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Isolation of a new gene (*SWA2*) encoding an α-amylase from *Schwanniomyces occidentalis* and its expression in *Saccharomyces cerevisiae*

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A new gene (*SWA2*) encoding a secretory α-amylase activity from *Schwanniomyces occidentalis* has been cloned from this yeast and then expressed in *Saccharomyces cerevisiae*. Both *Sw. occidentalis* and a transformant of *S. cerevisiae* incorporating *SWA2* contain a transcript of 2.1 kb which hybridizes to DNA carrying the *SWA2* gene. This indicates that the transcript is a product of the *SWA2* gene. Transcription of the *SWA2* gene seems to be regulated in both *Sw. occidentalis* and *S. cerevisiae*. Furthermore, the secretion of α-amylase activity is drastically repressed by glucose in both *Sw. occidentalis* and a transformant of *S. cerevisiae* containing *SWA2*.

α-Amylase; Gene cloning; Starch utilization; *Schwanniomyces occidentalis*; *Saccharomyces cerevisiae*

1. INTRODUCTION

The endoamylolytic enzyme α-amylase (EC 3.2.1.1,α-1,4-glucan-4-glucanohydrolase) catalyses the cleavage of α-1,4-glycosidic bonds within starch and related substrates releasing maltose and longer oligosaccharides and α-limit dextrins. A wide range of organisms produce α-amylases and several of the relevant genes have been cloned [1–4]. Species of the yeast genus *Schwanniomyces* totally hydrolyze starch to glucose by utilizing the enzymes α-amylase, glucoamylase and α-1,6-glucosidase [5]. In contrast, *Saccharomyces cerevisiae*, the main yeast in alcoholic fermentation, is generally devoid of extracellular amylase activity [6] and it would, therefore, be desirable to create genetically manipulated *S. cerevisiae* strains with amylolytic properties. Indeed, a gene (*SWA1* or *AMY1*) encoding an α-amylase from *Sw. occidentalis* (former *Sw. castellii* and *Sw. alluvius* [7]) has already been cloned and expressed in *S. cerevisiae* by several research groups, including our own [4,8,9]. In many eukaryotic organisms, including humans and plants α-amylases are encoded by isogenes. Moreover, the mycelial fungus *Aspergillus oryzae* contains at least three genes for α-amylases which are differentially transcribed [10]. The present work describes both the cloning of a novel gene (*SWA2*) from *Sw. occidentalis* that encodes an extracellular α-amylase and its expression in *S. cerevisiae*.

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2. MATERIALS AND METHODS

Schwanniomyces occidentalis was obtained as *Schwanniomyces castellii* ATCC 26077 from Dr M.A. Delgado (Cruz Campo S.A., Sevilla, Spain). The *Saccharomyces cerevisiae* strains MCCX1-5d (*a, leu2-3 2-112, his4, sta*, sta10*), and IM1-8b (*a, leu2-3 2-112, his4, sta*, sta10, MAL**) were obtained from our culture collection. *Escherichia coli* DH-5 was used for transformation and plasmid amplification. The *S. cerevisiae-E. coli* shuttle vector YEp13 has been described by others [11]. The plasmids pGEM1 and pGEM2 were purchased from Promega Biotech (Madison, WI) and used to prepare riboprobes. Plasmid bluescript was obtained from Stratagene (La Jolla, CA).

Yeast cells were grown on either YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or YNB medium (0.7% yeast nitrogen base w/o amino acids, supplemented with 1% (w/v) starch, 40 μg/ml amino acids and either 0.15% (w/v) glucose or 0.2% (w/v) maltose as required). Starch was purchased from American Society of Brewing Chemistry (St. Paul, MN).

