J. Microbiol. Biotechnol. (1999), **9**(5), 668–671

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Expression of *Schwanniomyces occidentalis* α-Amylase Gene in *Saccharomyces cerevisiae* var. *diastaticus*

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Received: May 6, 1999

Abstract The gene encoding *Schwanniomyces occidentalis* α-amylase (*AMY*) was introduced into *Saccharomyces cerevisiae* var. *diastaticus* which secreted only glucoamylase, by using a linearized yeast integrating vector to develop stable strains with a capability of secreting α-amylase and glucoamylase simultaneously. A dominant selectable marker, the geneticin (G418) resistance gene (Gt^r), was cloned into a vector to screen wild-type diploid transformants harboring the *AMY* gene. The amylolytic activities of transformants were about 3~7 times higher than those of the recipient strains. When grown in nonselective media, the transformants with the linearized integrating vector containing the *AMY* gene exhibited almost all of the mitotic stability after 100 generations.

Key words: *Schwanniomyces occidentalis* α-amylase gene, yeast integrating vector, mitotic stability, *Saccharomyces cerevisiae* var. *diastaticus*

Traditional conversion of starch into fermentable sugars by *Saccharomyces cerevisiae* lacking amylolytic activity depends on the addition of α-amylase and glucoamylase, which leads to liquefaction and saccharification of starch [1, 15, 16]. *S. cerevisiae* var. *diastaticus* (formerly *S. diastaticus*) produces only glucoamylase and is closely related to *S. cerevisiae*, which can be a suitable host for the expression of the heterologous α-amylase gene (*AMY*) [1, 8, 10, 14, 16]. A genetically manipulated *S. cerevisiae* var. *diastaticus* with heterologous *AMY* genes, such as the mouse *AMY* gene and the *Schwanniomyces occidentalis AMY* gene, could be useful in an efficient one-step starch utilization [8, 14]. Yeast integrating vectors or yeast centromeric vectors have been used for laboratory haploid strains in order to maintain the mitotic stability of the exogenous *AMY* gene [1, 7, 8, 16]. However, for the industrial production of ethanol or single-cell protein from starch materials, the yeast strains should be diploid or polyploid because a haploid strain susceptible to mutation is not stable in a long-term growth [2, 8]. Many attempts were made to transform industrial polyploid strains of *S. cerevisiae* with a circular integrating vector containing both the glucoamylase gene (*STA2*) and *AMY* gene. In addition, transformation of the diploid strain with a centromeric vector containing the *STA2* gene has not been successful [1, 16]. Recently, hybrid strains between a recombinant haploid of *S. cerevisiae* var. *diastaticus* secreting α-amylase and a polyploid strain of *Saccharomyces* without any detectable amylase activity or another haploid of *S. cerevisiae* var. *diastaticus* were constructed to obtain stable diploid or polyploid starch-fermenting yeast strains [8, 12]. In this study, we constructed a linearized yeast integrating vector carrying a *Sch. occidentalis AMY* gene and a geneticin (G418) resistance gene (Gt^r) to be directly introduced into the wild-type diploid strain of *S. cerevisiae* var. *diastaticus*. Starch utilization and mitotic stability of transformants were monitored and compared.

The bacterial strain of *Escherichia coli* JM83 [*ara*, *Δ* (*lac-proAB*), *rsp*, *Φ*80, *lacZ*ΔM15] was used for all bacterial transformation and plasmid preparations. The yeast strains and plasmids used are summerized in Table 1. All procedures for the plasmid manipulations and transformation of *E. coli* were performed by the method of Sambrook *et al.* [13]. *E. coli* was grown in LB medium supplemented with ampicillin (50 μg/ml) as required [13]. YPD medium (1% Difco yeast extract, 2% Difco peptone, and 2% dextrose) was used for culture of recipient yeast cell. Various concentrations of geneticin (0-250 μg/ml G418, Sigma) were added to YPD plates, and the cell suspension derived from one colony was inoculated onto each YPD plate with G418 added to a specific concentration. After an incubation period of 2-3 days at 30°C, the concentration of G418 in which the colony could not grow any further was determined [5]. Yeast cells were transformed according to the lithium acetate/DMSO method [4]. Transformed cells

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Table 1. Yeast strains and plasmids used.

