Construction of an efficient amylolytic industrial yeast strain containing DNA exclusively derived from yeast

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Abstract

An amylolytic industrial yeast strain of Saccharomyces cerevisiae containing the Schwanniomyces occidentalis SWA2 amylase gene was generated. The new strain contains DNA derived exclusively from yeast and expresses a high starch hydrolyzing activity. Yeast transformation was carried out by an integrative process targeted to a dispensable upstream region of the ILV2 locus, which determines sulfometuron resistance. The SWA2 enzyme was constitutively expressed under the ADH1 promoter. The growth, substrate utilization and fermentative capacity of this organism are described. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Industrial Saccharomyces cerevisiae are unable to metabolize starch, a relatively cheap carbon source. The induction of such a capability is a marked improvement in both, the baking and brewing industry as well as in producing ethanol, maltose syrups or single cell protein (SCP).

α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) catalyzes the endoamylolytic cleavage of α-1,4-glucosidic linkages of starch and similar substrates releasing maltose, oligosaccharides and limit dextrins. Typically, commercial amylases are crude preparations obtained from species of the filamentous fungus Aspergillus. These enzymes are commonly added to flour to enhance baked products. However, their use increases the cost of the final product, and those preparations act as potent allergens capable of sensitizing many of the workers who are exposed to them [1].

S. cerevisiae has been widely used to express and study amylases from different eukaryotic organisms and glucoamylases and α-amylases from different yeast genera have also been analyzed [2–4]. Previously, we isolated two different α-amylase genes (SWA1 and SWA2) from the yeast Schwanniomyces occidentalis and studied their expression in S. cerevisiae [5–7].

Recombinant DNA technology for industrial yeast modification requires a dominant selection marker, which can be used in a wild-type polyploid background. A one-step dominant selection procedure based on resistance to the sulfonylurea-herbicide sulfometuron-methyl (SM) has been previously developed [8]. This method provides a site of integration close to the ILV2 locus of S. cerevisiae, which encodes the enzyme acetolactate synthase. At least 10 phenotypically distinct alleles (named SMRI) of ILV2 mutants which determine resistance to SM have been described [9,10]. The SM marker has also been used in industrial strains to disrupt the sake yeast arginase gene [11], and to introduce the S. cerevisiae var. uvarum α-galactosidase MEL1 into baker’s yeast strains [12].

Previously, the α-amylase and glucoamylase genes from Aspergillus oryzae were used to construct a starch-utilizing baker’s yeast. α-Amylase cDNA was introduced into baker’s yeast via the episomal plasmid (YEp) [13] or by employing an integrative cassette targeted to the ribosomal rDNA locus [14]. Glucoamylase cDNA has also been introduced into baker’s yeast targeted to the δ-sequence of the yeast retrotransposon Ty1 [15].
In this paper, we describe the generation of a stable baker’s yeast strain able to grow in starch efficiently after SWA2 amylase integration, targeted to the upstream region of the ILV2 locus. The new strain, which contains genes derived exclusively from yeast, improves the starch hydrolytic rates of similar S. cerevisiae transformants previously constructed. We analyzed the growth, the amylase activity, the ability to hydrolyze starch and the fermentative capacity of this organism.

2. Materials and methods

2.1. Strains, transformation and culture conditions

Escherichia coli DH5α was used for transformation, and plasmid constructs were generated by using standard techniques. The industrial baker’s yeast strain DADY was obtained from Dr. M.A. Delgado (Cruz del Campo S.A., Sevilla, Spain). Yeast cells were grown in complete YEP medium (1% yeast extract, 2% peptone), supplemented with either 2% glucose (YPEP), 2% maltose (YEPM), 2% soluble starch (YESP) or 2% starch and 1% or 0.05% maltose (YEPSM). Minimal MM medium contained 0.7% yeast nitrogen base w/o amino acids, 2% starch and 0.05% maltose. Molasses medium contained 0.7% yeast nitrogen base w/o amino acids and 2% beet molasses. To quantify amylase activity, yeasts were cultured on media supplemented with 50 mM sodium phosphate pH 5.5. When necessary, media were solidified by the addition of 2% agar. Yeast cells were transformed by using a lithium acetate method. Transformant selection was made in a MM medium supplemented with 2-methylsulfometuron (SM) (30 μg ml⁻¹). SM was provided by Dr. S.C. Falco (Du Pont).

2.2. Vectors, constructions and DNA manipulations

Small-scale DNA isolations from E. coli were performed by alkaline lysis. Bluescript plasmid (pBS) from Stratagene was employed for cloning and sequencing experiments. pQX1B [10], including the SMR1B gene and pBs2 [7], containing the S. occidentalis SWA2 gene are described in Table 1.

