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Construction of a Secretory Expression Vector Producing an α-Amylase of Yeast, *Schwanniomyces occidentalis* in *Saccharomyces*

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Abstract Using a modified yeast secretory expression vector, α-amylase of *Schwanniomyces occidentalis* was produced from *Saccharomyces cerevisiae*. The expression vector contains the α-amylase gene (*AMY*) harboring its own promoter without the regulatory region and the adenine base at the -3 position from the ATG start codon, its own signal sequence, *CYC1* transcription terminator, and SV40 enhancer. The expressed α-amylase activity from cells carrying the plasmid was approximately 26 times higher than that from the cells harboring an unmodified plasmid. When *Saccharomyces diastaticus* was transformed with this modified vector, a 2.5 times higher level of amylolytic activity than that from *Sch. occidentalis* was observed.

Key words: Yeast secretory expression vector, *Schwanniomyces occidentalis* α-amylase gene, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*

The bioconversion of starch biomass to fermentable sugars requires a combined action of α-amylase and glucoamylase [2, 30]. α-Amylase is a key enzyme for the liquefaction of starch, but the brewing yeast *Saccharomyces* lacks an α-amylase gene (*AMY*). In an attempt to supply *S. cerevisiae* with α-amylase activity, various heterologous *AMY* genes derived from bacteria, wheat, mice, and humans [10, 18, 20, 21, 24, 25, 32] have been expressed in *S. cerevisiae*. Amylolytic yeasts such as *Lipomyces*, *Saccharomycopsis*, and *Schwanniomyces* [1, 3, 13, 29, 31, 33] are favorable candidates as donors for the *AMY* gene because of their close relationship to *S. cerevisiae*. However, the expression of heterologous *AMY* genes in *S. cerevisiae* did not always produce high starch-degrading activity with satisfaction. To enhance the expression of *AMY* genes in *S. cerevisiae*, promoters of alcohol dehydrogenase I gene (*ADC1*), yeast pheromone α-factor gene, and glucoamylase I gene (*STA1*), along with transcription terminators such as *URA3*, *TRP5*, and *GAL7* have been used as alternatives. In addition, the signal sequences of α-factor, glucoamylase I, and mouse salivary α-amylase have been translationally fused to secrete α-amylase from *S. cerevisiae* [2, 14, 15, 16, 28, 30]. However, it is still necessary to have an improved yeast expression system for the production of α-amylase in *S. cerevisiae*. We have tried to construct a new *AMY* gene expression vector having both increased transcriptional and translational efficiency to develop *Saccharomyces* with higher and faster amylolytic activity. In this study, we constructed a yeast secretory expression vector containing a *Sch. occidentalis AMY* gene harboring its own promoter with the regulatory region deleted and the adenine base replaced at the -3 position from the start codon, its own signal sequence, *CYC1* terminator, and SV40 enhancer to obtain the increased secretory production of α-amylase in *S. cerevisiae* and *S. diastaticus*.

Materials and Methods

Strains and Plasmids

The bacterial strain *Escherichia coli* JM83 [*ara*, Δ(*lac-proAB*), *rsp*, Φ80, *lacZ*ΔM15] was used for all bacterial transformation and plasmid preparations. The yeast strains and plasmids used are summerized in Table 1.

DNA Manipulation and Transformation

All procedures for the plasmid manipulations and preparations, and transformation of *E. coli* were performed by the methods of Sambrook *et al.* [26]. Yeast cells were transformed according to the lithium acetate/DMSO method of Hill *et al.* [12].

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Table 1. Yeast strains and plasmids used.

Strain or plasmid	Relevant properties	Source or reference
Sch. occidentalis	*AMY, GAM1*[a]	ATCC[b] 26077
S. cerevisiae SHY3	a *ste, ura3, trp1, leu2, his3, ade1, can1*	[5]
S. diastaticus K114	a *trp1, ura3Δ, ade6, his2, STA*[c]	[15]
Plasmid		
pUC19	Amp[r]. replicative *ori* of ColE1, *lacZ'*	[34]
pScAMY	Amp[r], Tet[r], replicative *ori* of pBR322, 2 μ *ori, TRP1, AMY* from *Sch. occidentalis*	[22]
YEp352	Amp[r]. replicative *ori* of pUC19, 2 μ *ori, URA3, lacZ'*	[11]
pYES2	Amp[r]. pUC19 *ori*, 2 μ *ori, GAL1* promoter, *CYC1* terminator, *URA3*	Invitrogen, U.S.A.
pYES2ΔGAL1	pYES2 with the deleted *GAL1* promoter	This work
pGL2-control vector	Amp[r] pUC19 *ori, luc,* SV40 early promoter and enhancer	Promega, U.S.A.
pSA1	YEp352 carrying *Sch. occidentalis AMY*	This work
pSA2	pSA1 with the deleted regulatory site of *AMY* promoter and its own terminator	This work
pSA3	pYES2ΔGAL1 carrying the *AMY* of pSA2	This work
pSA4	pSA3 with the adenine base at the -3 position from ATG of *AMY*	This work
pSA5	pSA4 carrying the SV40 enhancer	This work

