Journal of Microbiology and Biotechnology
Vol. 4, No. 1, 7-12, 1994

Construction of a Transformed Yeast Strain Secreting Both α-Amylase and Glucoamylase for Direct Starch-Fermentation

KIM, KEUN* AND JUNG-WHA LEE

Institue of Genetic Engineering, The University of Suwon, P.O. Box 77, Suwon 445-743, Korea

A yeast strain secreting glucoamylase was transformed with an expression vector (pMS12) containing the promoter of yeast alcohol dehydrogenase I gene *ADC1*, mouse salivary α-amylase cDNA, and a segment of yeast 2 μm plasmid. The transformed strain could produce ethanol from starch (4%, w/v) through a direct one-step process with the conversion efficiency of 93.2%, during 5 days of fermentation, while the original, untransformed strain exhibited a conversion efficiency of 38.1% under the same condition. When the regulatory site of the *ADC1* promoter region was removed, the production of ethanol increased to 29~37% in the presence of exogenous 3%(v/v) ethanol in the fermentation medium.

The production of industrial ethanol from starchy biomass commonly employs a three-step process: (i) liquefaction of starch with an endoamylase such as α-amylase, (ii) enzymatic saccharification of the liquefied starch to produce fermentable sugars such as glucose and maltose, and (iii) fermentation of the sugars by yeasts. The conventional yeasts belonging to *Saccharomyces* are devoid of α-amylase and glucoamylase, and the commercial enzymes used for starch degradation result in a significant cost in the production of fermentation alcohol. Therefore, the development of a yeast strain capable of fermenting starch directly in a one-step fermentation has attracted considerable attention in the recent years.

Saccharomyces diastaticus is very closely related to *S. cerevisiae* genetically, but unlike *S. cerevisiae*, *S. diastaticus* secretes glucoamylase and therefore is able to ferment starch. Glucoamylase secretion is determined by the presence of one or more unlinked glucoamylase structural genes in *S. diastaticus* (5, 11, 14, 18). Three such genes, *STA1*, *STA2*, and *STA3*, have been identified (5, 18) and cloned (5, 11, 13, 14, 23). Laluce and Mattoon (10) evaluated a variety of *S. diastaticus* strains for the direct conversion of starch and dextrins to ethanol. By using a selected hybrid strain, upto 80% conversion of Lintner starch was attained after 12 days of fermentation. The residual 20% carbohydrate represents some sort of limit dextrin which is refractory to the hydrolysis by *S. diastaticus* glucoamylase.

Several laboratories have introduced heterologous α-amylase genes derived from various organisms, into *S. cerevisiae* to produce transformants which secrets active heterologous α-amylase into the culture medium (6, 15, 19, 20, 23). However, there has been no report on efficient ethanol production directly from starch. Kim *et al.* (8) reported that the transformed *Saccharomyces* cells which secrete both yeast glucoamylase and mouse α-amylase were constructed and that the transformed cells could carry out direct, one-step assimilation of starch, with the conversion efficiency greater than 93% during a 5-day growth period. However, the report does not have any data on the direct ethanol production from starch.

The present report describes the construction of an efficient yeast strains which secretes both α-amylase and glucoamylase, and consequently is able to ferment starch directly in a single step.

MATERIALS AND METHODS

Yeast Strains

The yeast strains belonging to genus *Saccharomyces* are listed in Table 1 along with their genotypes and sources.

Plasmid

Plasmid pMS12 (20) containing the cDNA coding for mouse salivary α-amylase in yeast expression vector

*Corresponding author
Key words: transformed yeast, α-amylase, glucoamylase, starch-fermentation

Table 1. Genotypes and sources of yeast strains used

Yeast strain	Genotype	Source
SHU32a	*a leu2 trp1 ura3*	M. Breitenbach, Austria
SHU32α	*α leu2 trp1 ura3*	M. Breitenbach, Austria
1403-7A	*a ura3 trp1 MAL4*	J. Mattoon, USA
Spx11-22B	*a ade arg4 leu1 thr1 STA1*	J. Polania, USA
Spx15-3D	*a leu1 thr1 STA1*	J. Polania, USA
CL1-17B	*a ade6 his2 STAa*	J. Mattoon, USA
5301-17B	*α lys7 STA3*	H. Tamaki, Japan
SX4-6A	*a ura3-1 ura3-2 his3 trp1-289 ade2*	J. Johnson, USA
SD2-A8/C5	*α ade(1 or 2) STA*	J. Mattoon, USA

