Improvement of Cloned α-Amylase Gene Expression in Fed-Batch Culture of Recombinant *Saccharomyces cerevisiae* by Regulating both Glucose and Ethanol Concentrations Using a Fuzzy Controller

Sumihisa Shiba, Yoshio Nishida,* Yong Soo Park,† Shinji Iijima, and Takeshi Kobayashi‡

Department of Biotechnology, Faculty of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-01, Japan

Received March 7, 1994/Accepted June 17, 1994

The effect of ethanol concentration on cloned gene expression in recombinant *Saccharomyces cerevisiae* strain 20B-12 containing one of two plasmids, pNA3 and pNA7, was investigated in batch cultures. Plasmids pNA3 and pNA7 contain the α-amylase gene under the control of the *SUC2* or *PGK* promoter, respectively. When the ethanol concentration was controlled at 2 to 5 g/L, the gene expressions were two times higher than those at 20 g/L ethanol. To increase the gene expression by maintaining both the ethanol and glucose concentrations at low levels, a fuzzy controller was developed. The concentrations of glucose and ethanol were controlled simultaneously at 0.15 and 2 g/L, respectively, in the production phase using the fuzzy controller in fed-batch culture. The synthesis of α-amylase was induced by the low glucose concentration and maintained at a high level of activity by regulating the ethanol concentration at 2 g/L. The secretory α-amylase activities of cells harboring plasmids pNA3 and pNA7 in fed-batch culture were 175 and 392 U/mL, and their maximal specific activities 7.7 and 12.4 U/mg dry cells, respectively. These values are two to three times higher in activity and three to four times higher in specific activity than those obtained when glucose only was controlled.

Key words: α-amylase production • recombinant *Saccharomyces cerevisiae* • *PGK* promoter • *SUC2* promoter • fuzzy controller • on-line glucose–ethanol analyzer • effect of ethanol on cloned gene expression

INTRODUCTION

Widely used in fermentation, *Saccharomyces cerevisiae* has also been employed in a number of instances for the production of heterologous proteins, and there is a comprehensive body of knowledge on its genetic and biological attributes. Furthermore, this microorganism is very suitable as a host because of the absence of pyrogenic toxins. For these reasons, *S. cerevisiae* is an attractive host organism for the production of useful commercial proteins.

Among the factors that influence the production rate of a gene product in a recombinant yeast system is the interaction between the host cell and the plasmid. At the cellular level, an important consideration is the influence of gene expression on host growth. High expression is desired; however, the synthesis of a cloned gene product places additional stress on the cells. This can result in a lower growth rate, lower cellular yield, and plasmid instability. The combined effect is that the overall production rate of the gene product is sometimes reduced.[30] To lessen these negative effects of cloned gene expression, plasmids with inducible promoters may be effective in maximizing gene production, because the timing and level of cloned gene product synthesis can be controlled.[6] Inducible promoters such as *trp*,[12] *pho*, p_L,[27] p_R, *tac*,[3] *lac*,[5] and a runaway plasmid[28] are well known for *Escherichia coli*, whereas in *S. cerevisiae* systems, *SUC2*,[1] *PHO5*,[16] *ADH1*,[32] *GAL1*,[2] *GAL7*,[2] and *GAL10*[13] have been developed.

To maximize gene expression with some of these inducible promoters, several processes have been proposed—the use of a fermentor equipped with a crossflow filtration system,[10] an on-line glucose monitoring and controlling system,[17] and exchange of the carbon source.[19,24] From an industrial viewpoint, selection of the most suitable inducible promoter for a particular host vector system is very important in the production of useful gene products by recombinant yeast: A strong promoter, efficient gene transcription, and good secretion of the gene product in the host vector system are required. We have investigated the effect of the promoter on the production of a heterologous protein in this yeast,[17–19,24] as well as making a comparative study of the use of three promoters, *SUC2*, *PGK*, and *GAL7*, in the production of mouse salivary α-amylase with the same signal sequence for secretion and terminator.[26]

Research[17–19,24,26] on cloned gene products from recombinant yeasts has revealed that an inhibitory metabolite, ethanol, accumulates in the culture broth during the aerobic fermentation of yeast. Production of the metabolite is due to

*On leave from Food Research Institute, Aichi Prefectural Government, Shinpukuji-cho, Nishi-ku, Nagoya 451, Japan.

† Present address: Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422, Japan.

‡ To whom all correspondence should be addressed.

CCC 0006-3592/94/091055-09

the Crabtree effect[4] and it inhibits both cell growth and product formation. It is therefore necessary to keep the concentration of the inhibitory metabolite at a low level by feeding glucose at the optimal value to restrict metabolite formation, while maintaining a relatively high growth rate. Using this strategy, with the aid of a glucose analyzer we controlled the glucose concentration at 0.15 g/L during the fermentation of recombinant *S. cerevisiae* cells, and obtained a 20-fold increase in the gene product compared with that when the glucose concentration was maintained at 10 g/L.[17] However, even when the glucose concentration was restricted at a low level in fed-batch culture, about 20 g/L of ethanol was found to accumulate in the culture broth,[26] indicating the difficulty of maintaining ethanol at a low concentration.

In recombinant *S. cerevisiae* culture, a high ethanol concentration may severely affect gene expression. However, until now there has been no control strategy available that can maintain both the ethanol and glucose concentrations at defined values simultaneously in fermentation processes, due to the fact that in many cases there are numerous difficulties in constructing an accurate mathematical model as well as to the time variation inherent in the nonlinear characteristics of a computer-controlled fermentation process. In these bioprocesses, because it is very difficult to describe simple relationships between fermentation state variables and control variables, the operation usually is successful only with experienced experts.