[a]glucoamylase gene of *Sch. occidentalis.*
[b]American Type Culture Collection
[c]glucoamylase gene of *S. diastaticus.*

Polymerase Chain Reaction (PCR)

PCR reactions were performed with Pre-Mix Top containing a mixture of *Taq* DNA polymerase, high salt buffer, dNTPs (Bioneer, Korea), 20 pmol of sense and antisense oligo primers, and 300 ng of template DNA in a total volume of 20 μl (reaction volume). The DNA was amplified in a DNA thermal cycler (Perkin Elmer 2400). Cycler conditions were initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50~55°C for 30 sec, and extension at 72°C for 10 sec.

Media and Culture

E. coli cells were grown in LB medium supplemented with 50 μg/ml of ampicillin when required [26]. YPD medium (1% Difco yeast extract, 2% Difco peptone, and 2% dextrose) was used as a complete medium for the culture of yeast cells. The minimal selective medium (SD) for the yeast transformants contained 0.67% Difco yeast nitrogen base (without amino acid), 2% dextrose, 2% bacto agar, and nutritional supplements as required [27]. The yeast transformants grown on SD agar plates were transferred onto YPS3 agar plates [YP containing 3% Lintner potato soluble starch (Sigma) and 2% bacto agar] or YPD1S3 agar plates (YPS3 containing 1% dextrose) to test the halo-forming ability as a result of amylolytic activity after incubation for 2 days at 30°C followed by refrigeration at 4°C for 2 days. The buffered starch or glucose medium containing 0.1 M sodium phosphate buffer (pH 6.0), 2% Lintner starch or 2% dextrose, 1% Difco yeast extract, and 2% Difco peptone (BYPS2 or BYPD2) was used to assay the amylase activity secreted by yeast transformants. Yeast cells previously grown on minimal media for 2 days were used to inoculate 50 ml of BYPS2 or BYPD2 medium in a 250-ml flask. The inoculated media were incubated aerobically on a shaking incubator (30°C) operated at 250 rpm for 4~5 days.

Assay of Amylolytic Activity

The reaction mixture for the enzyme assay contained 950 μl of 0.1 M sodium phosphate buffer (pH 6.0) containing 0.5% Lintner starch and 50 μl of centrifuged culture fluid as a crude enzyme. After a 10-min incubation at 40°C, the contents of the reducing sugars were measured by the DNS method [4]. One unit of amylolytic activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per ml per min.

RESULTS AND DISCUSSION

Construction of Recombinant Plasmids

Park *et al.* [22] have previously cloned the *AMY* gene from *Sch. occidentalis* into pYcDE-1, resulting in pScAMY. In this report, the *AMY* gene was expressed using the original *AMY* gene promoter and signal sequence. The *AMY* gene was recloned as a 3.8-kb *Eco*RI DNA fragment into the *Eco*RI site of YEp352, generating pSA1 (Fig. 1). A 1.8-kb *Dra*I-*Eco*RV DNA fragment containing the *AMY* gene was subcloned into the *Sma*I site of YEp352. In the resulting plasmid, designated pSA2 (Fig. 1), the region of the *AMY* gene promoter lying upstream from the *Dra*I site located at the position -172 from the

