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Maximum glucoamylase production by a temperature-sensitive mutant of *Saccharomyces cerevisiae* in batch culture

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In order to maximize the glucoamylase production by recombinant *Saccharomyces cerevisiae* in batch culture, first a temperature-controlled expression system for a foreign gene in *S. cerevisiae* was constructed. A temperature-sensitive *pho80* mutant of *S. cerevisiae* for the *PHO* regulatory system, YKU131, was used for this purpose. A DNA fragment bearing the promoter of the *PHO84* gene, which encodes an inorganic phosphate (P_i) transporter of *S. cerevisiae* and is derepressed by P_i starvation, was used as promoter. The glucoamylase gene connected with the *PHO84* promoter was ligated into a YEp13 vector, designated pKU122. When the temperature-sensitive *pho80*^{ts} mutant harboring the plasmid pKU122 is cultivated at a lower temperature, the expression of glucoamylase gene is repressed, but at a higher temperature it is expressed. Next the effect of temperature on the specific growth rate, μ, and specific production rate, ρ, was investigated. Maximum values of μ and ρ at various temperatures were at 30°C and 34°C, respectively. The optimal cultivation temperature strategy for maximum production of glucoamylase by this recombinant strain in batch culture was then determined by the Maximum principle using the relationships of μ and ρ to the cultivation temperature. Finally, the optimal strategy was experimentally realized by changing the cultivation temperature from Tμ (30°C) to Tρ (34°C) at the switching time, t_s.

Keywords: glucoamylase; *Saccharomyces cerevisiae*; *PHO* regulatory system; temperature-sensitive *pho80*^{ts} mutant; Maximum principle

Introduction

Glucoamylase (α-1,4-glucanglucohydrolase:E.C.3.2.1.3) catalyzes the release of glucose from starch and related malto-oligosaccharides [4]. The produced glucose is used as a substrate for the enzymatic production of fructose syrup and a feed source for fermentation of various microorganisms.

Aspergillus oryzae is a very important strain in food industries, such as those producing sake and soy sauce, and secretes vast quantities of hydrolytic enzymes, such as α-amylase, glucoamylase and various kinds of proteinase. Especially, in sake brewing, glucoamylase is considered to be most important, because the rate of fermentation is dependent on the activity of the glucoamylase.

The yeast *Saccharomyces cerevisiae* is probably the second most widely used host organism for the production of heterologous proteins. Although the maximum specific growth rate of yeast is much slower than that of *Escherichia coli*, it is still much faster than that of most mammalian and animal cell lines. *S. cerevisiae* is not susceptible to lytic phages and contaminating organisms at low pH, and has no known pathogenity with man and is considered a GRAS (generally recognized as safe) microorganism [3]. However, it appears that in most cases the overproduction of a foreign protein may repress cell growth and decrease the amount produced. To avoid this happening, two-stage fermentation systems for cell growth and protein production have been developed using controllable gene expression systems [3,9,14,15]. Expression at the production stage is occasionally controlled by the medium composition, but in batch cultures it has proved rather difficult in practice to control the medium composition for gene expression at the desired time, and such control usually limits cell growth [18]. An expression system regulated by the cultivation temperature [2,5,7–11,13] is thus clearly more attractive and economical.

One of the most important tasks in biochemical process synthesis using a recombinant gene expression system is to determine what host–vector and gene expression systems are most suitable for the given protein production objectives. In general, these systems should be selected when they show their ability in full, that is, under the optimal conditions [10,15]. Hata *et al* [4] reported the isolation and the characterization of the glucoamylase cDNA from *A. oryzae*, and its expression in *S. cerevisiae*. It has been shown that the host–vector system of *S. cerevisiae* was effective for expression of a cDNA encoding glucoamylase and for secretion of the enzyme with native signal peptide [16]. In this paper, we constructed a glucoamylase expression vector and a temperature-controllable expression system. We also described the maximum glucoamylase production strategy, realized the strategy experimentally and confirmed its validity.

