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The Construction of a Stable Starch-Fermenting Yeast Strain Using Genetic Engineering and Rare-Mating

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ABSTRACT

To develop a yeast strain that is able to produce ethanol directly from starch, α-amylase cDNA (originated from mouse salivary glands) was introduced into the hyploid *Saccharomyces diastiticus* cells secreting glucoamylase by using a linearized integrating vector. The integrating vector contains a *LEU2* gene and the inside of the *LEU2* gene was cut by *KpnI* to make the linearized vector. One of the transformants exhibited 100% mitotic stability after 100 generations of cell multiplication. To improve its ethanol-fermentability, the haploid transformant was rare-mated with a polyploid industrial strain having no amylase activity. The resulting hybrid RH51 produced 7.5 (w/v) ethanol directly from 20% (w/v) soluble starch and its mitotic stability was 100% at the end of fermentation.

Index Entries: Starch fermenting yeast; heterologous α-amylase gene; chromosomal integration; mitotic stability; integrating vector; rare-mating.

INTRODUCTION

To date, with the instability of petroleum supplies and shortage problem, many countries in the world have been interested in the production of ethanol from renewable biomass as a substitute for petroleum. Traditionally, ethanol fermentation has been carried out almost exclusively by

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Saccharomyces cerevisiae and *S. carlsbergensis* belonging to yeast. The conversion of starchy biomass to industrial and fuel ethanol by yeasts employs a three-step process (*1*): (1) liquefaction of starch with α-amylase; (2) enzymatic saccharification of the liquefied starch to produce fermentable sugars; and (3) fermentation of the sugars. The liquefaction and saccharification steps are required owing to the lack of starch-degradation capability of the yeast strains; these steps represent a significant expense in the production process. Therefore, new yeast strains of yeast have been sought for the direct conversion of starch material to ethanol (*2,3*). So far, *Saccharomyces diastaticus* is considered as one of the best yeast strains for that purpose. This yeast strain exhibited high tolerance for ethanol and high fermentation rates, and was very closely related to *S. cerevisiae* genetically (*4,5*). The primary difference between the two strains is that *S. diastaticus* secretes a glucoamylase (*6*), whereas *S. cerevisiae* lacks this ability. However, the use of *S. diastaticus* has not satisfied the complete fermentation of starch, since this strain secretes only glucoamylase, and not α-amylase (*4*).

Several laboratories have succeeded in introducing heterologous α-amylase genes (derived from various organisms, such as those of human origin [*7,8*], from animals [*9–11*], plant [*12*], and of bacillus origin [*13*]) into *S. cerevisiae* to produce active α-amylase into the culture medium. However, the conversion of carbohydrate to ethanol by these transformants secreting α-amylase is not efficient, varying from 10 to 50% (*14*). Another significant problem is that the exogenous α-amylase gene is lost as the cell multiples (*11*). It is generally observed that yeast transformants bearing plasmids carrying the 2-μm origin of replication exhibit various degrees of mitotic stability. That is, during many generations of growth under nonselective conditions, cells with plasmids are gradually diluted out of the population. To overcome this disadvantage of episomal vectors, therefore, many yeast investigators have researched the methods of introducing a vector into the host cell by chromosomal integration where the heterologous gene is integrated into the homologous sequence of the chromosomal loci for the improvement of the mitotic stability (*15–17*). These integration events take place by the homologous recombination into the homologous loci of the chromosome (*18–21*). Blomqvist et al. (*15*) reported that, by using the chromosomal integration of the heterologous gene into yeast chromosomal loci, the integrated strain was mitotically stable during the fermentation process. Recently, Sakai et al. (*22*) reported that, using the δ sequence of yeast retrotransposon Ty as a recombination site, they have constructed δ-integrative plasmid carrying heterologous genes such as mouse α-amylase.