start codon was removed. This part of the promoter may be responsible for the repression of the *AMY* gene expression by glucose [16]. For the enhanced expression of the *AMY* gene by *CYC1* transcription terminator, pYES2ΔGAL1 was used, which was constructed by self-ligation after the *GAL1* promoter region was deleted by digesting pYES2 containing 2 micron origin and *CYC1* terminator with *Spe*I. A 1.8-kb *Eco*RI-*Xba*I DNA fragment containing the *AMY* gene was isolated from pSA2, and ligated into the *Eco*RI-*Xba*I DNA fragment of pYES2ΔGAL1, generating pSA3 (Fig. 1). The *AMY* gene was thus inserted into the region upstream to the *CYC1* terminator of pYES2ΔGAL1. For the efficient translation of the mRNA of the *AMY* gene, the base guanine (G) and adenine (A) at the -3 and -2 positions from the ATG start codon were changed to adenine (A) and cytosine (C) by the PCR technique [7]. The region from the promoter to the start codon was amplified with oligonucleotides 5'-CAGGCTTTACACTTTATGCTTC-3' and 5'-TTTGGATCCATGGTTTGCTTTTATTTTAT-TTAGTA-3', and the coding region from the start codon to the *Sal*I site was amplified with oligonucleotides 5'-ATGAGAATTCATGAGATTTTCAACTGAAGG-3' and 5'-TCAAACAATAATCGTGG-3'. The amplified *Eco*RI-*Bam*HI DNA fragment containing the promoter region was then ligated into the *Eco*RI-*Bam*HI fragment of pUC19 containing the ampicillin resistant gene and the ColE1 replication origin (Fig. 2). The resulting 2.9-kb plasmid (pUC-αTA) was then linearized with *Nco*I and *Sal*I, and ligated with the 461-bp *Rca*I-*Sal*I amplified fragment containing the coding region from the start codon to the *Sal*I site, generating a 3.3-kb plasmid (pUC-αAC(N)) which contained the substituted bases. The sequence around the -3 and -2 positions of pUC-αAC(N) was 5'-AAGTTGAACACCATGAGATTT-3'. The 658-bp *Eco*RI-*Sal*I DNA fragment containing the substituted

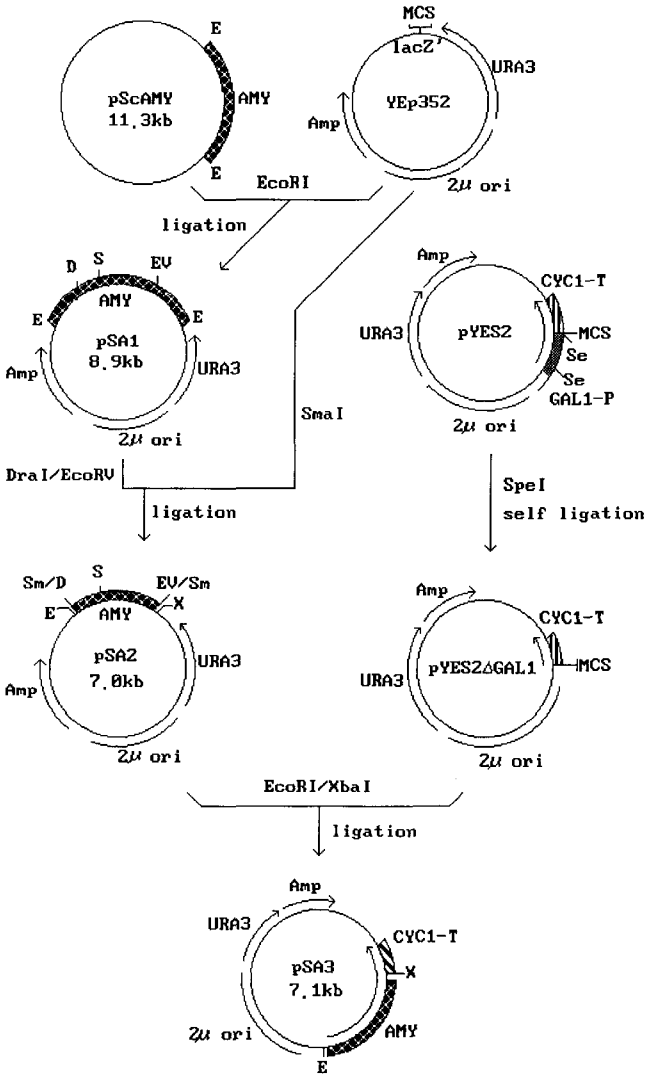

Fig. 1. Construction of recombinant plasmids pSA1, pSA2, and pSA3.

D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; S, *Sal*I; Se, *Spe*I; Sm, *Sma*I; X, *Xba*I.

