, glycerol.

ing the transition phase were higher than those during the production phase because not only methanol but also glycerol were fed as a carbon source (Fig. 3). The specific production rates during the transition phase were also higher than those during the early production phase (Fig. 4A, squares). The decrease in specific production rates in the late production phase was due to proteolytic degradation as described above. The specific methanol consumption rates during the transition phase were slightly higher than those during the production phase (Fig. 4B, squares). These results indicate that the glycerol feeding during the transition phase did not repress methanol consumption.

Effects of methanol concentration on the specific rates The specific rates were calculated using experimental data in the early production phase in which they were affected neither by the glycerol feeding in the transition phase nor by proteolytic degradation. The relationship between the methanol concentration and the specific rate is shown in Fig. 5. Although the specific growth rate decreased gradually as the methanol concentration increased up to $17 \text{ g} \cdot \text{l}^{-1}$, it decreased markedly to 0.0009 h^{-1} at a methanol concentration of $31 \text{ g} \cdot \text{l}^{-1}$. The specific methanol consumption rate also decreased with methanol concentration, however, the rate was still $0.042 \text{ g} \cdot \text{g dry-cell}^{-1} \cdot \text{h}^{-1}$ at a methanol concentration of $31 \text{ g} \cdot \text{l}^{-1}$. In contrast, the specific production rate increased with the methanol concentration. At high methanol concentrations, cell growth was markedly inhibited and the consumption of methanol was inhibited slightly, but $\beta_2\text{GPIdV}$ production increased. The remarkable inhibition of cell growth at a methanol concentration of $31 \text{ g} \cdot \text{l}^{-1}$ appeared to be mainly due to the toxic effects of

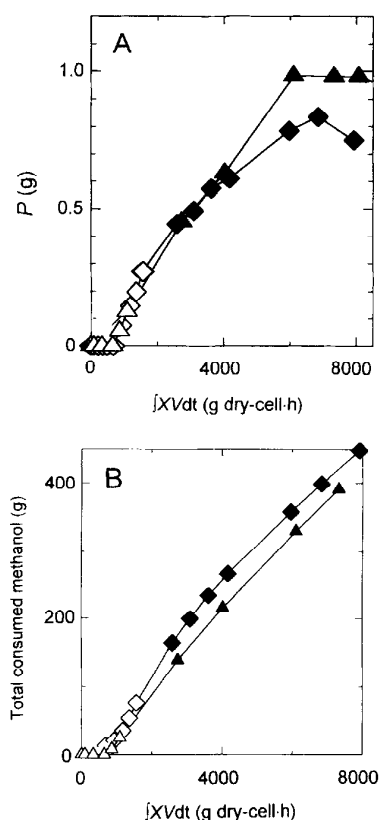


FIG. 4. Calculation of the specific rates. A, The specific production rate; B, the specific methanol consumption rate. Symbols: squares, methanol concentration controlled at $10 \text{ g} \cdot \text{l}^{-1}$; triangles, methanol concentration controlled at $5.5 \text{ g} \cdot \text{l}^{-1}$ and glycerol feeding at the rate $5.0 \text{ ml} \cdot \text{l}^{-1} \cdot \text{medium}^{-1} \cdot \text{h}^{-1}$ during the production phase. Filled symbols indicate the production phase.

methanol on DNA replication and membrane synthesis, because metabolism and protein biosynthesis were not significantly inhibited. The following three interpretations are proposed to explain the increase in $\beta_2\text{GPIdV}$ production in the presence of the high methanol concentrations.

The first is an increase of $\beta_2\text{GPIdV}$ gene transcription, the second is an increase of $\beta_2\text{GPIdV}$ permeability across the cell membrane, and the third is an increase in energy supply for $\beta_2\text{GPIdV}$ production. The transcription of $\beta_2\text{GPIdV}$ and alcohol oxidase, a key enzyme for methanol utilization, are both under the control of the *AOX1* promoter. It was reported that the specific alcohol oxidase activity decreased at methanol concentrations of greater than $20 \text{ g} \cdot \text{l}^{-1}$ (17), and the specific methanol consumption rate decreased at such high methanol concentrations (Fig. 5). Accordingly, the increase in $\beta_2\text{GPIdV}$ production could not be due to increase in the level of transcription. Although it is known that solvents including methanol permeabilize cell membranes, if permeabilization of the membrane promotes the secretion of $\beta_2\text{GPIdV}$ of a large molecular size, low-molecular-size metabolites should leak also from the cell and such leakage could seriously damage cell activities. Therefore, $\beta_2\text{GPIdV}$ production is increased neither due to an increase in the level of transcription nor by the permeabili-

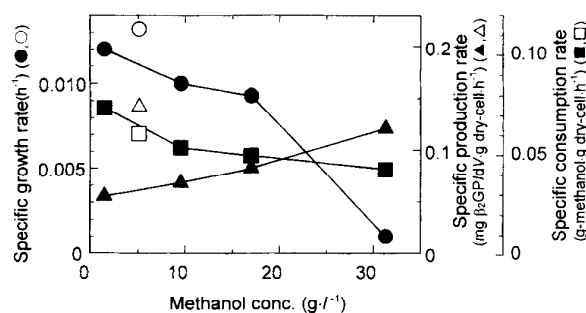


FIG. 5. Effects of methanol concentration on the specific rates. Symbols: circles, the specific growth rates; squares, the specific methanol consumption rates; triangles, the specific production rates. Clear symbols indicate the case under which the glycerol solution was fed at $5.0 \text{ ml} \cdot \text{h}^{-1} \cdot \text{l}^{-1} \cdot \text{medium}^{-1}$ during the production phase.