The halo-forming (SWA$^+$) phenotype was scored on plates containing YNB medium supplemented with 2% (w/v) starch and 0.15% glucose (for non-maltose-consuming strains) or 0.2% maltose (for maltose consuming strains). Colonies were transferred to either media and incubated for 7–10 days at 30°C before starch was precipitated by keeping the plates at 4°C for 1–2 days. A clear halo appeared around the SWA$^+$ colonies. Liquid medium for amylase production was YNB buffered with 50 mM phosphate buffer, pH 5.5 [12]. L medium [13] was used for culturing bacterial cells and, when appropriate, it was supplemented with 100 μg/ml ampicillin. Yeast transformation was carried out as described by Burgers et al. [14]. Genomic and plasmid DNA from yeast were isolated as described by Sherman et al. [15]. Bacterial transformation and plasmid isolation were performed as described by Hanahan et al. [16]. Poly(A)$^+$ RNA was prepared by chromatography through oligo(dT)-cellulose of total RNA [17] previously obtained by vortexing yeast cells with glass beads. Southern blots and colony hybridization were performed as described elsewhere [18]. DNA probes were labelled with ^{32}P by nick translation [18]. Northern blots and hybridization with ss-RNA probes were both carried out as previously reported [13]. The RNA dot-blot technique was performed as described by others [19].

Preparation of a genomic library of total DNA from *Sw. occidentalis* in plasmid YEp13 was carried out as described elsewhere [13]. Yeast spheroplasts from strain MCCX1-5d (a non-starch-fermenting strain) were transformed with this gene bank. LEU$^+$ and SWA$^+$ transformants were selected on plates containing 0.7% yeast nitrogen base w/o amino acids, 0.4% YEPD, 2% soluble starch, 0.5% glucose, 1 M sorbitol and 3% agar. Starch-fermenting colonies were detected by observing the clear haloes formed by starch solubilization.

The α-amylase activity from culture filtrates was assayed by the Simoes-Mendes method [20], which was modified as described previously [4]. One unit of α-amylase activity was defined as a decrease of absorbance at 595 nm of 0.1.

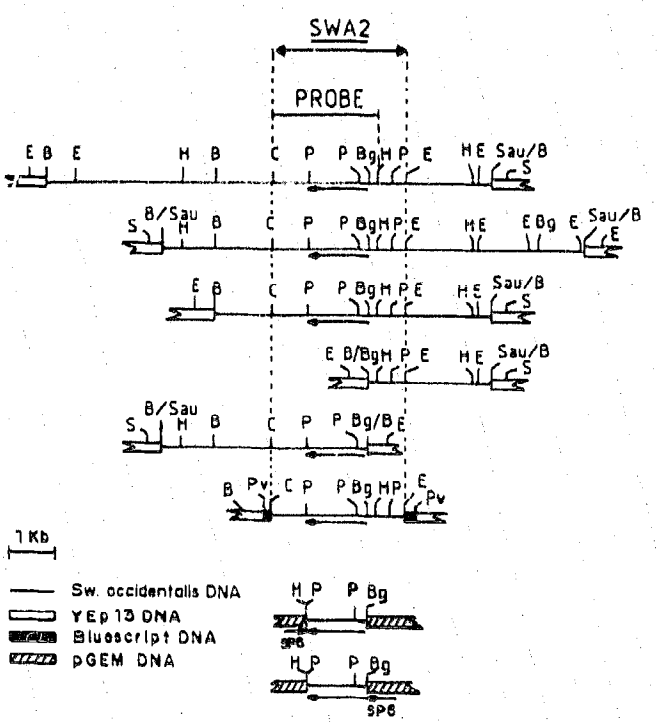
3. RESULTS AND DISCUSSION

3.1. *Isolation of the* SWA2 *gene*

In addition to YEp13-derived plasmids carrying the *SWA1* gene [4], the *Sw. occidentalis* gene library gave rise to halo-forming, Leu$^+$ *S. cerevisiae* transformants harboring the plasmids pFH2 and pFH3 (Fig. 1). The similar restriction maps of the DNA inserts (9.5 and 9 kb, respectively) of these plasmids were unrelated to that of a DNA fragment carrying the *SWA1* (*AMY1*) gene and previously described [4,8,9]. When *S. cerevisiae* spheroplasts were transformed with either pFH2 or pFH3, all LEU$^+$ transformants were also SWA$^+$. In the absence of selection, this phenotype had mitotic instability indicating that the SWA$^+$ character was conferred by a gene (named *SWA2*) which was present in these plasmids. A series of plasmids were obtained by subcloning experiments (Fig. 1). Of these, all plasmids expressing a SWA$^+$ phenotype contained the same 2.9 kb DNA fragment. Therefore, this sequence must contain the *SWA2* gene. The 2.2 kb *ClaI-Hin*dIII fragment from plasmid pFH2.111 was used as a probe to hybridize to a Southern blot that contained genomic DNA from *Sw. occidentalis* and *S. cerevisiae* and plasmid DNA carrying either *SWA1* or *SWA2*. The results of this experiment indicated that the DNA inserts from plasmids pFH 2 and 3 (Fig. 1) were derived from *Sw. occidentalis* and that no homologous DNA was present in either the inserts of plasmids carrying *SWA1* [4] or in several *S. cerevisiae* strains (results not shown).