Under such circumstances, these kinds of processes seem to be most appropriate for the application of fuzzy control theory. Recently, fuzzy reasoning has been applied to the control of certain bioprocesses: the production of glutamic acid,[14,15] and a precursor of pravastatin sodium.[8] Also, in a previous study, we measured culture parameters such as the cell, dissolved oxygen, ethanol and glucose concentrations on-line during baker's yeast culture,[21] and using the data obtained, constructed a fuzzy controller to regulate the concentrations of both ethanol and glucose, simultaneously.[25] However, because the control performance did not come up to our expectations, we have worked on making improvements in the fuzzy controller.

This fuzzy controller was used to express a cloned gene efficiently in the cultivation of a recombinant yeast by regulating the concentrations of both glucose and ethanol at low levels. Cell growth, gene expression, and secretion of the cloned gene under the control of both the glucose and ethanol concentrations are compared with those under the control of only the glucose concentration.

MATERIALS AND METHODS

Computer-Controlled Fermentation System

The computer-controlled fermentation system was fully described in our previous article.[25] Concentrations of cells and dissolved oxygen in the culture broth were measured on-line by a turbidimeter (SSB-50, DKK Co., Tokyo)[11] and a dissolved oxygen meter (ABLE Co., Tokyo), which were interfaced with an A/D converter [AD 12-4(98), Contec Co., Tokyo] to transmit signals to a personal computer (PC9801, NEC Co., Tokyo). The time intervals for measurement of the cell and DO concentrations were 2.5 and 1.25 min, respectively (i.e., two cell and four DO measurements ever 5 min). The glucose and ethanol concentrations in the broth were measured on-line every 5 min by a glucose–ethanol analyzer (dual-cell type, PM-1000DC, Asahi Kasei Co., Tokyo), as reported previously.[29] For the simultaneous measurement of glucose and ethanol concentrations, immobilized enzymes (glucose oxidase for glucose and alcohol oxidase for ethanol) were used as biosensors. The operation of the glucose–ethanol analyzer was controlled by the personal computer interfaced with PIO 16/16(98), with signals transmitted between them through a RS232C cable. The on-line data were passed to the controller, where the feed rate of the concentrated glucose solution (200 g/L) was calculated every 1.25 min. The concentrated glucose solution was provided by a programmable peristaltic pump (AC-2120, Atto Co., Tokyo) interfaced with a D/A converter [DA 12-4(98), Contec Co., Tokyo].

Fuzzy Control Strategy

In recombinant *S. cerevisiae* cultivations, because it is very difficult to describe the simple relationship between fermentation state and control variables, the operations usually depended only upon experience. From these points of view, these cultivations seem to be the most appropriate process for applying fuzzy control theory. The control strategy, which consisted of feedforward and feedback control, is illustrated in Figure 1 and described as follows.

Feedforward Control of Glucose Feed Rate

From the mass balance equation between the feeding and consumption of glucose, the feedforward glucose feed rate (normal glucose feed rate, F^*) can be determined in accordance with the increase in the cell concentration as follows,

$$F^* \cong \frac{\mu XV}{Y_{x/s}S_0} \tag{1}$$

where μ, X, S_0, V, and $Y_{x/s}$ denote the specific growth rate, cell and feed glucose concentrations, culture volume, and cellular yield from glucose, respectively. To obtain a precise feedforward glucose feed rate, the specific growth rate was calculated from on-line data from the turbidimeter. The turbidity values measured by the turbidimeter were converted to OD_{660} values. The cell concentration, X (dry cell weight, in grams per liter), was calculated from the OD_{660} value by multiplying it by a conversion factor. μ and $Y_{x/s}$ were calculated from the data of a serial cell concentration and glucose consumption within 20 min. The initial cellular yield from glucose was assumed to be 0.5.

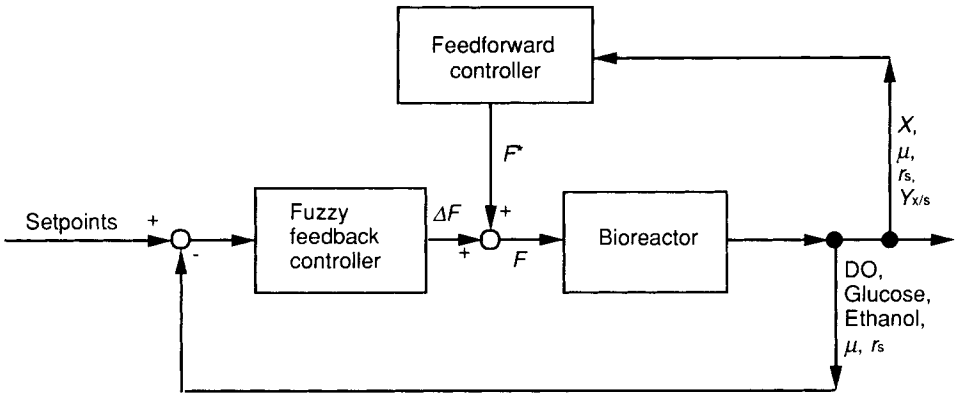

Figure 1. Schematic representation of the fuzzy control strategy: F^*, normal glucose feed rate; F, real glucose feed rate; ΔF, corrected glucose feed rate; X, cell biomass; μ, specific growth rate; r_s, specific glucose consumption rate; $Y_{x/s}$, cellular yield: DO, dissolved oxygen concentration.

Feedback Control of Glucose Feed Rate Using Fuzzy Control Algorithm

The previous determination of the normal glucose feed rate, F^*, is only a rough approximation. Because of changes in cell activity and environmental conditions, the feed rate has to be recalculated. This correction, ΔF, was determined by the fuzzy control algorithm. As a result, the real glucose feed rate was adjusted thus:

$$F = F^* + \Delta F \tag{2}$$

The environmental conditions obtained from on-line data are converted to linguistic data. For example, 0.1, 0.2, and 0.3 g/L for glucose and 1, 2, and 3 g/L for ethanol were set as "small," "medium," and "big" concentrations, respectively. Here, ΔF was decided from the production rules[25] judged by the environmental conditions written by the linguistic data. For example, if DO concentration is "big" and concentrations of glucose and ethanol are both "small," then ΔF is "positive big." To make an inference for the linguistic data like "big," "small," and "positive big" membership functions for inputs and outputs of on-line data are required. The minimum and maximum ΔF were fixed as $-F^*$ and $3F^*$, respectively, of which boundaries were based on experiences. From previous experimental results with our feedforward and feedback fuzzy controller, we found that the lower the ethanol control level, the greater the fluctuation of glucose concentration,[25] the reason being that, because the maximum ΔF is fixed, the fuzzy controller has no minor adjustment function with respect to the glucose feed rate. Because the measuring interval of our on-line analyzer could not be shortened to less than 5 min, we tried to substitute a flexible maximum (αF^*) for a fixed one ΔF ($3F^*$).