On an industrial scale of ethanol production, the yeast strain should have such valuable fermentation characteristics as high efficiency and rate in ethanol fermentation, high ethanol-tolerance, high temperature-tolerance, and sugar-tolerance (*23*). Also, the strain should be diploid or polyploid, since the haploid yeast strain is susceptible to mutation and

Table 1
List of Yeast Strains

Strain	Relevant properties	Source
K35(ρ^-)	Polyploid peptite mutant of *Saccharomyces* industrial yeast	Laboratory collection
K114	a *trp*1 *ura*3Δ *ade*6 *his*2 STA[a]	Laboratory collection
K114/pMS12ΔR (*LEU*2)	K114 carrying pMS12ΔR (*LEU*2)	This work
K114/ YIpMSΔR (*LEU*2/*URA*3)	K114 carrying YIpMS12ΔR (*LEU*2/*URA*3)	This work
RH51	a hybrid strain between K114/ YIpMSΔR (*LEU*2/*URA*3) and industrial yeast K35(ρ^-)	

[a]STA, gene of glucoamylase.

Table 2
List of Plasmids

Plasmid	Relevant properties	Source
pMS12	Amp^r, *ADC*1 promoter, *TRP*1, 2 μ ori replicative ori of pBR322, mouse salivary α-amylase cDNA	K. K. Thomsen (*14*)
pMS12ΔR	pMS12 with the deleted regulatory site of *ADC*1 promoter	K. Kim and J. W. Lee (*24*)
pMS12ΔR (*LEU*2)	pMS12ΔR carrying *LEU*2 gene of YEp13 in the *Sal*1 site	K. Kim, this laboratory
YIpMSΔR (*LEU*2)	pMS12ΔR (*LEU*2) with deleted 2 μ ori gene	this laboratory
YIpMSΔR (*LEU*2/*URA*3)	YIpMSΔR (*LEU*2) carrying *URA*3 gene of YEp24 in the *Hind*III site	From this work
YEp24	Amp^r, replicative ori of pBR322 2 μ ori, *URA*3	D. Botstein (*25*)

does not give good stability in fermentation in long-term use. The yeast strain used in the laboratory is generally haploid owing to its easier genetic manipulation, but the haploid laboratory strain does not have fermentation characteristics as good as the industrial strain has.

Based on the foregoing, the object of the present investigation is to develop a yeast strain that will provide a stable secretion of both α-amylase and glucoamylase, and has good fermentation characteristics for industrial use.

MATERIALS AND METHODS

Bacterial Strains, Yeast Strains, and Plasmids

The bacterial strains *E. coli* C600SF8 and DH5α were used for all bacterial transformations and plasmid preparations. The yeast strains and plasmids used are shown in Tables 1 and 2. The plasmid used in this