3.2. *Biochemial analysis*

Culture filtrates from *S. cerevisiae* transformants that incorporate the *SWA2* gene had α-amylase activity (Fig. 2) and totally lacked any glucoamylase or debranching activities. Moreover, small quantities of maltose and high amounts of higher molecular-weight oligosaccharides, but no glucose, were detected by thin-layer chromatography from reactions containing both starch and culture filtrates (results not shown). It appears, therefore, that the *SWA2* gene from *Sw. occidentalis* encodes an extracellular α-amylase activity. As was the case with *SWA1* gene [4], *S. cerevisiae* MAL$^+$ strains



PLASMID	INSERT SIZE (kb)	SWA PHENOTYPE
pFH2	9,5	+
pFH3	9,0	+
pFH2.1	5,9	+
pFH2.2	2,6	–
pFH3.1	4,4	–
pFH2.111	2,9	+
pSWA21	1,3	ND
pSWA22	1.3	ND

Fig. 1. Restriction maps of selected plasmids. Only the *Sal*I, *Bam*HI and *Eco*RI restriction sites from the YEp13 vector are shown in order to indicate the orientation of the DNA inserts. To construct pFH2.111, its DNA insert was taken from pFH2.1, introduced in Bluescript plasmid, taken back by *Pvu*II digestion and then introduced into the unique *Pvu*II site of YEp13. Arrows indicate the direction of transcription. SP6, promoter of the RNA polymerase from bacteriophage SP6. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; S, *Sal*I; Sau, *Sau*3AI. Probe indicates the DNA fragment used as a probe in Fig. 3.

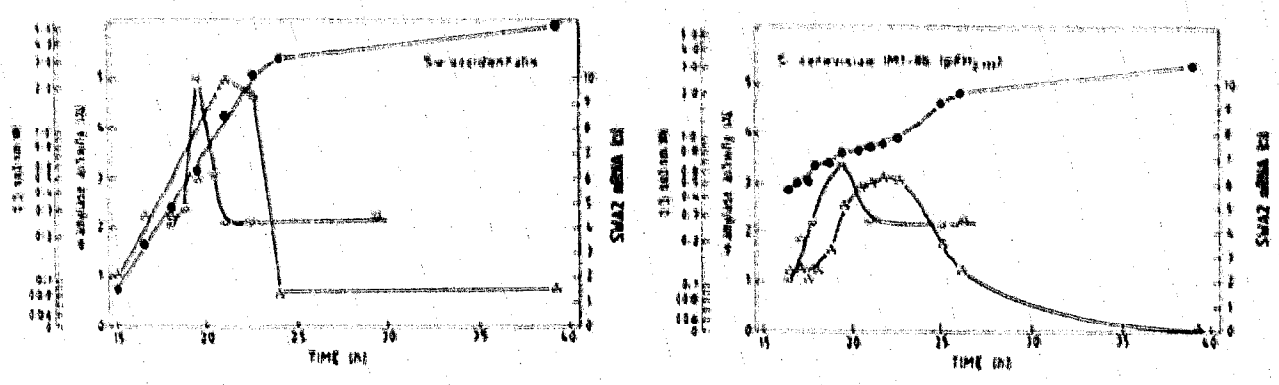


Fig. 2. Expression of the *SWA2* gene in *Sw. occidentalis* and *S. cerevisiae* (pFH2.111). Strains were grown in minimal YNB medium buffered with 50 mM phosphate, pH 5.5, buffer and supplemented with 1% (w/v) starch and 0.2% (w/v) maltose. At the indicated times, samples were taken to estimate optical density, α-amylase activity and the content of α-amylase RNA specific for *SWA2* gene. The latter was determined by the dot blot technique using, as a probe, an (α-^{32}P)dCTP-labelled 2.2 kb *ClaI-HindIII* fragment from pFH2.111 (Fig. 1). Poly(A)$^+$ RNA samples (30 μg) were serially and identically diluted up to 2 μg and then hybridized to the probe. The spots were quantitated and then integrated in a model 300A Molecular Dynamics computing densitometer. The amount of *SWA2* mRNA is given in arbitrary units relative to a constant amount of total poly(A)$^+$ RNA. The amylase activity is given in units as described in section 2.

that incorporate *SWA2* showed low levels of growth on culture media containing starch as the sole carbon source. This effect could result from the production of small amounts of maltose. Although the *SWA2* gene has a similar activity at all different temperatures studied to that of the *SWA1* gene product, it is much more labile to pH changes (results no shown).