zation. In contrast, the third interpretation is reasonable. The energy for cell growth and $\beta_2\text{GPIdV}$ production is supplied by methanol oxidation, and the growth and production compete with each other for the energy in the production phase. When the cell growth is inhibited at high methanol concentrations, the energy for cell growth could be diverted for $\beta_2\text{GPIdV}$ production, resulting in an increase in the productivity. This interpretation is supported by the observation that the specific production rates in the transition phase (clear squares in Fig. 4A) were higher than those in the production phase (filled squares in Fig. 4A). Since glycerol was fed in addition to methanol during the transition phase, the energy supply for $\beta_2\text{GPIdV}$ production increased. Methanol is not only an inducer of but also an energy source for the production of foreign proteins. However, these results indicate that the energy from the added methanol was insufficient for the production.

Mixed feeding in the production phase The simplest method to increase the energy supply to recombinant cells is to increase the concentration of the carbon sources in the culture broth. However, if the methanol concentration is increased, cell growth would be inhibited in the case of recombinant *P. pastoris*. Although glucose was reported to repress the *AOX1* promoter (2), glycerol does not repress its expression as stated above. Therefore, the glycerol solution was fed at $5.0 \text{ ml} \cdot \text{l}^{-1} \cdot \text{medium}^{-1} \cdot \text{h}^{-1}$ throughout the production phase during which the methanol concentration was controlled at $5.5 \text{ g} \cdot \text{l}^{-1}$. The specific rates during the early production phase were almost the same (clear symbols in Fig. 5) although the glycerol feed rate was constant. The specific methanol consumption rate was almost the same as that in the absence of glycerol feeding (see also Fig. 4B). The specific growth rate at a methanol concentration of $5.5 \text{ g} \cdot \text{l}^{-1}$ as estimated from the data in the absence of glycerol feeding (filled circles in Fig. 5) was 0.011 h^{-1} . The specific growth rate in the presence of glycerol feeding (clear circles in Fig. 5) was about 20% higher than that in the absence of glycerol feeding. The specific production rate of $0.14 \text{ mg} \cdot \text{g dry-cell}^{-1} \cdot \text{h}^{-1}$ (clear triangle in Fig. 5) was 2.3 times higher than that in the absence of glycerol feeding (filled triangles in Fig. 5). In the absence of glycerol feeding, the specific production rates in the early production phase were lower than those in the transition phase (squares in Fig. 4A). In contrast, in the presence of glycerol (triangles in Fig. 4B),

the specific production rate in the early production phase was almost same as that in the transition phase. These results demonstrated that the glycerol feeding increased the energy supply and increased the productivity of foreign proteins in *Pichia* fermentation.

Conclusions The glycerol feed rate and the methanol concentration should be optimized individually for the production of each foreign protein, because the optimum conditions should depend on the species of the protein, the copy number of the gene, and how the gene(s) is introduced into the chromosome of the host cell. For example, when a gene of interest is introduced by replacement or integration, the recombinants have the *aox1* or *AOX1* genotype, that is, low or high alcohol oxidase activity, respectively. Therefore, the glycerol feed rate for replacement-type recombinants would be higher than that for integration-type recombinants, because methanol is oxidized by only the weak *AOX2* product. Furthermore, it is experimentally known that the tolerance of integration-type recombinants to methanol is usually higher than that of the replacement-type, indicating that the tolerance varies with the specific activity of alcohol oxidase.

In conclusion, (i) a simple, practical and inexpensive methanol control system was constructed, (ii) the effect of methanol concentration on the specific growth, methanol consumption and production rate in recombinant *P. pastoris* culture was first studied and (iii) it was demonstrated that the specific production rate of foreign proteins could be increased by glycerol feeding during the production phase.

NOMENCLATURE

a : cell volume per dry-cell, $l \cdot g \text{ dry-cell}^{-1}$
 F : methanol feed-rate, $g \cdot h^{-1}$
 P : β_2 GPIdV concentration in the culture, $g \cdot l^{-1}$
 S : methanol concentration in the culture, $g \cdot l^{-1}$
 S_f : methanol concentration of the feed, $g \cdot l^{-1}$
 V : volume of the culture, l
 V_s : volume of the culture supernatant, l
 X : dry cell concentration, $g \cdot l^{-1}$
 μ : specific growth rate, h^{-1}
 ν : specific methanol consumption rate, $g \text{ methanol} \cdot g \text{ dry-cell}^{-1} \cdot h^{-1}$
 ρ : specific β_2 GPIdV production rate, $g \beta_2\text{GPIdV} \cdot g \text{ dry-cell}^{-1} \cdot h^{-1}$
 Subscripts indicate the time, h

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