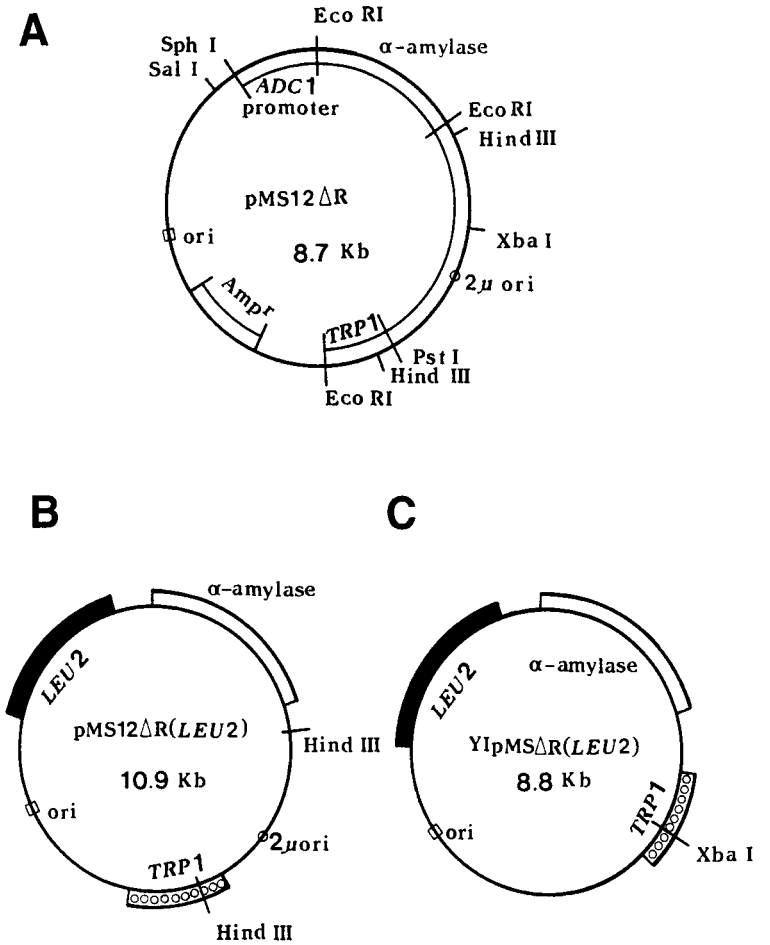

Fig. 1. The genetic and restriction maps of recombinant plasmids.

experiment was a YIpMSΔR (*LEU2*) yeast integrating vector, that was constructed from pMS12 (*14*) in our laboratory. The pMS12 plasmid, a yeast-*E. coli* shuttle vector, contained mouse salivary α-amylase cDNA, an *ADC1* promoter of alcohol dehydrogenase I gene, a replicative origin of pBR322, an ampicillin resistance gene, a 2 μ *ori* site of yeast, and a *TRP1* gene for the yeast auxotropic marker (*14*). The pMS12ΔR (Fig. 1A) was constructed by self-ligating the pMS12 after the plasmid was deleted with a small fragment (1350 bp) containing the upstream of the regulatory site of an *ADC1* promoter region by digesting pMS12 with *Sph*I (*24*). This regulatory region of the *ADC1* promoter has a function that inhibits the expression of alcohol dehydrogenase in the presence of ethanol or glucose (*26*). Also, the pMS12ΔR (*LEU2*) (Fig. 1B) was constructed by digesting the pMS12ΔR with *Sal*I and ligating the digested pMS12ΔR with 2.2 kb of DNA fragment containing *LEU2* gene. The 2.2 kb DNA fragment of *LEU2* gene was derived from YEp13 (*27*) by digesting the plasmid with *Sal*I and

*Xho*I. The YIpMS12ΔR(*LEU2*) (8.8 kb) (Fig. 1C) was constructed by self-ligating the pMS12ΔR (*LEU2*) after the plasmid was deleted with a 2.1-kb *Hind*III fragment containing 2 μ *ori* region.

Media and Culture

LB broth (*28*) was used for the culture of *E. coli* strains for the amplification of plasmids. A selection media for *E. coli* transformants was the LB medium supplemented with ampicillin (50 μg/mL). YPD medium (1% Difco yeast extract [*Y*], 2% Difco peptone [*P*], and 2% dextrose [*D*]) was used as a complete medium for the culture of yeast cells. The minimal selective medium for the yeast transformants was SD media (0.67% Difco yeast nitrogen base without amino acid, 2% dextrose, supplemented amino acid) (*29*). For ethanol fermentation, YPS4 containing 4% Lintner potato soluble starch (Sigma) or YPS20 containing 20% Difco soluble starch was used. For the test of halo-forming ability as the starch digesting activity of the yeast transformants, YPD1S3 media (YPD plus 3% Lintner potato soluble starch) were used. The incubation temperatures for bacteria and yeasts were 37 and 30°C, respectively. If the microbial cells had to be grown aerobically, the culture flasks were incubated in a shaking incubator operated at 200 rpm.

Transformation

Transformation of *E. coli* cells was performed according to the procedure described by Sambrook et al. (*30*). Yeast cells were transformed according to the lithium acetate method of Ito et al. (*31*). The yeast transformants obtained were grown on SD agar plates and transferred onto YPD1S3 agar plates followed by incubation at 4°C for 2-3 d. Any colonies showing a clear and large halo zone around the colonies owing to the degradation of starch were selected as the desirable transformant secreting the α-amylase.

Plasmid Construction

All procedures for the plasmid manipulations and plasmid preparations were performed according to the method described by Sambrook et al.(*30*).