Materials and methods

Strains and media

S. cerevisiae, YKU76 [*MATa ura3-52 trp1 leu2 his3 pho80-69*^{ts}] was used as the host strain. The *S. cerevisiae* YKU131 is a transformant of YKU76 with glucoamylase

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expression vector, pKU122 (Figure 1), which is a YEp-type vector bearing the fusion gene of the *PHO84* promoter and glucoamylase structural gene. The *PHO84* gene encodes an inorganic phosphate (P_i) transporter [19] and its expression is controlled by the *PHO* regulatory system [6,12,17,20]. In the wild-type cells, its transcription is derepressed when the P_i concentration in the medium is lower, but repressed at a higher inorganic phosphate concentration. Since the strain used in this study was a temperature-sensitive *pho80* mutant for the *PHO* regulatory system, YKU131 could be regulated by the cultivation temperature. That is, under high P_i concentration, *PHO84* gene expression of YKU131 was controlled by the cultivation temperature. Its expression at a lower temperature was repressed and at a higher temperature it was expressed. Glucoamylase production could thus be controlled by the cultivation temperature because its expression was controlled by the *PHO84* promoter.

The YPDA medium (1% yeast extract, 2% polypeptone, 2% glucose and 0.04% adenine) used for the general cultivation of yeast cells, Uracil and leucine auxotrophic test media for selection of yeast transformants with YIp5, YEp13, and their derivative plasmids were prepared with a basal medium composed of 2% glucose, 0.67% yeast nitrogen base without amino acids (Difco Laboratories, USA) supplemented with appropriate nutrients. A high-P_i nutrient and a low-P_i nutrient media based on YPDA medium were prepared as reported previously [6]. YPDA plate supplemented with 2% soluble starch was prepared