Strain or plasmid	Relevant properties	Source or reference
S. cerevisiae var. *diastaticus* K100	*αleu2*, *trp1*, *ura3Δ*, *STA1*[a]	[9]
S. cerevisiae var. *diastaticus* K114	a *trp1*, *ura3Δ*, *ade6*, *his2*, *STA*	[9]
S. cerevisiae var. *diastaticus* ATCC28338	diploid, homothallic, *STA*	ATCC[b]
plasmids		
pYES2	Amp^r, pUC19 *ori*, 2 μ *ori*, *GAL1* promoter, *CYC1* terminator, *URA3*	Invitrogen, U.S.A.
pYIS2 (*URA3*)	pYES2 with deleted 2 μ *ori* and *GAL1* promoter	This work
pSA4	pYES2 carrying *Sch. occidentalis* *AMY* with deleted *GAL1* promoter	[14]
pUC4K	Amp^r, pBR322 *ori*, Kan^r(Gt^r), *lacZ'*	Pharmacia Biotech., Sweden
YIpSA4 (*URA3*)	pYIS2 (*URA3*) carrying the *AMY* of pSA4	This work
YIpSA4 (*URA3*/Gt^r)	YIpSA4 (*URA3*) carrying Gt^r gene	This work
pSA4 (*URA3*/Gt^r)	pSA4 carrying Gt^r gene	This work

[a]Glucoamylase gene.
[b]American Type Culture Collection.

were then incubated in a YPD medium for 12 h, and plated on YPD plates containing G418 (200 μg/ml). The transformants grown on YPD plates containing G418 were transferred onto YPD1S3 plates [YP containing 1% dextrose, 3% Lintner potato soluble starch (Sigma), and 2% bacto agar] to test the halo-forming ability as a result of amylolytic activity after incubation for 3 days at 30°C, followed by refrigeration at 4°C for 2 days. The buffered starch medium containing 0.1 M sodium phosphate buffer (pH 6.0), 2% Lintner starch, 1% Difco yeast extract, and 2% Difco peptone (BYPS2) was used to assay amylase activity secreted by transformants. Yeast cells previously grown on YPD containing G418 (20 μg/ml) for 2 days were used to inoculate 50 ml of BYPS2 medium in a 250-ml flask. The inoculated media were incubated aerobically on a shaking incubator (30°C) at 250 rpm for 4–5 days. The amylolytic activity was determined by the method of Shin *et al.* [14]. The pH of the enzyme reaction mixture and the temperature employed were 6.0 and 40°C, respectively. One unit of amylolytic activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per ml per min. The mitotic stability of an *AMY* gene was measured by the method of Kim and Kim [8] according to the following equation:

$$\text{Mitotic stability (\%)} = \frac{\text{number of colonies showing halo on YPD1S3 plate}}{\text{total number of colonies on YPD1S3 plate}} \times 100$$

A linearized integrating vector was constructed for the expression of the *AMY* gene of *Sch. occidentalis* in *S. cerevisiae* var. *diastaticus*. The pYIS2 (*URA3*) was constructed by self-ligation after a 2 micron origin and *GAL1* promoter were deleted by digesting pYES2 with *Cla*I and *Ssp*I, and by treating with the Klenow fragment. A 1.8-kb *Eco*RI-*Xba*I DNA fragment containing the *AMY* gene isolated from pSA4 [14] was ligated into the *Eco*RI-*Xba*I DNA fragment of pYIS2 (*URA3*) to generate YIpSA4 (*URA3*) (Fig. 1). The *AMY* gene contained its own signal sequence and promoter without a regulatory region. The wild-type diploid recipient strain lacked selective genetic markers and, thus, could only be transformed with vectors containing a positive selectable marker, which is the geneticin resistance gene (Gt^r) [16]. The YIpSA4 (*URA3*) was linearized with *Rca*I and the ends were blunted with the Klenow fragment. A 1.5-kb *Pvu*II DNA fragment containing the Gt^r gene was isolated from pUC4K, and then ligated with the linearized YIpSA4 (*URA3*) to generate YIpSA4 (*URA3*/Gt^r). In addition, the Gt^r gene was introduced into the *Rca*I site of pSA4, generating pSA4 (*URA3*/Gt^r) (Fig. 1). The YIpSA4 (*URA3*/Gt^r) has a unique restriction site for *Apa*I within the *URA3* gene. This vector was linearized by digesting with *Apa*I which would be integrated into a homologous sequence of the *URA3* or *ura3* loci on the chromosome of the recipient yeast cell by initiating the homologous recombination [3, 8, 11]. The resulting linearized plasmid had a left arm containing a 603-bp *URA3* fragment along with a right arm containing the other 504-bp (Fig. 1).

