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Expression of Mouse α-Amylase Gene in Methylotrophic Yeast *Pichia pastoris*

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Abstract The expression of the mouse α-amylase gene in the methylotrophic yeast, *P. pastoris* was investigated. The mouse α-amylase gene was inserted into the multi-cloning site of a Pichia expression vector, pPIC9, yielding a new expression vector pME624. The plasmid pME624 was digested with *SalI* or *BglII*, and was introduced into *P. pastoris* strain GS115 by the PEG1000 method. Fifty-three transformants were obtained by the transplacement of pME624 digested with *SalI* or *BglII* into the *HIS4* locus (38 of Mut$^+$ clone) or into the *AOX1* locus (15 of Mut^S clone). Southern blot was carried out in 11 transformants, which showed that the mouse α-amylase gene was integrated into the *Pichia* chromosome. When the second screening was performed in shaker culture, transformant G2 showed the highest α-amylase activity, 290 units/ml after 3-day culture, among 53 transformants. When this expression level of the mouse α-amylase gene is compared with that in recombinant *Saccharomyces cerevisiae* harboring a plasmid encoding the same mouse α-amylase gene, the specific enzyme activity is eight fold higher than that of the recombinant *S. cerevisiae*.

Keywords: methylotrophic yeast, *Pichia pastoris*, mouse α-amylase gene

INTRODUCTION

Yeast *Saccharomyces cerevisiae* has been used extensively as a host for the expression of foreign genes in a number of cases, especially in fermentation, and there is a comprehensive body of knowledge on its genomic and biological background. However, the product yields of secreted proteins are low, and there are difficulties in secreting some proteins [1]. Many of the secreted proteins of *S. cerevisiae* are not found free in the medium, but rather in the periplasmic space.

Although *S. cerevisiae* has been used as a host for the expression of recombinant proteins, the methylotrophic yeast *Pichia pastoris* has become an alternative host for the production of several valuable proteins because of the high expression levels of heterologous proteins, together with the ability of the yeast to grow to very high cell densities in simple defined minimal salt media [1]. In the case of the production of tick anticoagulant peptide (TAP), the yeast *S. cerevisiae* transformants have been found to secrete the TAP in the culture medium, reaching levels of 200-250 mg/L [2], but *P. pastoris* cells have been observed to accumulate in the medium to approximately 1.7 g/L [3]. This level of expression is due to the powerful methanol-inducible *AOX1* promoter. The *AOX1* gene is tightly repressed when cells are grown in glucose or glycerol. When cells are grown in inducer (methanol), however, a single copy of the *AOX1* gene can produce alcohol oxidase up to 30% of total cell proteins [4]. *P. pastoris* secretes heterologous proteins through a secretory pathway by holding α-factor prepro leader peptide in the upstream of a foreign gene [5]; moreover, it permits post translational modifications such as proteolytic maturation, glycosylation, native folding, and disulfide bond formation [6].

The secretary expression of the mouse α-amylase gene has been investigated previously in recombinant *S. cerevisiae* [7]. In this paper, as a part of the strategy to express a foreign gene simply and efficiently, we describe an alternative secretory *P. pastoris* expression vector containing the mouse α-amylase gene. We also discuss differences in gene expression between *Pichia* and *Saccharomyces* expression systems using the same gene.

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MATERIALS AND METHODS

Yeast Strain Culture

The *P. pastoris* host strain used in all experiments was GS115 (*his4*) (Invitrogen Co., Carlsbad, CA, U.S.A.). Tube and flask cultures were grown in 10 and 50 mL of buffered minimal glycerol-complex (BMGY) medium, respectively. For the induction of recombinant gene, buffered minimal methanol-complex (BMMY) medium was used. The BMGY medium contained the following (per liter): 10 mL glycerol, 6.7 g yeast nitrogen base w/o amino acids (Difco Laboratory Inc., De-

troit, MI, U.S.A.), 10 g yeast extract (Oriental Koubo, Tokyo, Japan), 20 g Polypepton (Nihon Seiyaku Co. Ltd., Tokyo, Japan), and 0.4 mg biotin. The BMMY medium contained the same ingredients as the BMGY medium, but with 10 mL methanol instead of glycerol. The pH in the two media was adjusted to 6.0 prior to steam sterilization. Cells were grown in a 30°C reciprocal shaker at 110 strokes per min. For the induction of the recombinant gene, cells grown for 24 h in 10 mL or 50 mL of the BMGY medium were harvested and resuspended in the BMMY medium, and then the cultures were carried out at 28°C in a rotary shaker at 120 rpm.