Rare-Mating of Yeast Cells

The haploid K114 transformant and petite mutant of polyploid K35 have been mated by the crossing plate method (*29*). The colonies grown on the cross-region of the selective media containing glycerol and without required amino acids were selected as hybrids. To confirm that the colonies are true hybrids, the colonies were tested for sporulation and halo-forming ability.

Assay of Amylolytic Activity

The amylolytic activity was determined by the modified method of Kim et al. (*11*). The pH of the enzyme reaction mixture and reaction temperature used here were 7.5 and 45°C, respectively.

Ethanol Fermentation

Ethanol fermentation was performed in a cap tube or a fermenter. In cap-tube fermentation, a half loopful of yeast cells in stationary phase was inoculated into a cap tube containing 10 mL of YPS4 broth and then incubated at 30°C for 5 d, and then the ethanol formed was assayed.

A 2.5-L jar fermentor SY-250 equipped with an automatic temperature and pH control system (Korea Fermentor, Korea) was used. Twenty grams of yeast pellets were inoculated into the 800 mL of YPS containing 20% (w/v) soluble starch (Difco) in the fermentor. The fermentation was carried out at pH 5.0 and 34°C.

Ethanol Assay

The amount of ethanol in the fermented culture broth was assayed by using the modified method (*4*) described in Bernt and Gutman (*32*), where the enzymatic method was based on the reaction of alcohol dehydrogenase and β-NAD.

The Assay of Residual Starch

The amount of residual starch in the fermented media was determined by the method of Kim et al. (*11*).

Mitotic Stability Test

0.1 mL (10^5 cells/mL) of culture broth of stationary phase was inoculated into 100 mL of YPD broth. After incubation for 10 generations of cell-multiplication until the cell number reached 2^{10} or 1024 cells (10^8 cells/mL), 0.1 mL of the culture broth was reinoculated into 100 mL of a fresh YPD broth. These procedures were repeated for 100 generations of cell division. Cells were grown aerobically in a shaking incubator and the 0.1 mL of the appropriately diluted cell suspension from the 100 mL of YPD broth after each 10 generations was plated onto YPD1S3 plates and the plates were incubated for 2–3 d at 30°C. To observe the halo formation, the plates were stored for 3–4 d at 4°C. Each plate contained 200–400 colonies and the colonies were so small that the halo formed by glucoamylase was not visible or hard to see, but the halo formed solely by α-amylase was larger and clear enough to count. The mitotic stability of a α-amylase gene was determined by using the following equation:

Mitotic stability (percent) = 100 × (number of a halo-forming colonies on YPD1S3 plate ÷ total number of colonies on YPD1S3 plate)

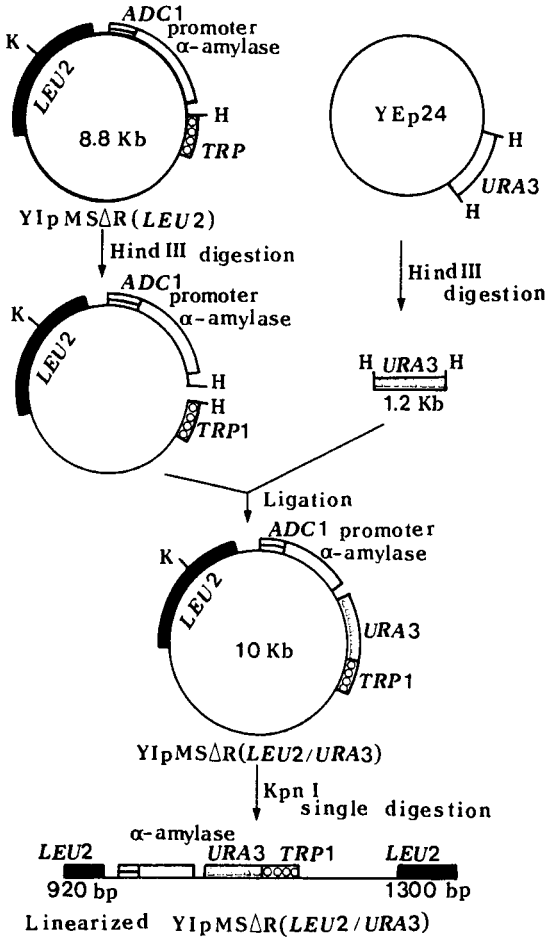

Fig. 2. Construction of YIpMSΔR (*LEU2*/*URA3*) and linearized YIpMSΔR (*LEU2*/*URA3*). H indicates restriction site of *Hind*III; K, *Kpn*I.