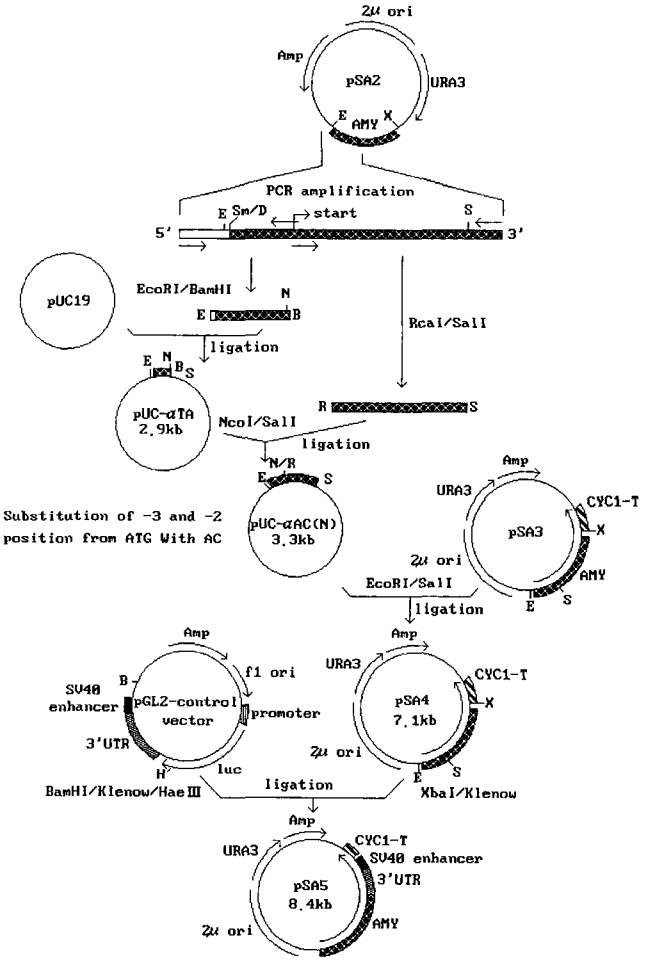

Fig. 2. Construction of recombinant plasmids pSA4 and pSA5.

B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hae*III; N, *Nco*I; R, *Rca*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

bases was isolated from pUC-αAC(N) and ligated into the *Eco*RI-*Sal*I fragment of pSA3 containing 2 micron origin and *CYC1* terminator, generating pSA4 (Fig. 2). Finally, to increase the expression of the *AMY* gene, SV 40 enhancer was introduced at the downstream of the *AMY* gene. pSA4 was linearized with *Xba*I and the ends were blunted with the Klenow fragment. A 1.3-kb *Hae*III-*Bam*HI DNA fragment containing SV40 3'UTR and the enhancer was isolated from pGL2-control vector, treated with the Klenow fragment, and then ligated with the linearized pSA4, generating pSA5 (Fig. 2).

Amylolytic Activities Secreted by Transformants and Recipient Strains

S. cerevisiae SHY3, and *S. diastaticus* K114 that has a relatively strong glucoamylase activity [15] were transformed to Ura^+ Amy^+ with plasmids constructed from this work (pSA1~pSA5). K114, transformant SHY 3/pSA1, and K114/pSA1 formed small halos, whereas SHY3/pSA2~pSA5 and K114/pSA2~pSA5 produced larger and clearer halos (Fig. 3). While the halo formed by glucoamylase was hardly detectable, that produced by α-amylase was large and clear [15]. Cell-free culture fluids from various transformants and recipient strains were examined for amylolytic activity (Table 2). As shown in Table 2, the α-amylase activity of SHY3/pSA2 was approximately 7 times higher than that of SHY3/pSA1. When SHY3/pSA1 and SHY/pSA2 were grown in glucose media (BYPD2), SHY3/pSA1 exhibited no detectable hydrolysis of starch, but the α-amylase activity produced by SHY3/pSA2 was 0.65 U/ml (data not shown). Wang *et al.* [33] have noted that the *Sch. occidentalis AMY* gene is negatively regulated by glucose. In pSA2, the regulatory site of the promoter responsible for the repression of *AMY* gene expression by glucose appeared to have been removed. This result corresponds to that of Kim and Lee [16] who reported that *ADC1* gene promoter with the deleted regulatory site continuously expressed