To select His$^+$ transformants a SD plate containing 20 g/L of glucose and 6.7 g/L yeast nitrogen base w/o amino acids was used.

Screening for methanol utilization plus (Mut$^+$) and methanol utilization slow (Mut^S) strains was performed by streaking single colonies on minimal methanol agar (MM) plates containing 0.5% methanol and 6.7 g/L yeast nitrogen base w/o amino acids.

Vector Construction

The 1.6-kbp fragment encoding the mouse α-amylase gene was isolated from plasmid pNA3 [7]. The pNA3 plasmid was digested with both *Sal*I and *Xba*I, and the resulting termini were converted to blunt ends with the Klenow enzyme and deoxyribonucleotides. On the other hand, an expression vector pPIC9 (Invitrogen Co., San Diego, CA, U.S.A.) was digested with *Eco*RI, and the termini were also converted to blunt ends. The vector pPIC9 is composed of the *AOX1* promoter, 3'*AOX1* transcriptional terminator (TT), and a multi-cloning site into which a foreign gene is inserted. Moreover, the *P. pastoris* histidinol dehydrogenase gene (*HIS4*) and 89 amino acid prepro leader sequences of α-mating factor from *S. cerevisiae* were used for selection in *P. pastoris his4* hosts and for the direct secretion of heterologous protein from *P. pastoris*, respectively. The resulting blunt-ended fragments containing the α-amylase gene were ligated with pPIC9, previously blunt-ended, using T4 ligase, and yielding α-amylase expression vector pME624. Conditions recommended by the suppliers were used for the endonuclease and ligase.

Transformation of *P. pastoris*

E. coli cells were transformed using the pME624 plasmid, and four strains resistant to ampicillin (100 μg/mL) were then selected. The presence of the α-amylase gene in the four transformants was confirmed by colony hybridization. Linearized pME624 fragment digested with *Sal*I or *Bgl*II was transformed into the *P. pastoris* strain GS115 by the PEG1000 method [8]. Five to twenty μg sample of DNA was added into a still-frozen tube of competent *P. pastoris* cells and incubated at 37°C for 5 min. 1.5 mL sample of buffer A containing 40% polyethylene glycol 1000 (Sigma, U.S.A.) and 2 M bicine (pH 8.35) was added gently and incubated in a 30°C water bath for 1 h. The mixture was centrifuged at 3,000 rpm for 10 min; the supernatant was then discarded, and 1.5 mL of buffer B containing 15 M NaCl and 10 mM bicine (pH 8.35) was added. The resulting solution was then rinsed several times. Finally, the obtained cell pellet was resuspended in 0.2 mL of buffer B, spread onto a SD plate and incubated at 30°C for 2-3 days. The His$^+$ transformants were recovered on SD plate.

Screening for Mut$^+$ and Mut^S strains was performed by spreading colonies on minimal methanol agar (MM) plates, and incubating at 30°C for 3 days. Because the Mut^S transformants do not produce alcohol oxidase, they can not efficiently metabolize methanol as a carbon source and therefore grow poorly on MM plate. This slow growth on methanol can be used to distinguish His$^+$ transformants in which the *AOX1* gene has been disrupted (His$^+$ Mut^S) from His$^+$ transformants with an intact *AOX1* gene (His$^+$ Mut$^+$).