RESULTS AND DISCUSSION

Construction of Integrating Vector, YIpMSΔR (*LEU2*/*URA3*)

The strategy employed in the construction of the integrating vector, YIpMS12ΔR (*LEU2*/*URA3*) is illustrated in Fig. 2. The plasmid YIpMS12ΔR (*LEU2*) (Fig. 1C) was digested with *Hind*III and ligated with a 1.2-kb *Hind*III fragment containing *URA3* gene. The *URA3* gene is for a selectable marker derived from YEp24 (25). The resultant plasmid YIpMS12ΔR (*LEU2*/*URA3*) was the circular form and the size was 10 kb. This plasmid was linearized by digestion of the plasmid with *Kpn*I. There is a unique restriction site for *Kpn*I within the *LEU2* gene of the plasmid. From the original 2220 bp of the *LEU2* gene fragment, the resulting linearized plasmid has a left arm containing 920 bp *LEU2* fragment and a right arm containing the other 1300 bp (Fig. 2). The right and left arms of plasmid containing the *LEU2*

Table 3
Amylolytic Activity and Ethanol Production of Various Transformants

Yeast strains	Amylolytic activity, U/mL	Ethanol concentration, %, w/v[a]
K114	0.36	0.03
K114/pMS12ΔR (*LEU2*)	1.19	1.30
K114/YIpMSΔR (*LEU2/URA3*)		
clone 1	1.12	1.30
2	1.18	1.70
9	1.04	1.30
23	1.17	1.70
24	1.16	1.45

[a]Half loopful of cells of each strain was inoculated into 10 mL of YPS24 and fermented for 5 d at 30°C.

gene fragment are capable of integrating into a homologous sequence of *LEU2* or *leu2* loci on the chromosome of the recipilent yeast cell by homologous recombination (*19*).

Selection of Transformants Secreting α-Amylase

The recipient yeast strain for introducing the linearized plasmid YIpMSΔR (*LEU2/URA3*) was K114 (Table 1), which has a relatively strong glucoamylase activity. Two hundred twenty-six clones of the transformants of K114 were obtained. By comparing the halo size of the transformants, five transformants with the largest halo size were selected. These selected transformants were tested for their amylolytic activities and ethanol production, and the results are shown in Table 3.

The untransformed strain showed definitely lower amylolytic activity and lower ethanol production compared to the transformed strains either by episomal or integrating vector containing the α-amylase gene. This result indicates that the action of the α-amylase in addition to glucoamylase is necessary for the more complete hydrolysis of starch and consequently higher ethanol production. It also has been shown previously that the α-amylase works cooperatively with glucoamylase for complete hydrolysis of starch (*11*). However, the selected transformants containing the integrating vector showed as much amylolytic activity and ethanol production as the transformant haboring the episomal vector. Generally it has been known that the episomal vector is present in a cell as multicopy and the integrating vector exists as one copy. However, the results of Table 3 show that the two transformants of K114 containing the episomal or integrating vector exhibited almost the same amylolytic activity. Several copies of the integrating vector might be integrated into the chromosome (*17,33,34*). It is also known that an increase in copy number does not lead automatically to higher expression levels, especially where protein secretion is intended (*35*). Even higher yields of secreted product (in the yeast

Table 4
Mitotic Stability[a] of α-Amylase Gene in the Cell of Yeast K114 Transformant After Different Number of Cell-Multiplication

Vector		Recipient yeast	Clone number	Mitotic stability, % 0 G[b]	20	40	80	100
Episomal	pMS12ΔR (*LEU2*)	K114		100	92.6	59.0	17.3	0.0
Integrating	YIpMSΔR (*LEU2/URA3*)	K114	1	100	100	100	100	100
			2	100	100	100	100	98.5
			9	100	100	100	99.3	96.9
			23	100	100	100	100	99.5
			24	100	100	100	99.6	91.9

[a]The halo-forming ability of each transformant was expressed as the presence of the α-amylase gene in the cell of the transformant.
[b]G, number of generations of cell multiplication.

transformant with an integrated vector) than the one with episomal vectors have been reported; four integrated copies of a prochymosin expression unit resulted in similar overall expression levels but high secretion yields than were achieved with a multicopy vector (*36*). The reason for this difference is not clear.