[a]Contains one or more *STA* gene of undefined genetic locus.

pMA56 (22), was generously provided by Karl K. Thomsen, Carlsberg Laboratory, Valby, Denmark. The expression vector contained the *Escherichia coli* origin of replication and the β-lactamase gene of pBR322, a segment of yeast 2 μm DNA containing the origin of replication, the yeast *TRP1* gene, and the promoter of the alcohol dehydrogenase I gene *ADC1* (Fig. 1). In chimeric plasmid pMS12, the α-amylase cDNA had been inserted by means of an *Eco*RI linker downstream of position −14 of the *ADC1* gene. The cDNA included the mouse α-amylase signal peptide, which is 15 amino acid residues long. Plasmid pMS12 was maintained in *E. coli* C600 SF8 grown in an LB medium with an ampicillin concentration of 50 μg/ml. Amplification and extraction of the plasmid were performed as described by Sambrook *et al.* (16).

Construction of pMS12ΔR Plasmid

From pMS12, the region of ADHI gene promoter lying upstream from a *Sph*I site located at position −410 from the initiation codon was removed and named pMS 12ΔR. This part of the promoter is responsible for the repression of ADHI gene expression by glucose or ethanol (1).

To construct a pMS12ΔR without the regulatory region, the pMS12 was completely digested with *Sph*I, which resulted in a 1350 bp and a 8650 bp DNA fragment. The 8650 bp DNA fragment was separated by electroelution, purified, and self-ligated using T4 DNA ligase. All the procedures for DNA manipulation were done following by the methods described by Sambrook *et al.* (16).

Media

Maltose color-indicator agar (MCIA) was composed of 1% yeast extract, 2% Difco peptone, 2% maltose, 1.5% agar and an appropriate amount of NaOH plus an appropriate amount of 1% bromothymol blue in 95% ethanol.

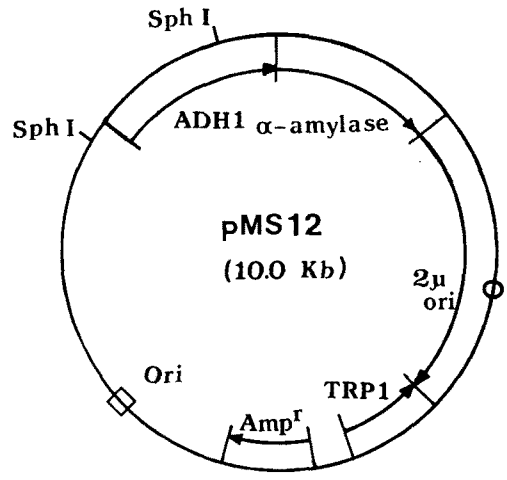

Fig. 1. Genetic map of pMS12 plasmid.

Genetic Methods

Since the pMS12 plasmid contains a *TRP1* transformation selection marker, the recipient yeast should carry a *trp1* auxotrophic marker. Construction of the recipient yeast strains containing both *STA* (glucoamylase) gene and the *trp1* marker was done by crossing *S. cerevisiae* SHU32a, SHU32α, or SX4-6A with various strains of *S. diastaticus*, as described by Mortimer and Hawthorne (12). After the treatment of asci with glusulase (Du Pont Co., Wilmington, Del., USA), spores were isolated by micromanipulation or by plating them on a YPD medium, incubating for 2 days, and then selecting those which were significantly smaller than the diploid colonies as putative haploid colonies. Haploids containing the *trp1* marker were then tested for the presence of the *STA* gene by the ability of colonies to produce halos on YPD1S3 plates after incubation for 5 days followed by refrigeration at 4°C for 2 days. Glucoamylase-producing strains produced clear halos in a turbid background, as described by Laluce and Mattoon (10). Mating-type tests and auxotrophic marker analysis were performed as described by Sherman *et al.* (17).

Maltose Assimilation Test

Since the main product of starch-hydrolysis by α-amylase is maltose (3, 20), the yeast strains which are able to assimilate maltose very efficiently were selected. The assimilation of maltose is dependent on maltose and the maltose uptake system.

The various yeast strains were tested for maltose-assimilation by plating the cells (size of inoculum, 5 mm) on MCIA and incubating the plate for 6 days to see the color change from blue to yellow.

Transformation

Yeast cells were transformed by the method of Ito *et al.* (7), and the transformants were selected on a

minimal medium lacking tryptophan.