DNA Anaylsis

Chromosomal DNA from *Pichia* transformants was prepared as described for *Saccharomyces* [9]. PCRs were performed under the general reaction conditions recommended by the supplier using a PTC System (PC-800, ASTEC, Hukuoka, Japan). Primer 5'*AOX1* (GACTGGTTCCAATTGACAAGC) and 3'*AOX1* (GCAAATGGCATTCTGACATCC) were purchased from Hitachi Instruments Service Co. Ltd. (Tokyo, Japan).

Five to ten micrograms of chromosomal DNA was restricted with *Sal*I or *Bgl*II and electrophoresed on a 0.9% agarose gel. The DNA was blotted onto a nylon membrane. Prehybridization, hybridization, detection, and randomly primed DIG-labeled probe preparation were carried out according to the Boehringer Mannheim Genius System (Indianapolis, IN, U.S.A.). The DNA probe for Southern analysis was the 1.6-kb *Sal*I or *Bgl*II segments of pME624 containing the α-amylase gene and the *AOX1* promoter, and was labeled randomly with the DIG DNA labeling and detection kit from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The hybridization was carried out using the DIG-labeled probe. The probe hybridization, washing, and the development of autoradiograms were carried out as recommended by the supplier (Cat. No. 1093-657, Boehringer Mannheim, Indianapolis, IN, U.S.A.).

α-Amylase Assay

The culture broth was centrifuged at 6,000 rpm for 5 min. The supernatant was used as an enzyme source. α-Amylase activity was determined by a modified method described by Nishizawa *et al.* [10]. 0.25 mL of enzyme sample diluted by phosphate buffer was added to 0.25 mL of 2% soluble starch, and the reaction mixture was incubated at 30°C. At 5 min, the reaction mixture was withdrawn and added to an equal volume of dinitrosalicylic acid reagent. The tubes were placed in a boiling water bath for 5 min and cooled to room temperature, at which point 4 mL of water was added to each tube. The amount of reducing sugar was then determined by measuring the absorbance at 540 nm using a spectrophotometer U-2001 (Hitachi Co. Ltd., Tokyo, Japan). One enzyme unit corresponds to the amount of

enzyme producing one micromole of glucose per min.

To determine the molecular weights of recombinant protein, SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli [11] with a 12% separating gel and a 4% stacking gel. A mid-range protein molecular weight marker was purchased from Promega (Madison, WI, U.S.A.).

Methanol Concentration

The methanol concentration in flask cultures was measured by gas chromatography with a flame-ionized detector (Shimadzu GC-14B, Shimadzu, Kyoto, Japan). A glass column (3 mm × 2 m) was packed with PEG-20M 20% uniport O HP 60/80 (Shimadzu, Kyoto, Japan). The temperatures of the column, injection port, and detection chamber were 200, 130, and 130°C, respectively. The gas pressures of nitrogen, hydrogen, and air were 140, 65, and 75 kPa, respectively.

RESULTS

Construction of Mouse α-Amylase Gene Expression Vector and Transformation

The α-amylase gene was inserted between the 89-amino acid *S. cerevisiae* prepro α-mating factor secretion signal and the *AOX1* (TT) gene of pPIC9 to give rise to pME624. The vector also carried the *Pichia HIS4* gene for selection in a *his4⁻* host strain. Fig. 1 shows two ways in which the α-amylase gene-expression cassette can be inserted into the host genome using this vector. By digesting pME624 with *Bgl*II located in the 3'*AOX1* region, the *AOX1* promoter, α-amylase gene, 3'*AOX1*(TT), *HIS4* and sequences even further downstream of 3'*AOX1* were transplaced into the chromosomal *AOX1* locus of *P. pastoris* GS115 (Fig. 1(a)). The chromosomal *AOX1* gene was disrupted by this recombination, and therefore only Mut^S mutants should have occurred. Another method involved linearizing pME624 with *Sal*I located in the *HIS4* region. Since the gene-insertion event occurs between the *his4* locus in the chromosome and the *HIS4* gene on pME624, Mut^+ recombinants were generated (Fig. 1(b)).