The haploid strains of K100 and K114 and the diploid strain of ATCC28338 were unable to grow at the concentration of 200 μg/ml of G418. Both K100 and K114 were transformed to Ura$^+$ Amy$^+$ and Gt^r (>200 μg/ml of G418) with YIpSA4 (*URA3*/Gt^r). ATCC28338 was transformed to Amy$^+$ and Gt^r (200 μg G418/ml) with YIpSA4 (*URA3*/Gt^r). The recipient strains of K100, K114, and ATCC28338 which secreted only glucoamylase formed small halos, whereas all transformants secreting both glucoamylase and α-amylase produced larger and clearer halos (Fig. 2). Cell-free culture supernatants obtained from various transformants and recipient strains were examined and analyzed for any amyloytic activity (Table 2). As shown in Table 2, the

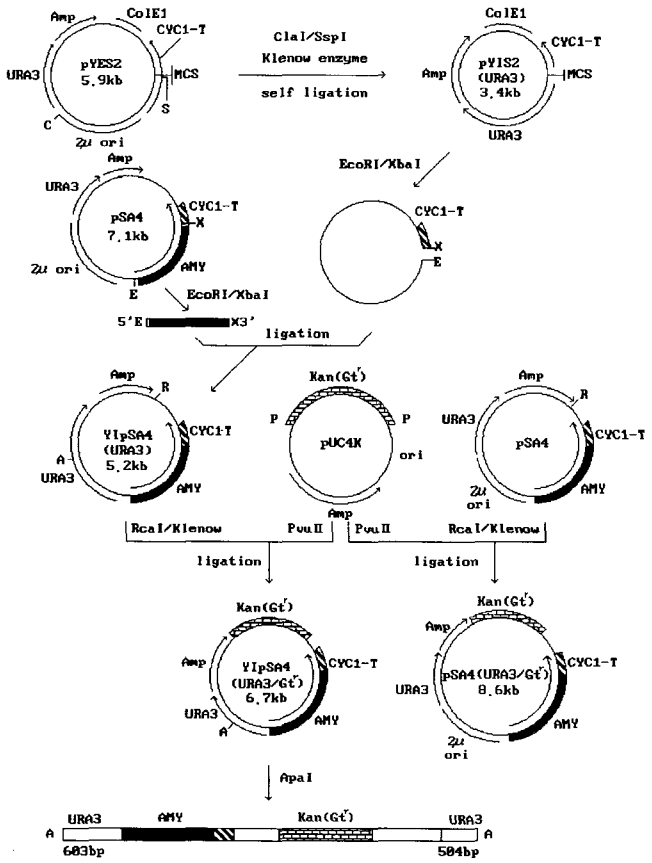

Fig. 1. Construction of the linearized integrating vector YIpSA4 (*URA3*/Gt^r) and the episomal vector pSA4 (*URA3*/Gt^r).
A, *Apa*I; C, *Cla*I; E, *Eco*RI; P, *Pvu*II; R, *Rca*I; S, *Ssp*I; X, *Xba*I.