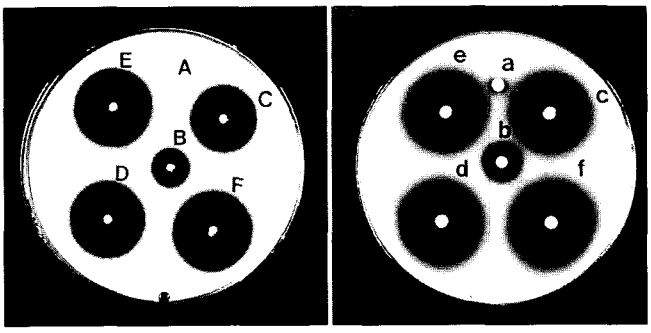

Fig. 3. The halo formed by SHY3, K114, and their transformants.
A, SHY3; B, SHY3/pSA1; C, SHY3/pSA2; D, SHY3/pSA3; E, SHY3/pSA4; F, SHY3/pSA5; a, K114; b, K114/pSA1; c, K114/pSA2; d, K114/pSA3; e, K114/pSA4; f, K114/pSA5.

Table 2. Amylolytic activities in cell-free culture fluids grown with various yeast strains.

Yeast strains[a]	Amylolytic activity (U/ml)
Sch. occidentalis ATCC 26077	1.75
S. cerevisiae SHY3	0.00
S. diastaticus K114	0.36
SHY3/pSA1	0.11
SHY3/pSA2	0.80
SHY3/pSA3	1.61
SHY3/pSA4	2.24
SHY3/pSA5	2.85
K114/pSA1	1.21
K114/pSA2	4.05
K114/pSA3	4.43
K114/pSA4	4.45
K114/pSA5	4.45

[a]Yeast cultures were grown in BYPS2 medium for 4 days.

the mouse salivary *AMY* gene in the medium containing either glucose or ethanol. In SHY3/pSA3, in which the *AMY* gene was linked to the *CYC1* terminator, the α-amylase activity was increased about 14 times higher than that of SHY3/pSA1. Thus, the α-amylase activity produced by SHY3/pSA3 was 2 times higher than that by SHY3/pSA2. Park *et al.* [23] reported that the expression level of *Bacillus subtilis* endo-β-1,4-glucanase gene in *S. cerevisiae* was increased about 2 times by inserting the *CYC1* terminator. The α-amylase activity produced by SHY3/pSA4 in which the G base at the -3 position from the start codon of the *AMY* gene was changed to A base was 1.4 times higher than that by SHY3/pSA3, as Kozak [19] suggested that A at the -3 position was the preferred base for the efficient translation of mRNA [7]. In SHY3/pSA5 containing SV40 enhancer which is able to activate transcription in yeast [6, 8, 9], the α-amylase activity was 2.85 U/ml. This value was 26 times higher than that of SHY3/pSA1, and was comparable with that of *S. diastaticus* YIY345 transformant secreting *Bacillus stearothermophilus* α-amylase (2.63 U/ml), using *STA1* promoter and signal sequence [14]. On the other hand, the amylolytic activity of K114/pSA1 (1.21 U/ml) was about 2.6 times higher than the sum of α-amylase activity produced by SHY3/pSA1 (0.11 U/ml) plus the glucoamylase activity produced by K114 (0.36 U/ml) (Table 2). This result is not consistent with those of Kim *et al.* [17] and Steyn and Pretorius [30] who reported that the effect of *STA* and *AMY* genes on the production of amylolytic activity appeared to be more or less additive. It is considered that the secreted α-amylase was in fact acting synergistically with the glucoamylase. In addition, the amylolytic activities produced by K114/pSA3~pSA5, albeit increased α-amylase activity, remained to be 4.45 U/ml. This result may be due to the expression of the *STA* gene located on the chromosome(s) of K114 under

the control of its own unmodified promoter. However, this value was 2.5 times higher than that obtained from a donor strain for the *AMY* gene, *Sch. occidentalis*, and 3.7 times higher than that of K114 transformant producing mouse α-amylase (1.19 U/ml) [15]. According to the previous report by Wang *et al.* [33], the amylolytic activity of *S. cerevisiae* transformant from their work was about 1.5 times higher than that of *Sch. occidentalis*. Further attempts are being carried out to increase the expression level of *STA* gene with the new vector that we reported here, and to make stable yeast strains producing both α-amylase and glucoamylase with high efficiency by integrating these genes into the chromosome.

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