A number of the transformants were then selected for a quantitative assay of the expression of the α-amylase gene. During screening from the transplacement transformations, 38 transformants of Mut^+ clone and 15 transformants of Mut^S clone were obtained (Fig. 2). Twenty-four transformants of the Mut^+ clone and three of the Mut^S clone showed higher than 15 units/mg cell in specific α-amylase activity, respectively. However, fourteen transformants of the Mut^+ clone and seven of the Mut^s clone were lower than 2 units/mg cells in specific α-amylase activity, respectively.

DNA Analysis

To confirm whether or not the *P. pastoris* transformants contained the α-amylase gene, PCR assay was performed (Fig. 3). The α-amylase gene was not de-

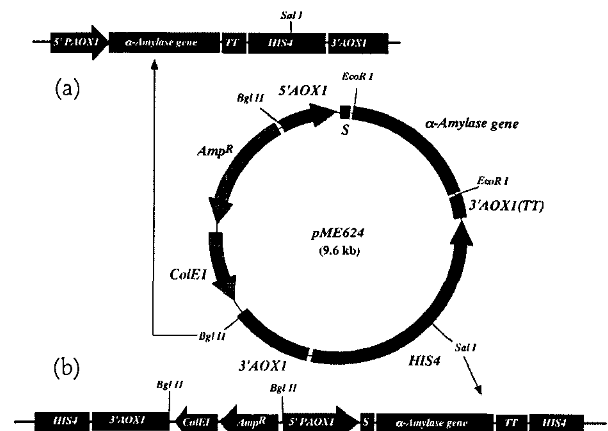

Fig. 1. Map of expression vector pME624 showing the two modes of chromosomal integrations: (a) transplacement of the expression cassette into *AOX1*, (b) integration of the whole vector into *HIS4*.

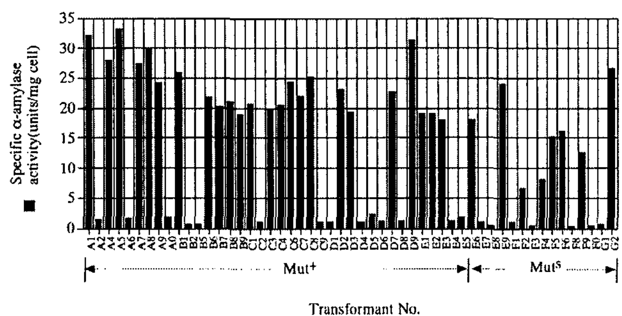

Fig. 2. Test-tube culture of transformants of pME624. Transformants were cultivated in 5 mL of BMGY medium for 2 d. Grown cells were harvested, resuspended in BMMY medium for induction of the cloned gene, and then cultivated at 28°C for 1 d. Transformants A1-E5 were the Mut^+ clone; while E6-C2, were the Mut^S clone.

tected in the genome of the A2 transformant. PCR assay revealed that transformants with a lower activity than 2 units/mg cells such as B5 and C9 did not show the expected band of the α-amylase gene. However, transformants higher in enzyme activity than 5 units/mg cells showed the expected bands, indicating the presence of the 1.6-kbp α-amylase gene in the chromosome (Fig. 3).

Analysis of genomic DNAs of seven transformants with α-amylase activity higher than 25 units/mg cell from the Mut^+ clone and four transformants with α-amylase activity higher than 15 units/mg cell from the Mut^S clone was carried out by Southern blotting. *Bgl*II-digestion of pME624 showed a 7.2-kbp band, while *Sal*I-digestion of pME624 showed a 9.6-kbp band, as expected. All the chromosomal DNA showed a 9.6-kbp band when digested with *Sal*I, except for the A2 transformants (negative control) and E9. The thick bands in the transformants A5, A7, A8, and D9 might be due to the recombination in more than two sites or tandem repeat on the chromosome of *P. pastoris*. However, two