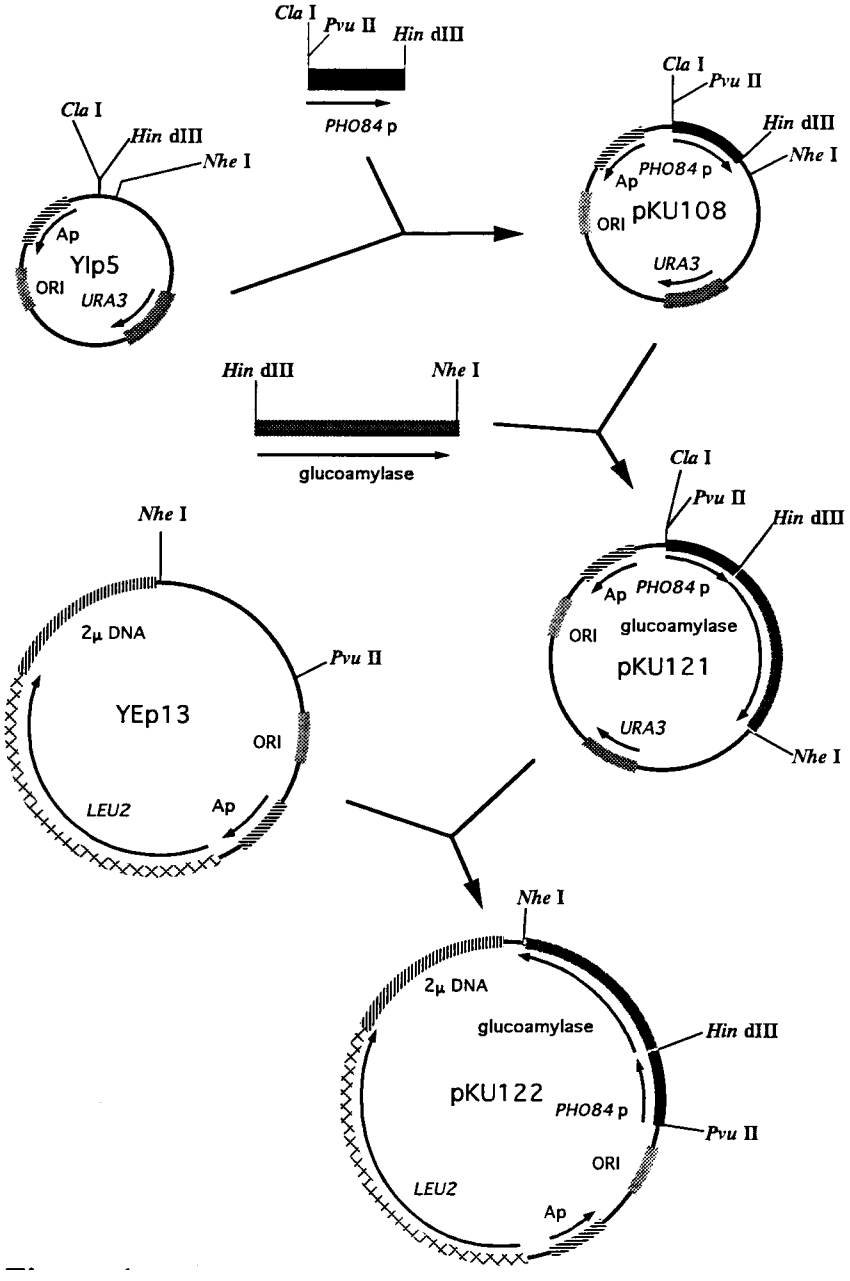

Figure 1 Construction of the glucoamylase expression vector pKU122. pKU122 contains the yeast *PHO84* promoter, 2 μm ori, *LEU2*, glucoamylase cDNA fragment, and part of the pBR322 plasmid.

as a selection medium for a starch-clearing plate assay [5]. Agar medium for plating was prepared by the addition of 2% agar.

Assay

The optical density (OD) of each culture sample was measured at 660 nm using a spectrophotometer (UV-150-02, Shimadzu Co, Kyoto, Japan) or a UV-VIS spectrophotometer (UV-1200, Shimadzu Co, Kyoto, Japan). The dry cell mass concentration was calculated from the correlation between optical density and cell mass at various temperatures. The glucose concentration was determined by using a glucose analyzer model 2700 SELECT, Biochemical analyzer, Yellow Spring Instruments, USA) after centrifugation at 3055 × *g* for 10 min. For assay of glucoamylase activity [11], the culture supernatants were incubated in the presence of 2% soluble starch (Nakarai Tesc Co, Japan) with 10 mM potassium succinate buffer (pH 5.0) for 2 h at 60°C. For a starch-clearing plate assay of glucoamylase expressed in *S. cerevisiae*, the strains grown onto YPDA plate were transferred to selection plates containing 2% soluble starch. The selection plates were stained with iodine solution containing 0.02% I_2 and 0.2% KI (w/v). The glucose generated by hydrolysis of starch was measured by the glucose analyzer. One unit of glucoamylase activity corresponds to the formation of 10 mg of glucose h^{-1}.

Culture conditions for glucoamylase production

Permanent cell stocks, in 15% glycerol, were stored at –80°C. Several days before the start of an experiment, cells were streaked on an appropriate selection plate (Leu^-). Cells from this plate were inoculated in liquid Leu^- medium for precultivation, and the main cultivation was then performed in YPDA medium. All batch culture experiments at various temperatures were conducted in 1000 ml in sakaguchi flasks (working volume: 200 ml) with a water bath shaker (MM-10, Taitec Co, Japan) at 120 rpm.

Results and discussion

Construction of a glucoamylase expression vector

The construction procedure of a glucoamylase expression vector was shown in Figure 1. A 0.9-kilobase (kb) fragment of *PHO84* gene covering nucleotide positions from –886 to –1 (the A residue of the ATG codon was taken as +1 [1]) was prepared by polymerase chain reaction (PCR; Program Temp Control System PC-700; ASTEC, Japan) with plasmid p373 [1] for a template and two oligonucleotides as primers. Forward primer is 5′-CTCATCGATCCCGGG TCTAGAAAAGTGTCA-3′ corresponding to nucleotide positions from –886 (5′ end) to –868 (3′ end) of the *PHO84* coding strand with an additional 6-bp *Cla*I and *Pvu*II restriction sequence and CTC at the 5′ end. Reverse primer is 5′-CTCAAGCTTTTGGATTGTATTCGTGGAGT-3′ of the *PHO84* DNA from positions –1 (5′ end) to –20 (3′ end) with a 6-bp *Hin*dIII sequence and CTC at the 5′ end. Then, the plasmid pKU108 was obtained (Figure 1). A 2.1-kb *Hin*dIII-*Nhe*I fragment that includes the entire glucoamylase structural gene (which was obtained from the plasmid pYGA-F [4]) from *A. oryzae* was introduced into the *Hin*dIII-*Nhe*I site (just downstream of the *PHO84* promoter (*PHO84*p)) of pKU108, and the YIp-type expression vector, pKU121, was obtained. And then a 3.0-kb *Pvu*II-*Nhe*I fragment of pKU121 that includes the *PHO84*p and the glucoamylase structural gene was introduced into the *Pvu*II-*Nhe*I gap of YEp13, designated pKU122.