Fermentation

The cells from slant stock culture were activated on the YPD or SD agar plate for 3 days. Two loopfuls of cells were inoculated in 10 m*l* of YPD or SD broth and incubated for 1~2 days. The culture broth was transferred to 90 ml of YPS4 broth which was then fermented anaerobically.

Ethanol Determination

Ethanol production was determined enzymatically by a modified method of Bernet and Gutman (2). Each reaction mixture (3.22 m*l*) contained 75 mM sodium PP_i, 21 mM glycine, 75 mM semicarbazide·HCl, 1.35 mM β-NAD(Sigma No. N-7004), ethanol (0 to 0.0012%, w/v), and 0.16 mg of lyophilized alcohol hydrogenase (Sigma. No. A-7011). The pH of the reaction mixture was 9.0, and the reaction temperature was 37°C. The reaction was initiated by adding 0.1 m*l* of an appropriately diluted ethanol-containing sample to the reaction tube. After a 25-min incubation, the reduced NADH produced was determined by measuring the absorbance at 340 nm. Prior deproteinization of the fermentation fluid by perchloric acid was not required for measuring ethanol. A standard curve was made by using standard ethanol solution (Sigma, No. 330-20) for each set of assays performed. The conversion rate of ethanol from starch was calculated by using the following equation:

$$\text{Conversion rate}(\%)=\frac{\text{Ethanol formed (g)}}{\text{Starch(g)}\times 1.05\times 0.511}\times 100$$

Table 2. The recipient yeast strain constructed

Strain		Genotype	Parent strains
K82	__[a]	*lys7 trp1 ura3 STA2*	*SHU32a*×5301-17B
K83	__	*lys trp1 STA2*	〃
K84	__	*lys STA2*	〃
K87	__	*his2 leu2 trp1 ura3Δ STA*[b]	SHU32α×CL1-17B
K88	__	*his2 leu2 STA*	〃
K91	__	*ade2 his3Δ lys7 ura3Δ trp1-289 STA3*	SX4-6A×5301-17B
K92	__	*lys7 trp1-289 STA3*	〃
K94	a	*leu2 trp1 ura3Δ STA*	SHU32a×SD2-A8/C5
K95	a	*leu2 trp1 STA*	〃
K97	__	*ade6 trp1 ura3Δ*	SHU32α×CL1-17B
K98	__	*ade6 his2 trp1 ura3Δ STA*	〃
K99	__	*leu2 trp1 STA1*	SHU32α×Spx11-22B
K100	α	*leu2 trp1 ura3Δ STA1*	〃
K102	α	*leu1 or 2 trp1 STA1*	SHU32α×Spx15-3D
K109	__	*lys7 tra1 STA3*	SHU32α×5301-17B
K114	a	*ade6 his2 trp1 ura3Δ STA*	SHU32α×CL1-17B

[a] The mating type was not determined, and the strain is probably homothallic.
[b] Contains one or more *STA* gene of undefined genetic locus.

The amount of glucose which could have been formed from starch after a complete hydrolysis was calculated by multiplying 1.05 to the amount of starch. And the amount of ethanol which could have been formed from glucose was calculated by multiplying 0.511 to the amount of glucose fermented. Therefore, starch (g) ×1.05×0.511 represents the amount of ethanol which can be formed from starch after a 100% hydrolysis and subsequent 100% conversion to ethanol.

RESULTS AND DISCUSSION

Consruction of Recipient Yeast Strain

Various yeast strains containing both *STA* (glucoamylase) gene and the transformation selection marker, *trp1*, were obtained. Their genotypes were determined and the results are shown in Table 2.

Selection of Maltose-assimilating Strains

Various yeast strain secreting glucoamylase were subjected to maltose-assimilation tests and the results are shown in Table 3. 1403-7A with *MAL4* gene was used as the positive control. The different abilities in maltose-assimilation were determined by the degree of the color

Table 3. The degree of the color-change in maltose color-indicator agar(MCIA) by various yeast strains containing *STA* gene[a]

Yeast strain	Color change
SHU32a	+[b]
1403-7A[c]	+++
SPx11-22B	−
Spx15-3D	−
CL1-17B	+++
K82	−
K83	−
K84	−
K87	+
K88	−
K91	+
K92	−
K94	+++
K95	++
K97	+
K98	+++
K99	+
K100	+++
K102	+
K109	++
K114	+++

[a] Each yeast strain was inoculated onto MCIA and incubated for 6 days at 30°C.
[b] Where +++ represent excellent color change, ++ good, + slight, and − no change.
[c] This strain is positive control containing MAL4 gene.