The same amylolytic activity of the transformant haboring either episomal or integrating plasmid was also observed in *Bacillus;* two copies of integrated α-amylase produced α-amylase in quantities comparable with that of the multicopy plasmid carrying the same α-amylase gene (*17*). One possible explanation for this is that more mRNA molecules are transcribed from the multicopy plasmid than would be expected or the cell can utilize (*17,37*).

The Mitotic Stability of Yeast Transformants Haboring Integrated α-Amylase Gene

The mitotic stability of various yeast transformants with the integrating vector containing the α-amylase gene were examined and the results are shown in Table 4. The integrating vector exhibited higher mitotic stability than the episomal vector. The stability of episomal plasmid decreased continuously with the time of cell-multiplication, and the transformants lost the plasmid completely after 100 generations. However, the integrated vector exhibited almost 100% stability even after 100 generations.

Some variations in the mitotic stability after the integration of the vector into the chromosome were observed even among the transformants of the same recipient. The mitotic stability of clone 1, 2, and 23 was 100% after 80 generations, while that of clones 9 and 24 was less than 100%. Clone 1 of K114 transformant was 100% even after 100 generations (Table 4). Therefore, to obtain the transformant with good mitotic stability of the integrated gene, it is necessary to use a large number of transformants in the screening. The K114/YIpMSΔR (*LEU2/URA3*) clone 1 was

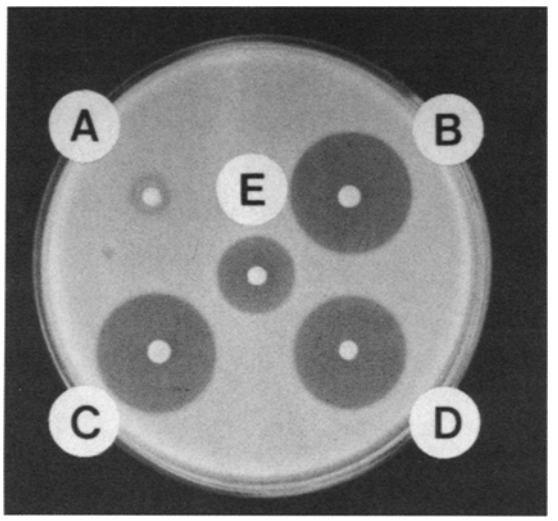

Fig. 3. The halo formed by K114 and its transformants. A, K114; B, K114/YIpMSΔR (*LEU2*/*URA3*)—clone 1; C, K114/YIpMSΔR (*LEU2*/*URA3*)—clone 2; D, K114/YIpMSΔR (*LEU2*/*URA3*)—clone 23; E, K114/pMS12ΔR (*LEU2*).

selected as the best strain. Figure 3 illustrated the halo-forming abilities of the K114 and their various transformants.

The Mitotic Stability of α-Amylase Gene in the Cells of the Hybrid Yeast

To combine the amylolytic activity of the K114/YIpMSΔR (*LEU2*/*URA3*) and the good ethanol fermentability of industrial K35 strain, the two strains were rare-mated and 66 hybrids were obtained. Halo-forming abilities and ethanol-producing activities of the hybrids were examined and RH 51 was selected as the best strain (data not shown). The RH 51 can form spores and has amylolytic activity, and therefore the strain is a true hybrid.

The mitotic stability of RH51 was examined and the results showed that RH51 exhibited 100% stability after 100 generations of cell-multiplication and did not lose the α-amylase gene at all. On the other hand, the transformant harboring the episomal plasmid pMS12ΔR (*LEU2*) continuously lost the plasmid with a lapse of the time and did not show any α-amylase activity after 100 generations of cell multiplication. The results indicate that the integrated α-amylase gene is also very stable in hybrid cells formed by the rare-mating between haploid and polyploid yeast strain.