Table 1 Comparison of glucoamylase activity in test tube culture at 34°C for 24 h

Strain	Glucoamylase activity (U L^{-1})
YKU76	0
YKU130 (YKU76-pKU121)	9.5
YKU131 (YKU76-pKU122)	57.5

Selection of the strain secreting glucoamylase

The recombinant plasmids pKU121 and pKU122 were introduced into *S. cerevisiae* YKU76, and YKU130 and YKU131 were obtained from the transformants, respectively. The transformants were transferred to selection plates supplemented with 2% soluble starch. After incubation at 30°C for 3 days, YKU130 and YKU131 stained with iodine solution formed a halo around the colony, while YKU76 and those transformants with plasmid YIp5 and YEp13 used as control did not (data not shown).

Then these transformants were cultivated in 10 ml of liquid YPDA medium at 34°C for 24 h with shaking, and glucoamylase activity in the culture broth was measured. The glucoamylase activity of YKU131 (ie, secretion of a 57.5 Unit glucoamylase per liter of the culture broth) was much higher than that of 9.5 U L^{-1} from YKU130, whereas no glucoamylase activity was detected in control (Table 1). For comparison of glucoamylase production at different temperatures, these transformants were streaked and grown on selection plates containing 2% soluble starch at 25°C and 37°C, respectively. The result was shown in Figure 2. When YKU131 was grown at 37°C, glucoamylase was secreted better than at 25°C in high-P_i nutrient medium as designed, but at 25°C in low-P_i nutrient medium, the

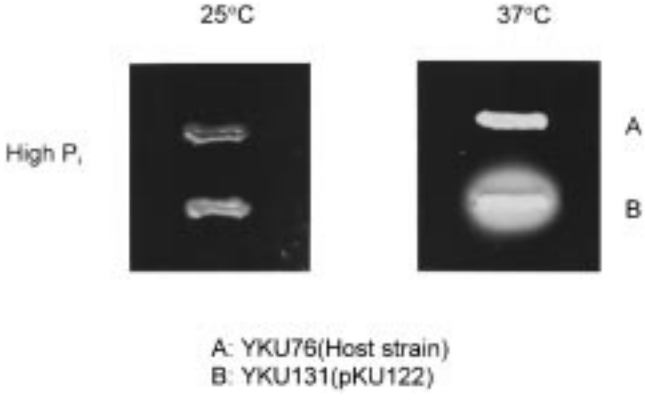

Figure 2 Temperature-dependent glucoamylase expression on selection plates of *S. cerevisiae* at 25°C and 37°C, respectively. For a starch-clearing plate assay of glucoamylase expressed in *S. cerevisiae*, the strains grown onto YPDA plates were transferred to selection plates containing 2% soluble starch. The selection plates were stained with iodine solution containing 0.02% I_2 and 0.2% KI (w/v). The clear zone indicates starch hydrolysis.

amount of glucoamylase was lower. In this experiment, glucoamylase production was under the control of the *PHO84* promoter, and the *PHO84* promoter was derepressed by the *PHO80* gene at low-P_i or *pho80*^{ts} gene at a higher temperature. Generally, cell growth of *S. cerevisiae*, YKU131, was higher at 37°C than at 25°C, and glucoamylase activity of YKU131 grown at 37°C was higher than at 25°C in the same culture volume.

Effects of temperature on the specific growth rate and the specific production rate in batch culture

Glucomylase production in YKU131 could be regulated by the cultivation temperature, as designed. In order to investigate the effects of temperature on the specific growth rate, μ, and the specific glucoamylase production rate, ρ, many experiments were performed in batch culture for strain YKU131 at various temperatures. The relationship between the temperature and specific growth rates, and the temperature and specific production rates of strain YKU131 was shown in Figure 3. Maximum values of μ and ρ at various temperatures were at 30 and 34°C, respectively.