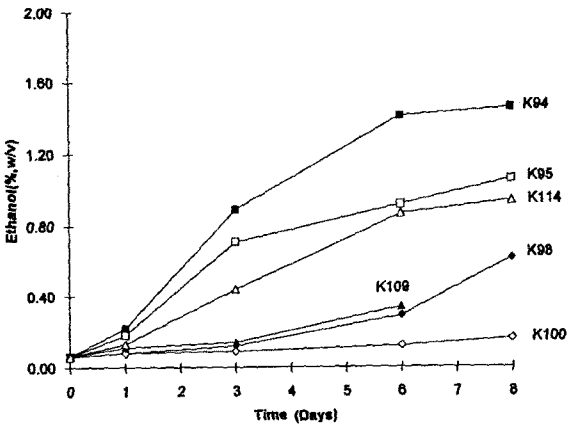

Fig. 2. Time course of starch fermentation by various strains of yeast secreting glucoamylase.
The fermentation broth (YPS4) initially contained 4%(w/v) soluble potato starch.

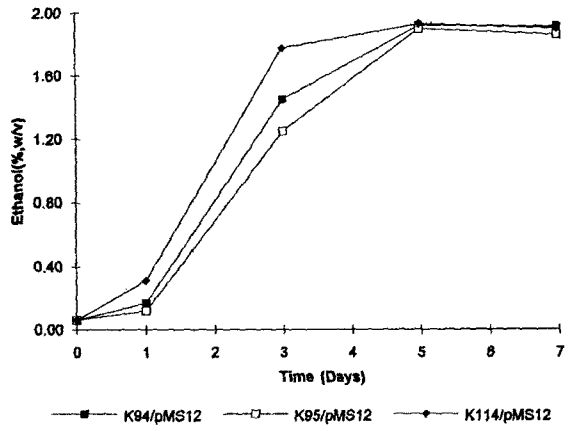

Fig. 3. Time course of starch fementation by various strains of yeast transformant secreting both α-amylase and glucoamylase.
The fermentation broth (YPS4) initially contained 4%(w/v) soluble potato starch.

change in MCIA agar plates. Among the strains tested, K94, K98, K100, and K114 showed excellent maltose-assimilating ability, and K95 and K109 showed good ability.

The fast maltose-assimilating yeast strains will be more useful in ethanol fermentation, since the fast maltose-assimilation rate will shorten the fermentation time (21).

Starch-fermentation by Yeast Secreting Glucoamylase

Except CL1-17B with no *trp1* selection marker, all of the strains showing good and excellent maltose-assimilating ability were tested for starch-fermentability which is dependent on their glucoamylase activity (Fig. 2). K94 produced 1.5% (w/v) ethanol from 4% soluble starch during 8 days and therefore showed best starch-fermentability. K95 was second and the next were K114, K109, and K98. K100 strain produced the smallest amount of ethanol from starch.

Starch-fermentation by Yeast Transformants Secreting Both α-Amylase and Glucoamylase

Three strains, K94, K95, and K114, were selected and transformed by pMS12. The results for starch-fermentation by the three yeast transformants secreting both α-amylase and glucoamylase simultaneously are shown in Fig. 3. Although all of the three transformants produced 2.0% (w/v) ethanol during 5 days, their initial fermentation rates were different. K114/pMS12 showed the fastest fermentation rate and the next one was K94/pMS 12 and the third one was K95/pMS12. Previously K114 secreting only glucoamylase, produced less ethanol than K94 and K95 (Fig. 2). However the result in Fig. 3 shows that K114/pMS12 secreting both α-amylase and glucoamylase exhibited a faster ethanol production rate than K94/pMS12 and K95/pMS12. The reason behind the results seems to be that the K114 strain has more α-amylase activity than K94 and K95, and therefore is more appropriate as a recipient for the pMS12 and expression of α-amylase gene.

The difference in fermentability between K94 and K95 seems to be caused by the different glucoamylase activity between the two strains, since the two strains were originated from same parents and the K94 shows better fermentability with or without α-amylase than K95 strain.

All of the three transformants in Fig. 3 produced 2.0% (w/v) ethanol from 4% starch with 93.2% conversion rate, and there was no residual starch in fermentation broth when judged by the iodine-test. It is clear that the introduction of α-amylase gene into the yeast strain significantly increased the rate of starch-fermentation, since the original untransformed strain exhibited only 38.1% of starch-conversion rate under the same condition, as shown in Fig. 2. Kim *et al.* (8) also reported that α-amylase and glucoamylase acted cooperatively and the transformants which secreted both types of amylase could utilize starch much more completely than the strain which secreted either α-amylase or glucoamylase.