To correct the cell activity in addition to the environmental conditions rates of growth and glucose consumption according to growth phase were considered. For example, when cultures are in the late growth phase, the specific growth and glucose consumption rates usually decrease. Therefore, a high glucose feed rate resulted in an increase in the ethanol control level and a consequent fluctuation of the glucose concentration in the late phase of baker's yeast culture.[25] Because the glucose feed rate is affected by the cell growth and glucose consumption rates, we constructed membership functions for the rates of specific growth (μ) and glucose consumption (r_s), as shown in Figure 2. The α value in ΔF is varied from a to b by the production rule for the determination of α (Table I). The range of the a was selected as being from 0.5 to 1.0, while the value of b was fixed at 3. For example, although the glucose and ethanol concentrations are both at level "S," if both μ and r_s are at level "S," then α is determined as "NS" (see Table I). Fuzzy inferencing is carried out in the following manner (as an example):

If: {μ/μ_{max} is "S" and r_s/r_{smax} is "S"}
Then: α is "NS"

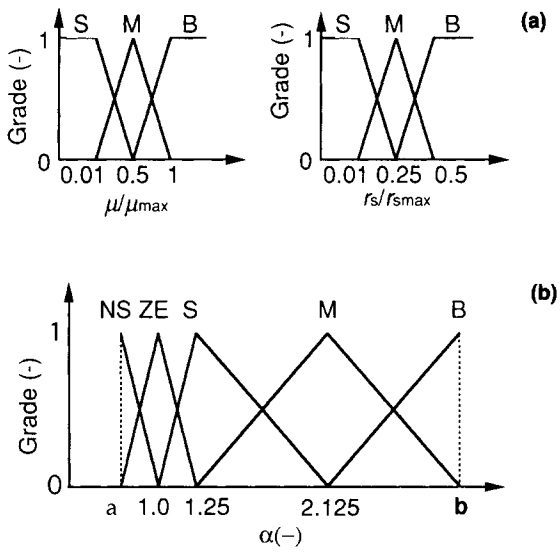

Figure 2. Membership functions of relative specific rates of growth and glucose consumption (a), and output (b). Simplified characters: B, big; M, medium; S, small; ZE, zero, NS, negative small.

Table I. Production rules for determination of α.

	r_s/r_{smax}		
	S	M	B
μ/μ_{max}			
S	NS	ZE	S
M	ZE	S	M
B	S	M	B

B, big; M, medium; S, small, ZE, zero; NS, negative small.

The inference procedure is taken from Mamdani's min–max algorithm.[20] The grade of output's fuzzy set is determined as the minimum value among the grades of input's fuzzy set by each selected rule. Using production rules, the corresponding fuzzy set and grades are determined. Defuzzification of each variable is accomplished using a simplified center-of-gravity method.[25]

Microorganism

Baker's yeast (Oriental Yeast Co., Tokyo) was used to develop the fuzzy control system. For application of the system to recombinant yeast culture, *S. cerevisiae* strain 20B-12 (*a, trp*-1, *pep*4-3) containing either of two different plasmids was used. These plasmids, pNA3 and pNA7, contain the α-amylase gene under the control of the *SUC*2 and *PGK* promoters, respectively. *SUC*2 is a regulatory gene switching over the synthesis of invertase, and *PGK* is a constitutive gene under the control of the yeast phosphoglycerate kinase. These promoters are repressed by a high glucose concentration in the growth medium and derepressed by a low glucose level. The *KIL*28 signal sequence encoded in the plasmids is the sequence from the 28-kDa killer toxin of *Klebsiella lactis* and secretes a cloned gene product into the culture medium.[31] The detailed structure was reported previously.[26]

Culture Media

The composition of the medium for baker's yeast was 10 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, and 2 g yeast extract (Difco, Detroit, MI). The glucose concentration in the medium was varied.

Three kinds of media were used for the cultivation of recombinant *S. cerevisiae* strain 20B-12 harboring the two plasmids. SD minimal medium (20 g glucose and 6.7 g yeast nitrogen base without amino acids [Difco] per liter) and YPD medium (10 g yeast extract, 20 g bacto-peptone, and 20 g glucose per liter) were used for test-tube culture and the measurement of plasmid stability, respectively. Synthetic medium contained 2 g KH_2PO_4, 2 g KCl, 1 g K_2HPO_4, 100 mg NaCl, 10 mg $MnSO_4 \cdot 4$ to $6H_2O$, 10 mg vitamin B_1, 10 mg calcium pantothenate, 5 mg inositol, 2 mg $ZnSO_4 \cdot 7H_2O$, 2 mg $Na_2MoO_2 \cdot 2H_2O$, 1 mg vitamin B_6, 0.5 mg H_3BO_3, 1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg biotin, 50 mg $CaCl_2$, 1 g $MgSO_4 \cdot 7H_2O$, 50 mg $FeSO_4 \cdot 7H_2O$, and glucose. $CaCl_2$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and glucose were added after being sterilized separately. The glucose concentration in the synthetic medium was varied. Two kinds of semisynthetic media were used for cloned gene production: the synthetic medium containing 5 g/L casamino acids (SSC medium), and the synthetic medium containing 10 g/L yeast extract (SSY medium).