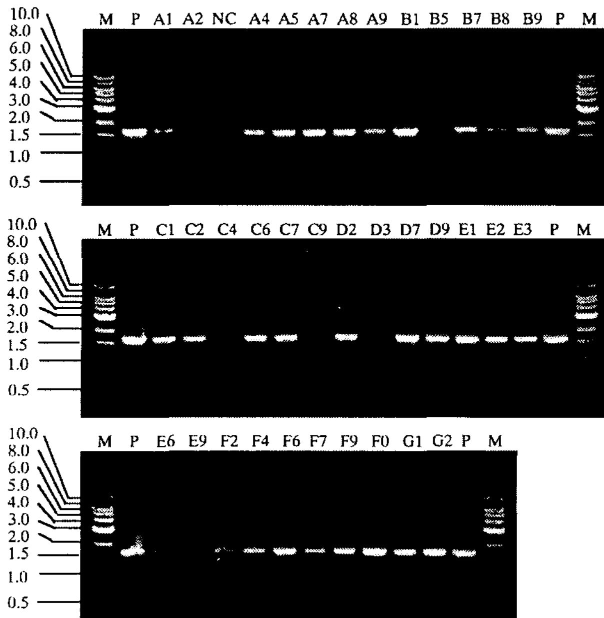

Fig. 3. PCR products show the expected presence of the α-amylase gene in the transformant. Lane M, marker; lane P, PCR product of pME624; A1-G2, transformants of pME624; NC (negative control), transformant of pPIC9.

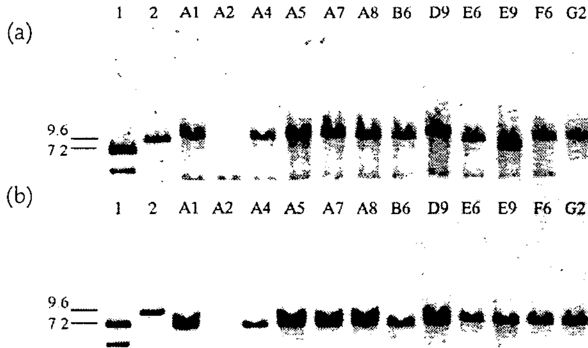

Fig. 4. Southern blot analysis of chromosomal DNA from the eight transformants of the Mut$^+$ clone and the four of the Mut^S clone. The DNA was cut with *Sal*I (a) or *Bgl*II (b), and the filter was hybridized with the α-amylase probe. Lanes 1 and 2 denote fragments of *Bgl*II-digested and *Sal*I-digested pME624, respectively. Lanes A1-D9 indicate the DNA from the Mut$^+$ clone and lanes E6-G2, from the Mut^S clone. Transformant A2 represents the negative control.

bands of 9.6 and around 7.2 kbp are present in the E9 transformant. Though the reason is not clear, the E9 transformant showed high α-amylase activity (Fig. 1). In the case of *Bgl*II-digested chromosomal DNA, except for A2, a 7.2-kbp band was observed. The A4 or E6 transformants might indicate recombination in a single site of the chromosome. The pattern in Fig. 4 demonstrates that the transformants contained the α-amylase gene in the chromosomal DNA.

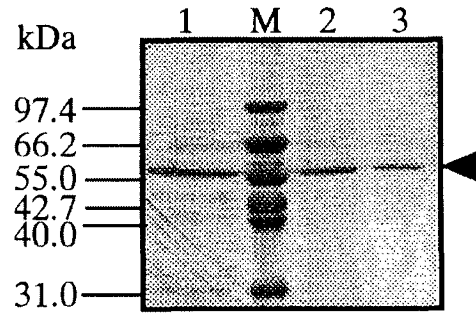

Fig. 5. SDS-PAGE stained with Coomassie blue. Culture broth of transformant G2 was analyzed by SDS-PAGE. Lane 1, supernatant 12.5 μL of transformant G2 culture was applied; lane 2, 10 μL; lane 3, 5 μL; lane M, size marker (kDa).