Ethanol Production by the Transformant Harboring pMS12ΔR

The alcohol dehydrogenase I (ADHI) is synthesized during fermentative growth and is repressed about five-fold during growth on an ethanol-containing medium (4). The region of the *ADC1* gene promoter lying upstream from an *Sph*I site located at position −410 from the initiation codon is known to be responsible for repression of *ADC1* gene expression by ethanol or glucose (1). Beier and Young (1) reported that with 3% ethanol present in the culture medium, the ADH activity before

Table 4. The production of ethanol from starch by the cells of amylolytic yeast K94 and its transformants harboring pMS 12 or pMS12ΔR *plasmids*

Plasmid	Ethanol(%, w/v) produced[a]
No plasmid	3.00
pMS12	3.42
pMS12ΔR	3.60

[a] Each yeast strain was grown in 10 ml of SD broth containing the necessary supplements for 2 days at 30℃ in a rotary shaker at 250 rpm and the 10 ml culture containing 13 mg cell (as dry weight) was inoculated into 100 ml of YPS10 and fermented anaerobically at 30℃ for 5 days.

Table 5. The production of ethanol from starch by the cells of yeast K94 and its transformants harboring pMS12 or pMS 12ΔR plasmids in the presence of ethanol[a]

Plasmid	Ethanol(%, w/v) produced[b] after		
	3 days	5	9
No plasmid	0.83	1.68	2.73
pMS12	1.58	2.88	4.14
pMS12ΔR	2.03	3.93	5.43

[a] Each yeast strain was grown in 300 ml of broth containing 3%(v/v or 2.78%, w/v) ethanol for 4 days at 30℃ in a rotary shaker at 250 rpm and 19 mg cell (as dry weight) was inoculated into 100 ml of YPS20 containing 3%(v/v) ethanol and fermented anaerobically at 30℃.
[b] Ethanol(%, w/v) produced = Ethanol content − 3%(v/v, or 2.78%, w/v)

and after the removal of regulatory site was 1,300 mU/mg and 8,300 mU/mg, respectively. Again, in the presence of 8% glucose the ADH activity before and after the removal of regulatory site was 2,200 mU/mg and 4,300 mU/mg, respectively.

From the above backgound, the regulatory site of *ADC1* gene was removed in order to continuously express the α-amylase gene in the medium containing either glucose or ethanol, or both.

The ethanol production from starch by the transformant containing pMS12 or pMS12ΔR in the presence or absence of 3% (v/v) ethanol was examined, and the results are shown in Table 4 and Table 5. With the ethanol absent in the fermentation medium, there was no distinct difference between the ethanol production of pMS12 and pMS12ΔR (Table 4). However, in the presence of ethanol, the transformant containing pMS12 ΔR produced 29~37% more ethanol than that harboring pMS12 during the entire 9 days of fermentation indicating that the expression of α-amylase gene in pMS 12ΔR was not inhibited by the exogenous 3% ethanol. Recently Kumagai *et al.* (9) reported that alcohol oxidase promoter from *Pichia pastoris* was used to express α-amylase in *S. cerevisiae* and the promoter was not inhibited either by ethanol or glucose. But the inhibition was tested only in low concentrations of ethanol (0.5%) or glucose (5%).

During the ethanol fermentation, in the presence of exogenously added ethanol and ethanol produced by yeast in the medium, α-amylase and glucoamylase still functioned well. After 9 days of fermentation, the residual starch contents in the fermentation medium for no plasmid, pMS12, and pMS12ΔR were 2, 0, and 0% (w/v), respectively, and at the same time, the ethanol concentrations were 5.1, 6.5, and 7.8%(w/v), respectively (Table 5). It would be interesting to test the amylase activity at higher concentrations of ethanol.

Acknowledgement

We thank K.K Thomsen for providing plasmid pMS12. This work was supported by "the R & D Program for Alternative Energy" of the Ministry of Trade, Industry, and Resources, Korea.