Flask Cultivation

Test-tube cultures containing 10 mL SD minimal medium were inoculated from glycerol stock (1.0% v/v) and incubated in a reciprocal shaker for 20 h. The reciprocation speed was controlled at 60 strokes/min and the temperature at 30°C. Because SD minimal medium does not contain tryptophan, and the host microorganism requires this amino acid for growth, only plasmid-bearing cells can grow in the medium. A T-shaped flask containing 100 mL SSC medium was inoculated from the test tube cultures (1% v/v), and the cells were cultivated for 18 h. Batch culture was carried out in a T-shaped flask containing 100 mL SSY medium by inoculation from the cells (1% v/v) grown in SSC medium. The initial glucose concentration in the SSY medium was 10 g/L.

The effect of the ethanol concentration on the synthesis of the cloned gene product was investigated in T-shaped flask cultures in SSY medium. The culture was performed until the glucose concentration was lower than 0.5 g/L (after about 12 h). All cultures were centrifuged aseptically, and then recovered cells were suspended in 100 mL SSY medium containing ethanol at 0, 2, 5, 10, and 20 or 30 g/L. The ethanol concentrations in the T-shaped flasks were measured every 5 h, and adjusted to the desired concentration by adding 95% ethanol solution manually. All these operations were carried out on a clean bench.

Fed-Batch Culture

Two kinds of fed-batch culture were carried out: one to investigate the control performance and the other for the application of the fuzzy controller. For the fed-batch culture of baker's yeast, the initial cell concentration was adjusted to an optical density of 10 at 660 nm (OD_{660}) by adding concentrated yeast solution (an OD_{660} of 100) to the medium. The initial glucose concentration in the medium was 2 g/L. The working volume of the fermentor was 1.2 L.

Fed-batch cultures of the recombinant *S. cerevisiae* strain 20B-12 harboring either of the two plasmids were also carried out to improve the expression of the cloned gene product by means of the fuzzy controller. Cell grown in 200-mL T-shaped flask cultures with SSC medium were transferred to a jar fermentor (Iwashiya Bioscience Co., Tokyo) containing 700 mL of SSY medium. The initial glucose concentration in the culture broth was 5 g/L.

In the cultures of baker's yeast and recombinant *S. cerevisiae* strains, the dissolved oxygen concentration was measured with a dissolved oxygen concentration meter

(ABLE, Co., Tokyo) and was maintained at 2 to 5 ppm by adjusting the agitation and aeration rates. The culture temperature was maintained at 30°C and the pH at 6.0 with 3 *M* sodium hydroxide solution and 1 *M* hydrochloric acid solution by means of a pH controller (Oriental Electric Co., Tokyo). Glucose and ethanol concentrations in culture broth were monitored automatically every 5 min by the glucose-ethanol analyzer and controlled at 0.15 g/L and 2 g/L, respectively, by the fuzzy control system as described as above. After the cloned gene was induced, concentrated yeast extract solution (200 g/L) was added at a constant feed rate of 3.5 mL/h to avoid depletion of the nitrogen source in the culture broth.

Analysis

The biomass concentration (dry cell weight, g/L) was determined from the OD_{660} with a spectrophotometer (UVIDEC-310, Jasco Co., Tokyo) and by multiplying by a conversion factor of 0.21 with baker's yeast (g dry cells per liter per unit of OD_{660}) or 0.22 with *S. cerevisiae* strain 20B-12. For off-line glucose measurement, a glucose analyzer (Model 2300 stat, Yellow Springs Instrument Co., Yellow Springs, OH) was used. Ethanol concentration was measured by a gas chromatograph (GC-7A, Shimadzu Co., Kyoto) with a Chromosorb 101 column (Gasukuro Kogyo Co., Tokyo).

The definition of an enzyme unit and the measurement of mouse α-amylase have been reported previously.[23] One enzymatic unit corresponds to the amount of enzyme producing one micromole of glucose from soluble starch per minute. The specific enzyme activity is calculated by dividing the enzyme activity by the dry cell concentration. The stability of recombinant plasmids in the yeast cells was defined as the ratio of the number of colonies (plasmid-bearing cells) that could be assessed on SD minimal agar plates to the total number of colonies (viable cells) on YPD agar plates.

RESULTS

Performance of Glucose and Ethanol Concentration Regulation by Fuzzy Controller

The improved fuzzy control system described in Materials and Methods was applied to baker's yeast culture. The lower (*a*) and upper (*b*) bounds of the α value were set at 0.75 and 3.0, respectively; the results are shown in Figure 3. The α value varied according to the fuzzy rules in Table I, and the glucose and ethanol concentrations were maintained at 0.03 ± 0.07 g/L and 2.39 ± 0.29 g/L, respectively. The maximum cell concentration was 31.8 g dry cells/L. A total of 62.4 g glucose was consumed by the yeast cells. The yield coefficients of cells and ethanol from glucose ($Y_{x/s}$ and $Y_{p/s}$) were calculated as 0.51 g cells/g glucose and 0.05 g ethanol/g glucose, respectively. The $Y_{p/s}$ value decreased compared with the previous result.[25] When the lower bound of α (*a*) was set at 0.5 or 1.0, the ethanol control level either decreased or increased, respectively (see Table II). By virtue of the small standard deviation and high cellular yield, the *a* and *b* values of α were determined to be 0.75 and 3.0, respectively, in the improved fuzzy control system.