Table 1. Cell growth and α-amylase production of transformants

Clone	Transformant	Cell concentration (g/L)	α-Amylase activity (U/mL)	Specific α-amylase activity (U/mg cell)
Mut$^+$	A1	7.1	115.4	16.3
	A4	7.1	88.5	12.5
	A5	7.1	53.9	7.6
	A7	7.1	115.9	16.3
	A8	6.9	112.0	16.2
	D9	6.9	72.2	10.5
	E6	7.1	84.4	11.9
Mut^S	E9	7.6	91.5	12.0
	F6	7.4	90.0	12.2
	G2	6.0	134.2	22.4

Cells grown in BMGY medium were inoculated into BMMY medium and cultivated in flask culture at 28°C in a rotary shaker. After 2 d of culture, 1% of methanol was added to the cultures for the induction of the cloned gene.

After removing *P. pastoris* cells from fermentation culture, supernatant containing α-amylase was analyzed by SDS-PAGE, and the gel was stained with Coomassie blue. As shown in Fig. 5, a single protein band (lanes 1, 2 and 3) was visualized. The size was 57 kDa, which coincided with the original band cloned in *S. cerevisiae* [10] and with the protein expressed specifically in the salivary gland [12].

Selection of *P. pastoris* Transformants with a High-level Expression of α-Amylase

Seven transformants among the Mut$^+$ clones and three among the Mut^S clones were subjected to a second screening, which was performed in a shaker flask. Although the cell concentrations were similar, the α-amylase activity depended on the individual transfor-

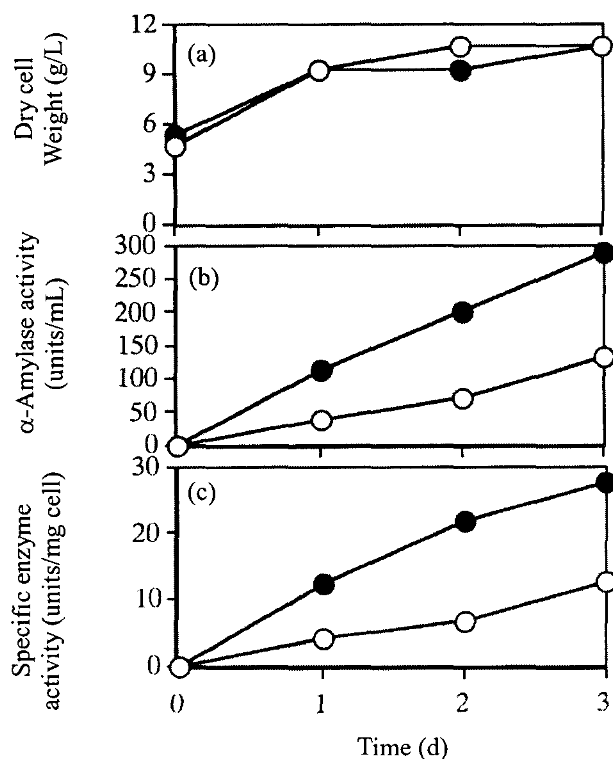

Fig. 6. Comparison of α-amylase production between A7 (O) and G2 (●) transformants. Cells grown in 50 mL of BMGY medium were centrifuged and resuspended in 50 mL of BMMY medium containing 3% methanol. Panels A, B, and C denote the time courses of cell concentration, α-amylase activity, and the specific activity, respectively.

mant (Table 1). Among the Mut^+ clones, A1, A7 and A8 exhibited higher activity than the others. In the case of the Mut^S clones, transformant G2 had the highest value. At this point, A7 and G2 transformants were chosen for further fermentation experiments.

The cells were grown in BMGY medium for cell production for 1 d and harvested and then transferred to BMMY medium for the induction of the α-amylase gene. As shown in Fig. 6(a), the difference in cell concentration between the two transformants was negligible. However, in the case of G2 the α-amylase activity reached 290 units/mL, while for A7 the activity was only 132 units/mL. The G2 transformant produced 2.2 times higher α-amylase activity than A7 did. The specific α-amylase activity of G2 was 27.4 units/mg cell, which was more than the value in the second screening and was also 2.2-fold higher than that of A7.