3.3. *Transcription studies*

The 2.2 kb *ClaI-HindIII* DNA fragment from plasmid pFH2.111 was used as a probe and hybridized, independently, to poly(A)$^+$ RNA from *Sw. occidentalis* and *S. cerevisiae* IM1-8b (YEp13) and (pFH 2.111). A 2.1 kb transcript appeared in *Sw. occidentalis* and *S. cerevisiae* (pFH2.111) but not in *S. cerevisiae* (YEp13) (Fig. 3). This transcript, which is of a different size to that of *SWA1* (1.55 kb) [4], should correspond to the *SWA2* gene. In addition, a 1.65 kb transcript was present in the poly(A)$^+$ RNA from *S. cerevisiae* (pFH2.111). A similar situation was encountered with the *SWA1* gene [4], and it could result from additional

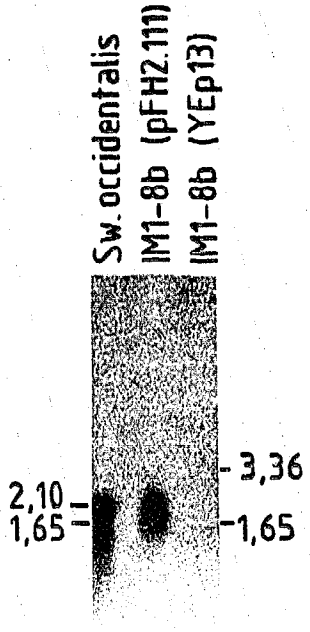


Fig. 3. Transcription studies. Poly(A)$^+$ RNA (5 μg) from *Sw. occidentalis* and the relevant *S. cerevisiae* strains were developed by denaturing gel electrophoresis and transferred to a nylon sheet that was hybridized to the 2.2 kb *ClaI-HindIII* DNA fragment from the pFH2.111 plasmid. Numbers indicate the size (in kb) of the RNA standards (right) and transcripts (left).

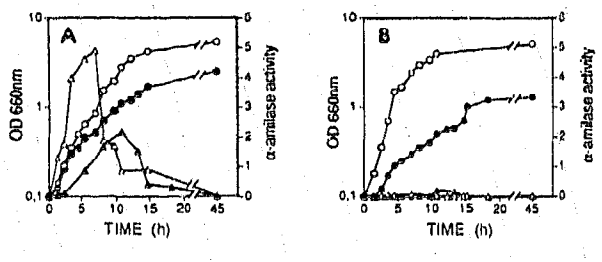


Fig. 4. Repression by glucose of α-amylase secretion. Inocula from *Sw. occidentalis* and *S. cerevisiae* (pFH2.111) were grown in YNB medium buffered with 50 mM phosphate pH 5.5 buffer supplemented with 2% glucose. The cells were washed in the final assay media and then inoculated into the relevant medium. (A) Medium containing 2% maltose as carbon source (○) and (●), OD_{660nm} from *Sw. occidentalis* and *S. cerevisiae* (pFH2.111), respectively. (△) and (▲) secreted α-amylase activity from *Sw. occidentalis* and S. cerevisiae (pFH2.111), respectively. (B) Medium containing 2% glucose as carbon source. Symbols as in (A). The α-amylase activity is given in units as described in section 2.

transcription sites involving initiation or termination from the *SWA2* gene in *S. cerevisiae*. Even, a RNase degradation could account for the presence of these smaller transcripts.

To determine the direction of transcription of *SWA2*, the 2.2 kb *ClaI-HindIII* fragment from pFH 2.111 was inserted downstream from the SP6 RNA polymerase promoter of plasmids pGEM1 and pGEM2 to generate plasmids pSWA21 and pSWA22, respectively (Fig. 1); these differed in the insert orientation. Both plasmids were used to prepare riboprobes, which were hybridized to poly(A)$^+$ RNA from *Sw. occidentalis* and *S. cerevisiae* IM1-8b (YEp13). Hybridization took place only with the riboprobe prepared from plasmid pSWA21, which indicated that the direction of transcription was from the *BglII* to the *PstI* site (Fig. 1) (results not shown).