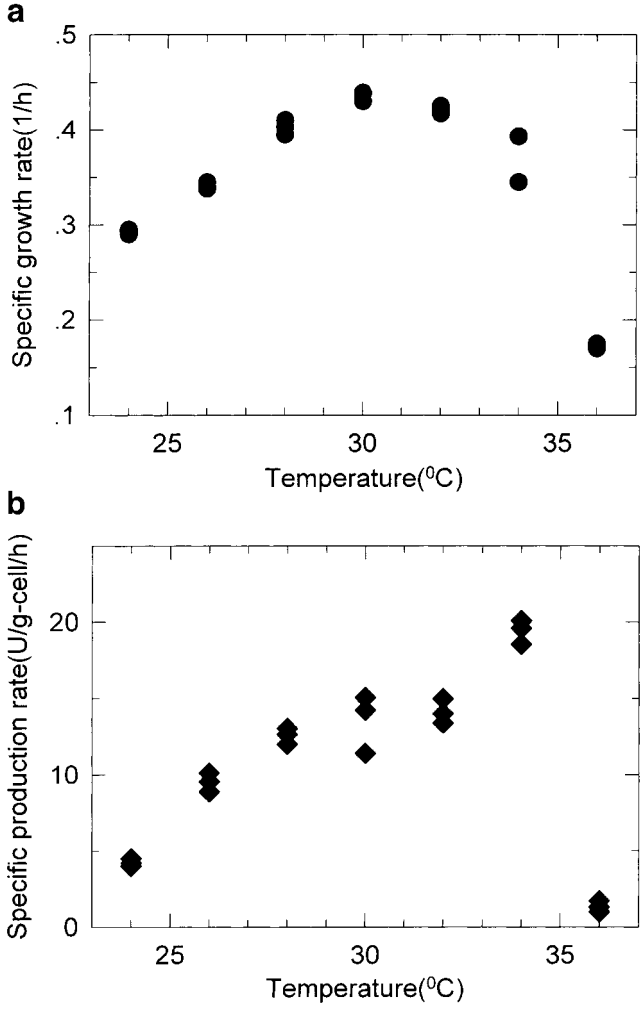

Figure 3 The effect of temperature on specific growth (a) and specific production rates (b).

Optimum cultivation temperature strategy for maximum glucoamylase production in batch culture

Finding an optimal control strategy of cultivation temperature is very important to attain the maximum production of glucoamylase. The optimal strategy was determined based on the mass balances, and the relationships of μ and ρ to the temperature, as follows.

For cell mass

$$\frac{dX}{dt} = \mu X \tag{1}$$

was satisfied, and for glucoamylase production

$$\frac{dG}{dt} = \rho X \tag{2}$$

was satisfied, X and G being the total cell concentration (g L^{-1}) and glucoamylase concentration (U L^{-1}), respectively. The specific growth and production rates were simplified as linear functions with respect to the temperature, as shown in Figure 4. The problem considered here is to maximize the amount of glucoamylase produced during the given operation times, t_f. The objective function, J, to be maximized, is the amount of glucoamylase produced in batch culture after fixed t_f hours, and can be written by integration of Eqn 2 as

$$J = \int_0^{t_f} \rho X dt \tag{3}$$

where t_f is a fixed value which should be given first.

Then, the optimal strategy for this optimization was obtained by Pontryagin's Maximum Principle. The associated Hamiltonian, H, can be represented by

$$H = \lambda \mu X + \rho X \tag{4}$$

because Eqn 2 is not required for this optimization. From the Maximum Principle, the adjoint variable, λ, should satisfy the following differential equation with the required boundary condition.

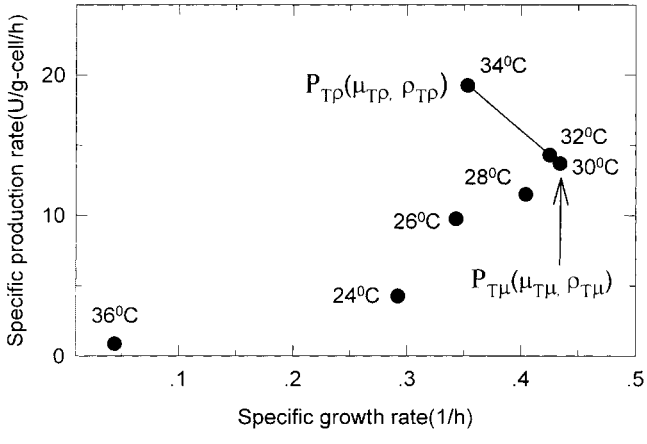

Figure 4 The relationship between specific growth and specific production rates. The data are rearranged from Figure 3.