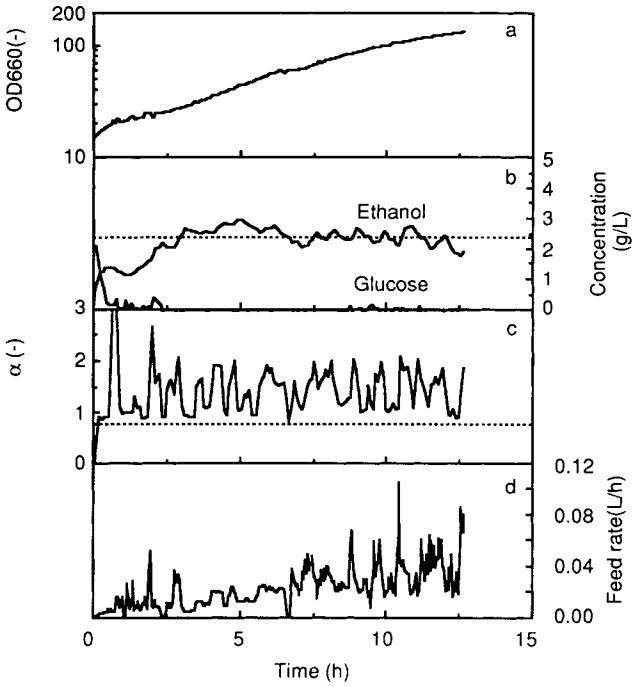

Figure 3. Cell growth (a), concentrations of glucose and ethanol (b), α value (c), and glucose feed rate (d) in fed-batch culture of baker's yeast with control of both glucose and ethanol concentrations. The culture was carried out using 0.75 as the lower bound of the α value. The dotted lines in (b) and (c) indicate the average concentration of ethanol and lower bound of the α value, respectively.

Effect of Ethanol Concentration on Cloned Gene Expression

As previously reported, when the glucose concentration was controlled at 0.15 g/L in fed-batch culture of recombinant *S. cerevisiae* 20B-12, the ethanol concentration increased to 17 ~ 20 g/L through the culture period.[26] Because this value is very high compared with that of glucose, we investigated the effect of the ethanol concentration in the production phase on cloned gene expression under the control of the *PGK* and *SUC2* promoter systems. The ethanol concentration was adjusted to 0, 2, 5, 10, and 20 g/L by adding 95% ethanol solution every 5 h in T-shaped flask cultures. In the case of the *PGK* promoter system, the cell growth, enzyme activity, and specific enzyme activity were investigated. The results are shown in Figure 4. The ethanol concentrations were relatively maintained at the desired levels. When the ethanol concentration was adjusted to 0 g/L, cell growth and enzyme production were low at 2 g/L and 18 U/mL, respectively, because of the limitation of the carbon source. The highest levels of cell growth and the

Table II. Comparison of control performance of fuzzy controller with various lower bounds of α values.

	Lower bound (a)		
	0.5	0.75	1.0
Cell concentration (g/L)	26.3	31.8	27.9
Consumed glucose (g)	62.4	62.4	62.4
Glucose control level (g/L)	0.10 ± 0.12	0.03 ± 0.07	0.03 ± 0.06
Ethanol control level (g/L)	1.95 ± 0.61	2.39 ± 0.29	2.63 ± 0.41
$Y_{cell/glucose}$	0.43	0.51	0.41
$Y_{ethanol/glucose}$	0.04	0.05	0.07

enzyme production, 9.5 g/L and 78 U/mL, respectively, were obtained when the ethanol concentration was maintained at 2 or 5 g/L.

The effects of ethanol concentration on cell growth and cloned gene expression in *S. cerevisiae* strain 20B-12 under the control of the *SUC2* promoter were also investigated, and the results are shown in Figure 5. The decrease in cell growth of *S. cerevisiae* 20B-12 at an ethanol concentration of 20 g/L was about 10%. However, enzyme activity was 13 U/mL, which was two times lower than at an ethanol concentration of 2 g/L. These results indicate that both cell growth and gene expression in the *SUC2* and *PGK* promoter systems are affected by high ethanol concentration in the production phase.

Fed-Batch Culture with Fuzzy Controller

Fed-batch culture of recombinant *S. cerevisiae* 20B-12 was carried out with the concentrations of glucose and ethanol controlled at 0.1 and 2 g/L, respectively, by the fuzzy controller. Figure 6 shows the results of the culture of recombinant yeast harboring plasmid pNA3. At a culture time of 4 h, 5 g/L of glucose was consumed, at which point the control of both glucose and ethanol began. Cell growth decreased at 5 h and was then maintained until 30 h. The ethanol and glucose concentrations were controlled at average values of 2.0 and 0.4 g/L, respectively, throughout the cultivation. The final cell concentration was 40 g/L. The maximum activity of α-amylase and specific enzyme activity were 175 U/mL and 7.7 U/mg dry cells, respectively.

Fed-batch culture of the recombinant yeast containing the *PGK* promoter was also performed and the results are shown in Figure 7. The glucose and ethanol concentrations were controlled at average values of 0.3 and 1.9 g/L, respectively. The final cell concentration was 55 g/L. The maximum α-amylase activity was 392 U/mL, and the specific enzyme activity 12.4 U/mg dry cells.

Comparison of Cloned Gene Expression in Fed-Batch Cultures with Control of both the Glucose and Ethanol Concentrations and with Control of Only the Glucose Concentration

Cloned gene expression was compared between cultures with both glucose and ethanol control and with only glucose