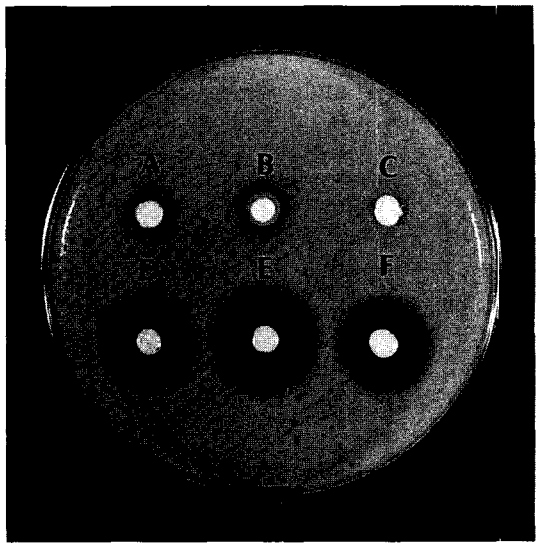

Fig. 2. The halo formed by K100, K114, ATCC28338, and their transformants.
A, K100; B, K114; C, ATCC28338; D, K100/YIpSA4 (*URA3*/Gt^r); E, K114/YIpSA4 (*URA3*/Gt^r); F, ATCC28338/YIpSA4 (*URA3*/Gt^r).

Table 2. Amylolytic activities in cell-free culture supernatants of various yeast strains.

Yeast strains	Amylolytic activity (U/ml)
S. cerevisiae var. *diastaticus* K100	0.17
S. cerevisiae var. *diastaticus* K114	0.36
S. cerevisiae var. *diastaticus* ATCC28338	0.25
K100/YIpSA4 (*URA3*/Gt^r)	1.18
K114/YIpSA4 (*URA3*/Gt^r)	2.40
ATCC28338/YIpSA4 (*URA3*/Gt^r)	0.76

amylolytic activities of K100/YIpSA4 (*URA3*/Gt^r) and K114/YIpSA4 (*URA3*/ Gt^r) were about 7 times higher compared to K100 and K114, respectively. The activity of K114/YIpSA4 (*URA3*/Gt^r) was twice greater than that of K114/YIpMSΔR (*LEU2/URA3*) producing a mouse α-amylase (1.19 U/ml) [8]. It is likely that several copies of the integrating vector might have been incorporated into chromosome of its recipient cells [6, 17]. On the other hand, the amylolytic activity produced by ATCC28338/ YIpSA4 (*URA3*/Gt^r) was 3 times higher than ATCC28338. So far, several laboratories were successful in constructing diploid or polyploid transformants of *S. cerevisiae* capable of secreting both α-amylase and glucoamylase. However, unfortunately, the results in relation to amylolytic activity have not yet been reported [8, 12, 16].

The mitotic stability of yeast transformants with the integrating vector containing the *AMY* gene was examined

Table 3. Mitotic stability of the *AMY* gene in various transformants after different numbers of cell-multiplication.

Vector	Recipient[b]	Mitotic stability[a](%)					
		0	20	40	60	80	100G[c]
YIpSA4 (*URA3*/Gt^r)	K100	100	100	100	100	100	100
	K114	100	100	100	100	100	100
	ATCC28338	100	100	100	100	100	99.8
pSA4 (*URA3*/Gt^r)[d]	ATCC28338	100	88.4	52.1	36.2	7.9	0.0

[a]The presence of the *AMY* gene was certified as the halo around each transformant.
[b]*Saccharomyces cerevisiae* var. *diastaticus* strain.
[c]G means the number of generations of cell multiplication.
[d]Yeast episomal vector.

and compared with the episomal vector (Table 3). The integrating vector exhibited a much higher mitotic stability than the episomal vector. The integrating vector exhibited 100% stability even after 100 generations (average generation time was approximately 95 min) in both haploid strains, and was also very stable in a wild-type diploid strain [1, 7, 8, 16]. According to an earlier report by Aguanno and Pretorius [1], the mitotic stability of the integrating vectors in haploid strains of *S. cerevisiae* was higher than that of the centromeric vectors (89~98%). Kim and Kim [8] reported that the integrated *AMY* gene in a haploid strain still exhibited 100% mitotic stability after 100 generations in hybrid cells formed by the rare-mating with polyploid strain. Further attempts are being made to introduce a glucoamylase gene into the integrating vector that carries the *AMY* gene, and to make stable industrial strains of *S. cerevisiae* capable of secreting both α-amylase and glucoamylase.

Acknowledgments

This study was supported by a research fund from Chonnam National University (1997). We thank Dr. Keun Kim at the University of Suwon for providing strains of *S. cerevisiae* var. *diastaticus* K100 and K114.

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