$$\frac{d\lambda}{dt} = -\frac{\delta H}{\delta X} = -\lambda\mu - \rho \tag{5}$$

and

$$\lambda(t_f) = 0 \tag{6}$$

For easy reduction of the solution, μ is taken as the manipulating variable instead of the temperature, T [16]. Thus, the optimal strategy of μ is chosen so as to maximize the Hamiltonian, H. When the data of Figure 3 were rearranged so as to show the relationship between μ and ρ, the result was shown in Figure 4. For maximum glucoamylase production, the switching occurred only once (30→34°C) from PT_μ (μT_μ, ρT_μ) to PT_ρ(μT_ρ, ρT_ρ) and all nomenclatures for μ and ρ correspond to those in Figure 5. An optimal profile of μ for maximum glucoamylase production is shown in Figure 5. From 0 time to switching time, t_s, in order to increase cell amount which is a maximum specific growth rate, μ at the t_s should be changed from μT_μ to μT_ρ. This specific growth rate gives the maximum specific production rate ρT_ρ. The strategy of optimal pattern of μ suggested that a two-stage cultivation for growth and production was best. The first stage was interpreted as cell growth and the second was for production formation. In strain YKU131, the t_s from μT_μ to μT_ρ can also be easily derived as previously demonstrated [16] using the conditions: $\lambda(t_s) = a_1$ and $\lambda(t_f) = 0$ such that

$$t_s = t_f = \frac{\ln(b_1/\rho T_\rho)}{\mu T_\rho} \tag{7}$$

where

$$b_1 = \frac{\mu T_\mu\, \rho T_\rho - \mu T_\rho \rho T_\mu}{\mu T_\mu - \mu T_\rho} \tag{8}$$

where $-a_1$ is the slope of the line which connects PT_μ (μT_μ, ρT_μ) and PT_ρ (μT_ρ, ρT_ρ) and b_1 in the above equation is the intercept of the line on the ρ axis in Figure 5. Parameters t_f, μT_ρ, ρT_ρ, are the final time of fermentation, specific growth rate which gives the maximum production rate, and the maximum specific production rate, respectively. For YKU131, the relationship between μ and ρ was represented by

$$\rho = -a_1\mu + b_1 \tag{9}$$

To rewrite the control strategy for μ in terms of the temperature profile, the following are easily deduced. The temperature condition is given as

$$T = \begin{cases} T_\mu & (0 \le t \le t_s) \\ T_\rho & (t_s < t \le t_f) \end{cases} \tag{10}$$

where t_s is given in Eqn 7.

In order to achieve this control strategy, we should control the cultivation temperature as the real manipulating variable. At first, we should control the temperature at T_μ and after switching time, T should be changed to T_ρ in Figure 5.