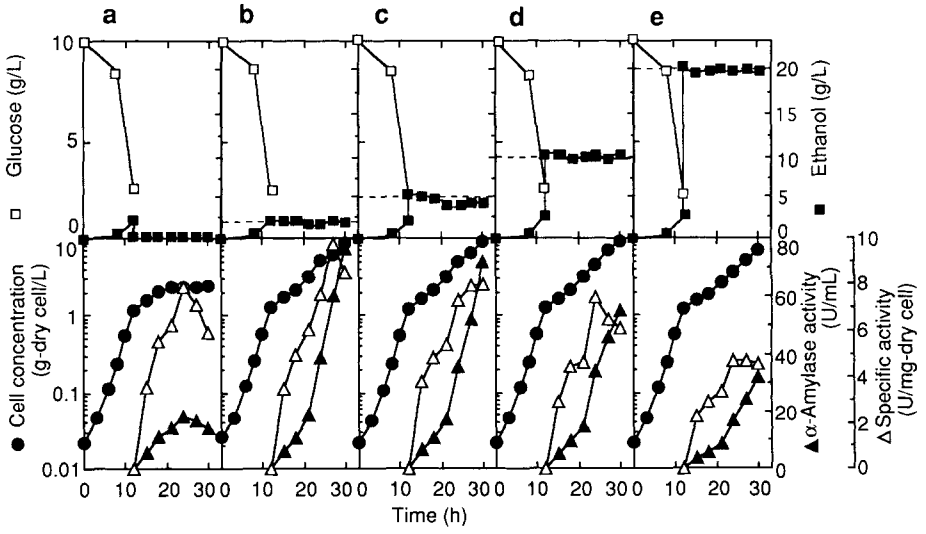

Figure 4. Batch cultures of *S. cerevisiae* 20B-12 harboring plasmid pNA7 with varying ethanol concentrations [0 in (a), 2 in (b), 5 in (c), 10 in (d), and 20 g/L in (e)]. Symbols: (□), glucose concentration (g/L); (■), ethanol concentration (g/L); (●), cell concentration (g/L); (▲), α-amylase activity (U/mL); (△), specific α-amylase activity (U/mg dry cells). Dotted lines denote the control levels.

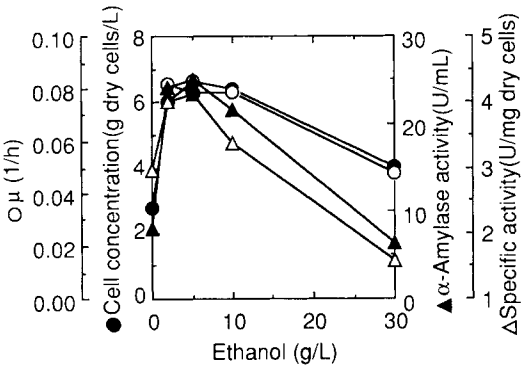

Figure 5. Effects of ethanol concentration in batch culture of *S. cerevisiae* 20B-12/pNA3. Symbols: (○), specific growth rate (h^{-1}); (●), cell concentration (g/L); (▲), α-amylase activity (U/mL); (△), specific α-amylase activity (U/mg dry cells).

concentration control[26] (Table III). With the *SUC2* promoter, the enzyme and specific enzyme activities using the fuzzy controller increased 1.9 and 3.3 times compared with those not controlling ethanol concentration, even though the cell growth was similar, whereas with the *PGK* promoter these activities were three and four times as high, respectively. The difference in the results between the two methods of control lies in the ethanol concentration. The ethanol concentration was controlled at 2 g/L in the culture using the fuzzy controller, whereas it increased to 17 or 19.8 g/L when ethanol concentration was not controlled. The fuzzy controller was thus shown to be very useful for controlling the concentrations of both glucose and ethanol.

DISCUSSION

A number of heterologous proteins expressed in *S. cerevisiae* are under preclinical or clinical trial or are on the market. To express gene products more efficiently, strong inducible promoters are employed, and the time over which product synthesis occurs is regulated to achieve a partial separation of growth and cloned gene expression. We previously compared the strength of three different promoters, *SUC2*, *PGK*, and *GAL7* with the same host strain.[26] Because *SUC2* and *PGK* are expressed at a low glucose concentration in the production phase, the glucose concentration must be controlled at a low level. However, in the bioprocess, although the glucose concentration was controlled at below 0.2 g/L, the ethanol concentration in fed-batch culture accumulated to 17 ~ 20 g/L in the strain *S. cerevisiae* 20B-12[17,18,21] and 25 g/L in the strain YNN27,[9] indicating the difficulty of restraining ethanol production. Wang et al.[33] reported that yeast cells aerobically grown in glucose-limiting medium began to produce ethanol at a glucose concentration of 0.13 g/L. On the other hand, Nanba et al.[22] found that both glucose and ethanol were metabolized at glucose levels lower than 0.07 g/L. This difference in the glucose concentration required for switching from respiration to ethanol formation appears to be due to the different strains or growth conditions used. Until now, there have been few studies on the effect of ethanol produced in recombinant yeast cultures on cloned gene expression. Our results clearly showed that α-amylase synthesis is affected by the ethanol concentration (Figs 4 and 5). Although the mechanism of the effect of ethanol has not yet been elucidated, the results indicted that control of both the glucose and ethanol concentrations at low levels is very important for improving α-amylase synthesis in recombinant yeast cultures.

As mentioned above, it was very difficult to control concentrations of both glucose and ethanol by means of control of glucose concentration alone, for the purpose of an improvement of cloned gene expression in cultivation of recombinant *S. cerevisiae* with a regulatory expression system. If it is possible to control the glucose concentration at

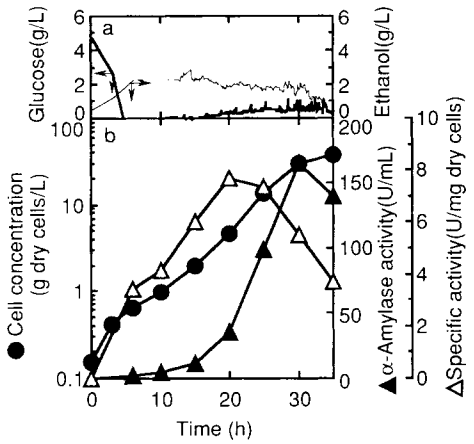

Figure 6. Expression of α-amylase gene of *S. cerevisiae* 20B-12 harboring plasmid pNA3 by controlling both the glucose and ethanol concentrations using the fuzzy controller. Bold and fine lines indicate the on-line concentrations of glucose and ethanol, respectively. Symbols: (●), cell concentration (g/L); (▲), α-amylase activity (U/mL); (△), specific α-amylase activity (U/mg dry cells).