REFERENCE

1. Beier, D. R., and E. T. Young. 1982. Characterization of a regulatory region upstream of the *ADR2* locus of *S. cerevisiae*. *Nature*. **300**: 724-728.
2. Bernet, E. and I. Gutmann. 1974. Ethanol Determination with alcohol dehydrogenase and NAD. p.1499-1502 In H. U. Bergmeyer(ed.), Methods of enzymatic analysis, vol. 3 Academic Press, Inc., New York.
3. De Mot, R., and H. Verachfert. 1985. Purification and Characterization of extracellular amylolytic enzymes from the yeast *Filobasidium capsuligenum*. *Appl. Environ. Microbiol.* **50**: 1474-1482.
4. Denis, C. L., J. Ferguson, and E. T. Young. 1983. mRNA levels for the fermentative alcohol dehydrogenase of *Saccharomyces cerevisiae* decrease upon growth on a nonfermentable carbon source. *J. Biol. Chem.* **258**: 1165-1171.
5. Erratt, J. A., and A. Nasim. 1986. Allelism within the DEX and STA genes families in *Saccharomyces diastaticus*. *Mol. Gen. Genet.* **202**: 255-256.
6. Filho, S. A., E. V. Galembeck, J.B. Faria, and A. C. S. Franscino. 1986. Stable yeast transformants that secrete functional α-amylase encoded by cloned mouse pancreatic cDNA. *Bio/Technology* **4**: 311-315.
7. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**: 163-168.
8. Kim, K., C. S. Park, and J. R. Mattoon. 1988. High-efficiency, one-step starch utilization by transformed *Saccharomyces* cells which secrete both yeast glucoamylase and mouse α-amylase. *Appl. Environ. Microbiol.* **54**: 966-971.
9. Kumagai, M., G. G. Sverlow, G. della-Cioppa, and L. K. Grill. 1993. Conversion of starch to ethanol in a recombinant *Saccharomyces cerevisiae* strain expressing rice α-amylase from a novel *Pichia pastoris* alcohol oxidase promoter. *Bio/Technology*. **11**: 606-610.
10. Laluce, C., and J. R. Mattoon. 1984. Development of rapi-

dly fermenting strains of *Saccharomyces diastaticus* for direct conversion of starch and ethanol. *Appl. Environ. Microbiol.* **48**: 17-25.
11. Mattoon, J. R., K. Kim., and C. Laluce. 1987. The application of the development starch-fermenting yeasts. *CRC. Cri. Rev. Biotechnol.* **5**: 195-204.
12. Mortimer, R. K., and D. C. Hawthorne. 1969. Yeast genetics, p.385-460. In A. H. Rose and J. S. Harrison (ed), The yeasts, vol. 1. Biology of yeasts. Academic Press. Inc., New York.
13. Pardo J. M., J. Polaina, A. Jimenez. 1986. Cloning of the *STA2* and *SGA* genes encoding glucoamylase in yeasts and regulation of their expression by the *STA10* gene of *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **14**: 4701-4718.
14. Pretorius I. S., T. Chow, and J. Marmur. 1986. Identification and physical characterization of yeast glucoamylase structural genes. *Mol. Gen. genet.* **203**: 36-41.
15. Rothstein, S. C., M. Lazarus, W. E. Smith, D. C. Baulcombe, and A. A. Gatenby. 1984. Secretion of a wheat α-amylase expressed in yeast. *Nature (London).* **308**: 662-665
16. Sambrook, J., E. F. Fritsch, T. Maniatis. 1989. Molecular cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor, New York.
17. Sherman, F., G. Fink, and J. B. Hicks. 1986. Methods in yeast genetics, laboratory course manual. Cold spring Harbor Laboratory. Cold Spring Harbor , N.Y.
18. Tamaki, H. 1978. Genetic studies of ability to ferment starch in *Saccharomyces*. Gene polymorphism. *Mol. Gen. Genet.* **164**: 205-209.
19. Thomsen, K. K. 1983. Mouse α-amylase synthesized by *Saccharomyces cerevisiae* is release into the culture medium. *Carlberg Res. Commun.* **48**: 545-555.
20. Thomsen, K. K. 1987. Production of α-amylase by yeast. *CRC. Crit. Rev. Biotechnol.* **5**: 205-215.
21. Tubb, R. S. 1984. Genetics of ethanol-producing microorganisms. *CRC. Crit. Rev. Biotechnol.* **1**(3): 241-261.
22. Valenzuela, P., A. Medina, W. J. Rutter, G. Ammeren, and B. D. Hall. 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature(London).* **298**: 347-350.
23. Yamashita, I., T. Itoh, and S. Fukui. 1985. Cloning and expression of Saccharomyces fibuligera α-amylase gene in *Saccharomyces cerevisiae*. *Agric. Biol. Chem.* **49**: 3089-3091.

(Received November 4, 1993)