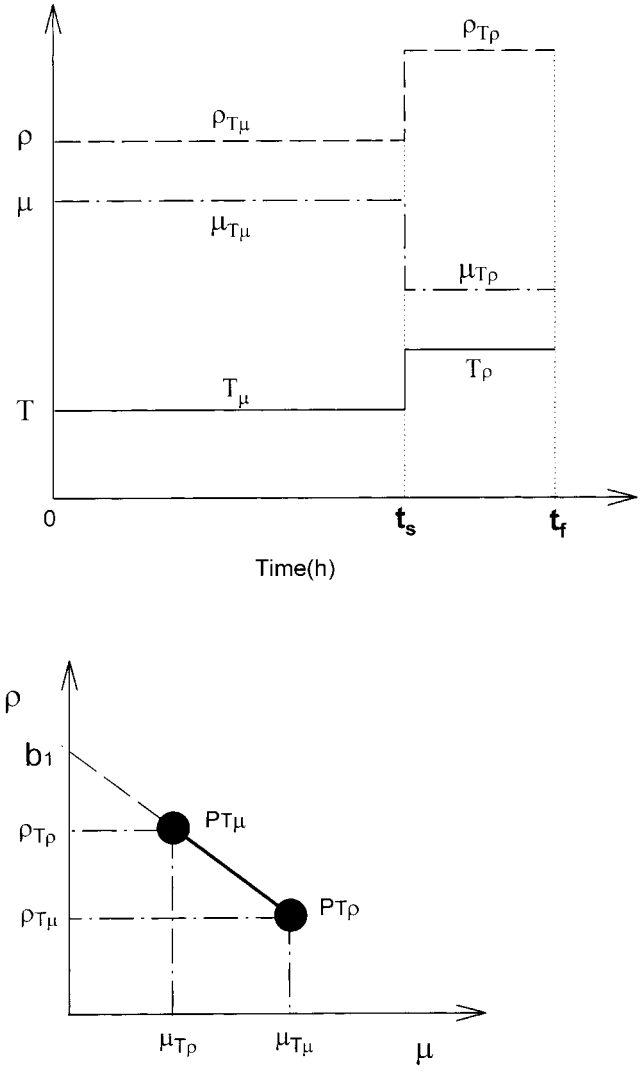

Figure 5 Optimal temperature control strategy taking account of the time of μ and ρ for maximum glucoamylase production by YKU131.

Realization of maximum glucoamylase production by experiments

The realization of the calculated optimal strategy was relatively simple and successful because the cultivation temperature could be changed easily. The result was shown in Figure 6. When the final operation time, t_f, is fixed at 10 h, the switching time, t_s, is at 8 h. The optimal cultivation time of cell growth and glucoamylase production phases was for 8 h and 2 h, respectively. After cultivation for 8 h at 30°C, the cultivation temperature was changed to 34°C. The optimal control gave 0.21 (g L^{-1}) higher cell concentration at t_s and 78.4 (U L^{-1}) higher glucoamylase production at t_f than in the case of control without a growth phase (the cultivation temperature was constant at 34°C).

Finally, the optimal solution obtained in these investigations was easily realized and was shown to be effective for maximum production.

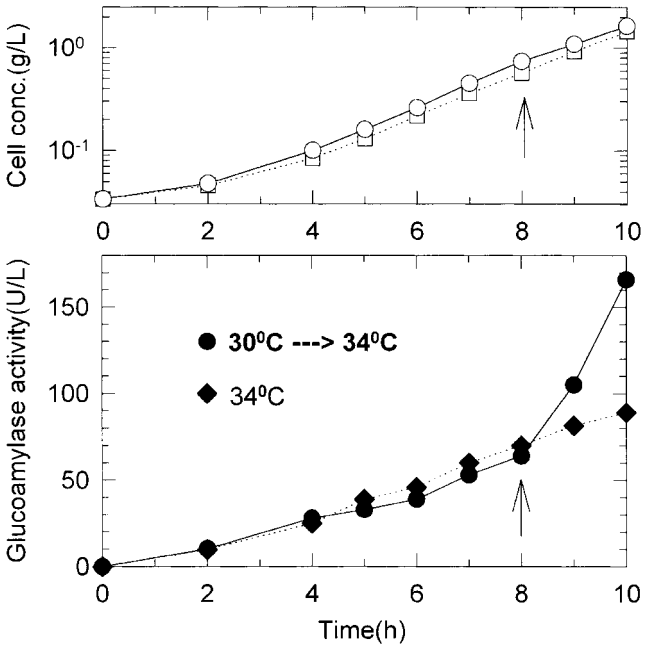

Figure 6 Time courses of glucoamylase activity and cell concentration in the experiment to realize the optimal operating strategy for YKU131. Under the optimal strategy, the temperature was changed from 30 to 34°C at 8 h. The cell concentration (○) and glucoamylase activity (●) under the optimal strategy are shown in comparison to constant temperature control at 34°C (□, cell concentration; ◆, glucoamylase activity).

Conclusion

When considering the selection and synthesis of a heterologous gene expression system, the optimal production strategy for the various candidates should be investigated and determined, because their true abilities should be compared under the best conditions. Here, a foreign gene expression system, using strain YKU131, in which expression could be controlled by the cultivation temperature, using a temperature-sensitive mutant for the *PHO* regulatory system, was constructed. With this strain, the optimal solution for maximum production in batch culture was determined based on the relationship of μ and ρ to the cultivation temperature by the Maximum Principle and was realized experimentally. Although the optimization was relatively simple because the relationships were assumed to be linear with respect to temperature, the solution is believed to be feasible, effective and sufficient to obtain the maximum production. Through this batch culture optimization, it was demonstrated that a gene expression system using a temperature-sensitive mutant is useful and easy to realize.

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