Both transcription from the *SWA2* gene and secretion of α-amylase activity were studied during the growth in liquid culture of *Sw. occidentalis* and *S. cerevisiae* IM1-8b (pFH2.111). The results of this experiment suggested that transcription from *SWA2* is regulated in these two yeasts (Fig. 2). The number of transcripts markedly increased at the middle of the log phase of growth to decrease dramatically at the late stages of growth. The α-amylase activity exhibited a similar pattern of variation (Fig. 2). In this respect, it has been shown that in *Sw. occidentalis* the α-amylase activity is induced by maltose and repressed by glucose [21]. This finding was confirmed in the experiment described in Fig. 4, where it is also shown that glucose drastically represses α-amylase secretion in a *S. cerevisiae* transformant containing *SWA2*. Whether that regulation is achieved by similar mechanisms in both organisms remains to be seen. In contrast, the expression of the *SWA1* gene, although it appears to be regulated in *Sw. occidentalis*, is constitutive in *S. cerevisiae* [4]. The reason for this different behaviour is presently unknown, but it is possible that these two genes are under different transcriptional control in *Sw. occidentalis*.

The nucleotide sequence of *SWA1* (*AMY1*) is already known [9] and that of *SWA2* is currently being determined. Initial results indicate a high degree of homology between both genes (unpublished observations), suggesting that they have each evolved from a single ancestor.

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REFERENCES

[1] Itoh, T., Yamashita, I. and Fukui, S. (1987) FEBS Lett. 219, 339–342.
[2] Emoni, M., Tojo, T. and Maruo, B. (1988) Agricul. Biol. Chem. 52, 399–406.
[3] Hoshiko, S., Makabe, O., Nojiri, C., Katsumata, K., Satoh, E. and Nagaoka, K. (1987) J. Bacteriol. 169, 1029–1036.
[4] Abarca, D., Fernández-Lobato, M., Claros, M.G. and Jiménez, A. (1989) FEBS Lett. 255, 455–459.
[5] Ingledew, W.M. (1987) CRC Crit. Rev. Biotechnol. 5, 159–176.
[6] Tamaki, H. (1978) Mol. Gen. Genet. 164, 205–209.
[7] Price, C.W., Fuson, G.B. and Pfaff, H.J. (1978) Microbiol. Rev. 42, 161–193.
[8] Wang, T.T., Lin, L.L. and Hsu, W.H. (1989) Appl. Environ. Microbiol. 55, 3167–3172.
[9] Strasser, A.W.M., Selk, R., Dohmen, R.J., Niermann, T., Bielefeld, M., Seeboth, P., Tu, G. and Hollenberg, C.P. (1989) Eur. J. Biochem. 184, 699–706.
[10] Wirsel, S., Lachmund, A., Wildhart, G. and Rutthovski, E. (1989) Mol. Microbiol. 3, 3–14.
[11] Broach, J.R., Strathern, J.N. and Hicks, J.B. (1979) Gene 8, 121–133.
[12] Oteng-Gyang, G., Moulin, G. and Galzy, P. (1980) Eur. J. Appl. Microbiol. Biotechnol. 9, 129–132.
[13] Pardo, J.M., Polaina, J. and Jiménez, A. (1986) Nucleic Acids Res. 14, 4701–4718.
[14] Burgers, P.M.J. and Percival, K.J. (1987) Anal. Biochem. 163, 391–397.
[15] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Methods in Yeast Genetics, Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[16] Hanahan, D. (1983) J. Mol. Biol. 166, 557–580.
[17] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[18] Maniatis, T., Fritsch, E.F. and Sambrook, K.J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[19] Constanze, C. and Gillespied, D. (1987) Methods Enzymol. 152, 582–587.
[20] Simoes-Mendes, B. (1984) Can. J. Microbiol. 30, 1163–1170.
[21] Dohmen, R.J., Strasser, A.W.M., Zitomer, R.S. and Hollenberg, C.P. (1989) Curr. Genet. 15, 319–325.