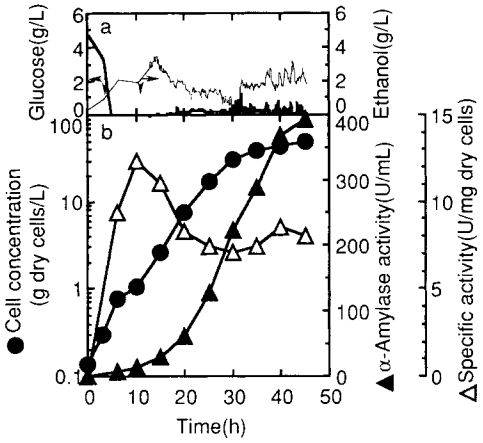

Figure 7. Expression of α-amylase gene of *S. cerevisiae* 20B-12 harboring plasmid pNA7 by controlling both the glucose and ethanol concentrations using the fuzzy controller. Lines and symbols are the same as in Figure 6.

Table III. Comparison of mouse α-amylase production in fed-batch cultures of recombinant *S. cerevisiae* 20B-12 with control of both glucose and ethanol, and of glucose only.

Promotor	Control	Glucose in culture (g/L)[a]	Ethanol in culture (g/L)	Cells (g/L)[b]	Activity (U/mL)[b]	Specific activity (U/mg cell)[b]	Reference
SUC2	Adaptive	0.26	17.0[b]	42.9	90.7	2.3	26
	Fuzzy	0.48	2.00[a]	40.0	175.0	7.7	This work
PGK	Adaptive	0.58	19.8[b]	40.3	128.6	3.2	26
	Fuzzy	0.30	1.89[a]	55.0	392.4	12.4	This work

[a]Average value.
[b]Maximal value.

as low as 0.13 or 0.07 g/L reported elsewhere,[22,33] the ethanol concentration will be maintained at a low level, but its control level will fluctuate. The reason is the metabolic time delay from glucose to ethanol, and the metabolic time delay leads to greater and greater fluctuations of glucose concentration. To overcome these problems, fuzzy reasoning was applied to control the concentrations of both glucose and ethanol under the condition of glucose control level being lower than ethanol. It is not necessary for ethanol concentration to be strictly controlled, since an ethanol concentration of 2 or 3 g/L in the culture did not inhibit gene expression (see Figs 4 and 5). The desired concentrations of glucose and ethanol may be different from strain to strain. Therefore, fuzzy reasoning appeared to be an appropriate control strategy for improving cloned gene expression in recombinant yeast cultures.

Our fuzzy controller was able to regulate the ethanol concentration at the set value in the control levels for recombinant yeasts as well as baker's yeast. In the case of different optimum levels, its control performance was successfully achieved only by setting "small," "medium," and "big" levels, respectively. Therefore, it is a very simple method to apply a different strain in the system not requiring tight control of each concentration. If it is necessary to control several substrates, products, or metabolites, a multicomponent control is available by this fuzzy reasoning with the aid of each biosensor. This fuzzy control system was thus shown to be very useful in overcoming the effect of glucose and ethanol on α-amylase synthesis in recombinant *S. cerevisiae* culture. Furthermore, we confirmed that it is also a useful means of improving the cellular yield from glucose in industrial yeast production by virtue of its good performance and simplicity.

CONCLUSION

We developed a fuzzy controller for regulating the concentrations of both glucose and ethanol, and its performance was investigated in the fed-batch culture of baker's yeast. This fuzzy controller satisfactorily controlled both the glucose and ethanol concentrations at set values. The fuzzy control strategy also was applied to the fed-batch culture of the recombinant strain *S. cerevisiae* 20B-12 containing either the *SUC2* or *PGK* promoter. The ethanol concentration was controlled at 2 g/L throughout the cultivation. Mouse α-amylase production was improved two- or three-fold compared with when only the glucose concentration was controlled. We confirmed that this system is very useful for improving cloned gene expression in recombinant *S. cerevisiae* by controlling both the glucose and ethanol concentrations.

This research was supported in part by a Grant-in-Aid for Scientific Research (No. 04453125) from the Ministry of Education, Science and Culture of Japan.

References

1. Carlson, M., Botstein, D. 1982. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. Cell **28**: 145–154.
2. Christopher, G. G., Donald, T. M., Kohno, T., Thomas, C. G. 1984. Expression of calf prochymosin in *S. cerevisiae*. Gene **27**: 35–46.
3. de Boer, H. A., Comstock, L. J., Vasser, M. 1983. The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. Proc. Natl. Acad. Sci. USA **80**: 21–25.
4. Deken, R. H. D. 1966. The Crabtree effect and its relation to the petite mutation. J. Gen. Microbiol. **44**: 157–165.
5. Edman, J. C., Hallesell, R. A., Valenznela, P., Goodman, H. M., Rutter, W. J. 1981. Synthesis of hepatitis B surface and core antigens in *E. coli*. Nature **291**: 503–506.
6. Georgiou, G. 1988. Optimizing the production of recombinant protein in microorganisms. AIChE J. **34**: 1233–1248.
7. Handa, H., Toda, T., Tajima, M., Wada, T., Iida, H., Fukasawa, H. 1987. Expression of the human adenovirus E1a product in yeast. Gene **58**: 127–136.
8. Hosobuchi, M., Fukui, F., Suzuki, T., Yoshikawa, H. 1993. Fuzzy control in microbial production of ML-236B, a precursor of pravastatin sodium. J. Ferment. Bioeng. **76**: 482–486.
9. Hsieh, J. H., Shih, K. Y., Kung, H. F., Shiang, M., Lee, L. Y., Meng, M. H., Chang, C C., Lin, H M., Shih, S C., Lee, S. Y., Chow, T. Y., Feng, T. Y., Kuo, T. T., Choo, K. B. 1988. Controlled fed-batch fermentation of recombinant *Saccharomyces cerevisiae* to produce hepatitis B surface antigen. Biotechnol. Bioeng. **32**: 334–340.
10. Iijima, S., Kawai, S., Mizutani, S., Taniguchi, M., Kobayashi, T. 1987. On–off regulation of gene expression from tryptophan promoter with cross-flow filtration. Appl. Microbiol. Biotechnol. **26**: 542–545.
11. Iijima, S., Yamashita, S., Matsunaga, K., Miura, H., Morikawa, M., Shimizu, K., Matsubara, M., Kobayashi, T. 1987. Use of a novel turbidimeter to monitor microbial growth and control glucose concentration. J. Chem. Tech. Biotechnol. **40**: 203–213.
12. Ikehara, M., Ohtsuka, E., Tokunaga, T., Taniyama, Y., Iwai, S., Kitano, K., Miyamoto, S., Ohgi, T., Sakuragawa, Y., Fujiyama, K.,