Ethanol Fermentaton by the Yeast Hybrid Containing the Integrated α-Amylase Gene

The performance of the hybrid RH51 for the ethanol fermentation directly from starch (20%, w/v) was examined by using a fermentor and

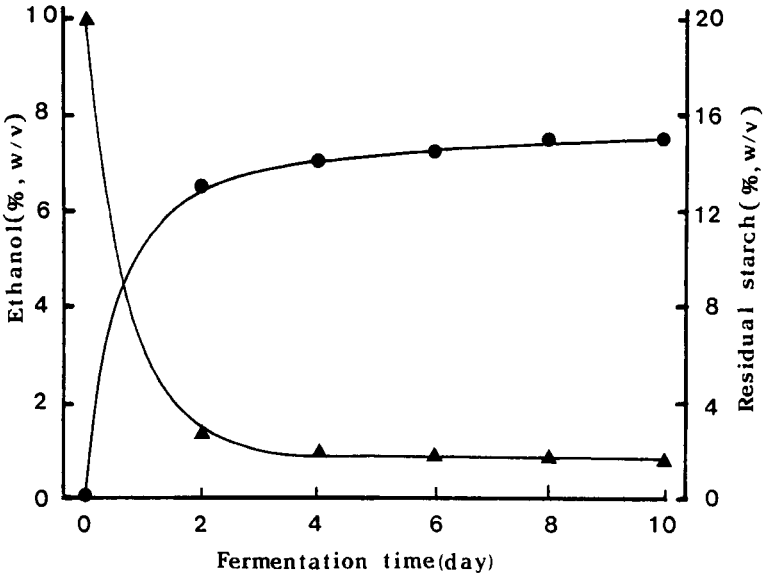

Fig. 4. The time course of ethanol production from starch by RH51 in a fermentor. (●); ethanol concentration; (▲): residual starch concentration.

the results are shown in Fig. 4. Most of the ethanol was formed after 2 d of the initial period of fermentation, and the ethanol production increased slowly to reach the maximum value of 7.5% (w/v) after 8 d. The residual sugar and the residual starch content were 1.28% (w/v) and 1.68% (w/v), respectively; these results indicate that the starch was almost completely degraded and converted to ethanol. In addition, the mitotic stability of the integrated α-amylase gene in the cell of the hybrid RH51 after 10 d of fermentation was examined; the results showed that the mitotic stability was 100% after the end of the fermentation. These results have proven that the method of the chromosomal integration of the heterologous α-amylase gene into the yeast chromosome using a linearized vector is a very valuable method for improving the strain of industrial yeast.

CONCLUSION

To develop a yeast that will be able to produce ethanol directly from starchy biomass, α-amylase cDNA (originated from mouse salivary) was introduced into the yeast *S. diastiticus* secreting glucoamylase. To sectete the α-amylase stably from the transformants, a linearized integrating plasmid YIpMSΔR (*LEU2*/*URA3*) that is able to integrate into chromosomal loci of yeast by a homologous recombination was constructed. The yeast transformant having the linearized and integrated plasmid vector exhibited higher mitotic stability than that of yeast transformant having a episomal plasmid vector.

The transformants having the YIpMSΔR (*LEU2/URA3*) plasmid showed, almost 100% stability in mitotic stability after 80 generations of cell multiplication. Especially, K114/YIpMSΔR (*LEU2/URA3*) clone 1 was superior in ethanol fermentation rate and amylolytic activity, and its mitotic stability was 100% after 100 generations of cell multiplication. In addition, the hybrid RH51 strain, which was rare-mated between a K114/YIpMSΔR (*LEU2/URA3*) clone 1 and an industrial strain K35 (petite mutant), exhibited 100% in the mitotic stability after the fermentation was finished.

These results suggest that the method of chromosomal integration of the heterologous gene into the yeast chromosome using a linearized vector is a very valuable method of the strain improvement for the industrial ethanol-producing yeast.

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