DISCUSSION

Several host-vector systems for the secreted production of heterologous protein in yeast have been reported. In yeast, since the culture techniques are well known, to obtain a high-level expression of a foreign gene, a choice of strong, regulated promoters is required. We have investigated secreted mouse α-amylase from *S. cerevisiae* in systems with the same host and gene construction except for the promoter [7].

Table 2. Comparison of mouse α-amylase production among various promoters used in the yeast

Strain	Recombinant *S. cerevisiae* 20B-12			Recombinant *P. pastoris*	
				G2	A7
Promoter	*SUC2*	*PGK*	*GAL7*	*AOX1*	
Plasmid	pNA3	pNA7	pNA9	non	
Induction method	Decreased glucose concentration		Switch carbon source to galactose	Addition of methanol	
Max. cell concentration (g/L)	8.8	8.5	9.7	10.6	10.6
Max. α-amylase activity (U/mL)	21	28	14	290	132
Max. specific α-amylase activity (U/mg cell)	3.4	3.5	2.8	27.4	12.5
Overall enzyme production rate (kU/L/h)[a]	0.7	0.9	0.5	4.0	1.8
Reference	7			This work	

Cited data are those of batch cultures.
[a] Maximum enzyme activity divided by culture time.

In this research, the recombinant *P. pastoris* and *S. cerevisiae* harboring the same mouse α-amylase gene were evaluated in the secreted production of heterologous protein. The results are shown in Table 2. In the case of *S. cerevisiae*, three strong promoters were used. Decreasing glucose concentration in the cultures induces *SUC2* and *PGK* promoters. Switching the carbon source to galactose and methanol, respectively, induces *GAL7* and *AOX1* promoters. The cell concentrations of *P. pastoris* were slightly higher than those of *S. cerevisiae*. The mouse α-amylase activity of *P. pastoris* G2 was ten times higher than that of the recombinant *S. cerevisiae*. *S. cerevisiae* has been found to have certain limitations as a host for heterologous protein expression. For example, product yields are usually low; except for a few notable exceptions, yields of heterologous proteins reach a maximum of 1-5% total protein, even with a strong promoter. Furthermore, because the synthesis of a cloned gene product places additional stress on the cells, the use of autonomously replicating expression plasmids has usually been found to result in poor plasmid stability during the production runs. This can result in lower growth rates and yield and reduce the overall production rate. To minimize the deleterious effects, inducible plasmid promoters are often employed,

which make it possible to separate partially cell growth and cloned gene expression. For example, during the period of gene induction, it is possible to increase the yield of protein production by maintaining the glucose concentration at a low level (0.15 g/L). We have reported that the α-amylase activity increased to 392 U/mL in a fed-batch culture in which the concentrations of glucose and ethanol were maintained at 0.1 and 2 g/L, respectively [13]. Although it is possible to achieve these low concentrations, process control units, on-line monitoring and control instruments, and robust software are required to control the concentrations of these nutrients. Until now, the instrumentation for the on-line control and monitoring of carbon sources has not been available, or, if it has been available, it is very expensive and not easily implemented on-line.

The recombinant *P. pastoris* is an attractive alternative host because it is easily grown to high cell density using minimal media, induction of gene expression is easily done by only the addition of methanol, moreover high-yield protein production can be achievable due to the strong *AOX* promoter. Many foreign genes have been successfully expressed in *P. pastoris* through secreted expression [14,15]. Difference in mouse α-amylase production yield between G2 (Mut^S) and A7 (Mut^+) transformants was negligible in test-tube cultures, but increased in flask cultures. The reason for this difference is not yet clear, although it is probably due to the difference in oxygen transfer between tube and shaker cultures.

We expect that the G2 transformant could be a powerful producer of heterologous gene product. Using the same gene, mouse α-amylase, the production level of heterologous protein from recombinant *P. pastoris* was ten times higher than that of recombinant *S. cerevisiae*. The high cell-density culture and improve-ments in heterologous protein production using this G2 should be studied further.

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