Ikari, T., Kobayashi, M., Miyake, T., Shibahara, S., Ono, A., Ueda, T., Tanaka, T., Baba, H., Miki, T., Sakurai, A., Oishi, T., Chisaka, O., Matsubara, K. 1984. Synthesis of a gene for human growth hormone and its expression in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81**: 5956–5960.

13. Jigami, Y., Muraki, M., Tajima, N., Tanaka, H. 1986. Expression of synthetic human-lysozyme gene in *Saccharomyces cerevisiae:* use of a synthetic chicken-lysozyme signal sequence for secretion and processing. Gene **43**: 273–279.
14. Kishimoto, M., Kitta, Y., Takeuchi, S., Nakajima, M., Yoshida, T. 1991. Computer control of glutamic acid production based on fuzzy clusterization of culture phases. J. Ferment. Bioeng. **72**: 110–114.
15. Kishimoto, M., Moo-Young, M., Allsop, P. 1991. A fuzzy expert system for the optimization of glutamic acid production. Bioproc. Eng. **6**: 163–172.
16. Kramer, R. A., Dechiara, T. M., Shauber, M. D., Hiliker, S. 1984. Regulated expression of human interferon gene in yeast: control by phosphate concentration or temperature. Proc. Natl. Acad. Sci. USA **81**: 367–370.
17. Lin, K. H., Iijima, S., Shimizu, K., Hishinuma, F., Kobayashi, T. 1989. Control of gene expression from the *SUC2* promoter of *Saccharomyces cerevisiae* with the aid of a glucose analyzer. Appl. Microbiol. Biotechnol. **32**: 313–316.
18. Lin, K. H., Iijima, S., Huang, S. Y., Hishinuma, F., Kobayashi, T. 1991. Effective production of heterologous protein using *Saccharomyces cerevisiae PGK* promoter. Kagaku Kougaku Ronbunsyu **17**: 680–686.
19. Lin, K. H., Iijima, S., Huang, S. Y., Hishinuma, F., Kobayashi, T. 1991. Optimization of the expression of a foreign gene from galactose-inducible promoter in a recombinant yeast. J. Ferment. Bioeng. **72**: 187–192.
20. Mamdani, E. H. 1976. Advances in the linguistic synthesis of fuzzy controllers. Int. J. Man–Machine Studies **8**: 669–678.
21. Mizutani, S., Iijima, S., Morikawa, M., Shimizu, K., Matsubara, M., Ogawa, Y., Izumi, R., Matsumoto, K., Kobayashi, T. 1987. On-line control of glucose concentration using an automatic glucose analyzer. J. Ferment. Technol. **65**: 325–331.
22. Nanba, A., Hirota, F., Nagai, S. 1981. Microcomputer-coupled baker's yeast production. J. Ferment. Technol. **59**: 383–389.
23. Nishizawa, M., Ozawa, F., Hishinuma, F. 1987. Construction of an expression and secretion vector for the yeast *Saccharomyces cerevisiae*. Agric. Biol. Chem. **51**: 515–521.
24. Ohshima, T., Zhang, X. L., Iijima, S., Kobayashi, T. 1992. Control of gene expression from the *SUC2* promoter of *Saccharomyces cerevisiae* using two carbon sources. Kagaku Kougaku Ronbunsyu **18**: 693–700.
25. Park, Y. S., Shi, Z. P., Shiba, S., Chantal, C., Iijima, S., Kobayashi, T. 1993. Application of fuzzy reasoning to control of glucose and ethanol concentrations in baker's yeast culture. Appl. Microbiol. Biotechnol. **38**: 649–655.
26. Park, Y. S., Shiba, S., Iijima, S., Hishinuma, F., Kobayashi, T. 1993. Comparison of three different promoter systems for secretory α-amylase production in fed-batch cultures of recombinant *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **41**: 845–861.
27. Remant, E., Stanssens, P., Fiers, W. 1981. Plasmid vectors for high-efficiency expression controlled by p_L promoter of coliphage lambda. Gene **15**: 81–93.
28. Remant, E., Tsao, H., Fiers, W. 1983. Improved plasmid vectors with a thermoinducible expression and temperature-regulated runaway replication. Gene **22**: 103–113.
29. Shi, Z. P., Shimizu, K., Iijima, S., Izumi, R., Matsumoto, K., Kobayashi, T. 1992. Development of dual on-line analyzer and its application to fed-batch lactic acid fermentation. J. Ferment. Bioeng. **73**: 22–25.
30. Stouthamer, A. H., van Verseveld, E. J. 1987. Microbial energetics should be considered in manipulating metabolism for biotechnological purposes. Trends Biotechnol. **5**: 149–155.
31. Tokunaga, M., Kawamura, A., Hishinuma, F. 1989. Expression of pGKL killer 28K subunit in *Saccharomyces cerevisiae:* identification of 28K subunit as a killer protein. Nucl. Acids Res. **17**: 3435–3446.
32. Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G., Hall, B. D. 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature **298**: 347–350.
33. Wang, H. Y., Cooney, C. L., Wang, D. I. C. 1977. Computer-aided baker's yeast fermentation. Biotechnol. Bioeng. **19**: 68–86.