The 520 nt (−517 to +3) fragment from pAH21 (Table 1) [16], containing the ADH1 promoter, was fused to the SWA2 coding region by polymerase chain reaction (PCR). For promoter amplification, the oligonucleotides A (−517 to −491), 5′-GGTACATAACGAATTCTATTACTCTA-GACC-3′, including EcoRI and XhoI recognition sequences (in bold), and B (−17 to +3), 5′-CATTGTATATAGATAGTTG-3′ were used. The pBs2 plasmid, the oligonucleotide C, 5′-CTATCCTCATATATGAAATT-TGCAACTATC-3′, which is homologous with the (−14 to −1) ADH1 promoter sequence (in bold), and the (+1 to +18) SWA2 coding sequence and the pBS universal −20, were used for SWA2 amplification. The amplified products were fused using PCR and the oligonucleotides A and the pBS universal −20 as primers. The fusion was verified by DNA sequencing by using standard techniques. To obtain the final construct (pADH-SWA2, Table 1), the EcoRI–ClaI 3.1 kb fragment was cloned in the pBS EcoRI–ClaI sites. Southern blot analysis was performed by using standard techniques. The DNA hybridization probe was labeled with [α-32P]dCTP using the prime A gene labeling system (Promega, Madison, WI, USA) and signals were recorded in a phosphorimager (Molecular Dynamics, Tina 2.0, D).

To construct the α-amylase integrative cassette (pSAS2, Table 1), the 3.1 kb EcoRI–ClaI pADH-SWA2 fragment, including SWA2 amylase fused to the ADHI promoter was blunt-ended and cloned into pQX1B plasmid previously digested with EcoRV.

2.3. Enzyme, starch hydrolyzed and fermentation test

α-Amylase was assayed at 40°C as described previously [5]. One unit of α-amylase activity was defined as a decrease in A95₄₅nm of 0.1 after 90 min incubation. The amylolytic phenotype was determined on MM medium plates. Cells were grown for 3 days at 28°C and then left at 4°C for 24 h. Cells, which expressed the amylase gene, were easily recognized because they were surrounded by a clear zone (halo) caused by starch hydrolysis in the medium. Starch hydrolysis was also evaluated in liquid medium by using 25 μl of culture supernatant as a loss of iodine (0.5% (w/v) KI, 0.15% (w/v) I₂) staining capacity [5]. For the fermentation test, Durham tubes of 100×12 mm, with insert tubes about 43×4.5 mm in size, were used. The tubes were filled with 4 ml of YEPSM medium and inocules.
ulated with 10 µl of a fresh yeast suspension of $5 \times 10^7$ cells per ml. Cells were incubated at 28°C for 12 h and then the volume of CO$_2$ produced estimated.

3. Results and discussion

3.1. Characterization and stability of transformants

The aim of the present study was to develop a stable, industrial baker’s yeast strain containing DNA derived exclusively from yeast, that was able to hydrolyze starch efficiently. To fulfill this objective, the α-amylase SWA2 gene from the yeast *S. occidentalis* was employed in an integrative transformation method that does not utilize plasmid sequences. The pSAS2 construction was *Pvu*I linearized and used to transform the DADY baker’s yeast strain. No background growth was observed on solid MM medium containing 30 µg ml$^{-1}$ of SM. Therefore, these conditions were used for selecting transformants. All amyloolytic transformants tested (10) were resistant to 250 µg ml$^{-1}$ of SM. To study mitotic stability, the four transformants with the largest starch hydrolysis halos were grown on non-selective liquid medium (YEPD) at 28 °C for 50 generations of growth. Cells were then plated on rich medium and incubated at 28°C for 2 days. Up to 2000 colonies were then replicated on selective YEPD medium containing 30 µg ml$^{-1}$ SM and more than 99% grew (data not shown). Transformant related as SAS2-1 was selected for detailed analysis. This new industrial organism is very stable. The high genomic stability of *S. cerevisiae* baker’s yeast growing in complete medium culture as reported here and previously recorded elsewhere [17], could suggest an industrial potential for this new strain.

Southern blot analysis of the transformant was carried out to determine if the amylase SWA2 integration was at the desired locus (Fig. 1). Using as a probe the pBs2 *Hin*cl–*Cla*I *S. occidentalis* DNA fragment that included part of SWA2 gene, the appropriate hybridizing bands appeared in the transformant, in agreement with the previously calculated size. The 11.8 kb band visualized in the *EcoRV* digested DNA from the SAS2-1 strain DNA indicates that the *ILV2* locus upstream region is correctly integrated, as the *EcoRV* site was not regenerated. As expected, no hybridization signal was obtained with total wild-type yeast DNA. No signal was obtained after rehybridization of this blot using the pBS plasmid as a probe (results not shown), confirming that the new strain (SAS2-1) is totally free of this bacterial plasmid DNA.

3.2. Expression of SWA2 amylase in the baker’s yeast strain

The α-amylase activity was examined in culture supernatants of strain SAS2-1 grown in media containing different, below indicated, carbon sources. As expected, the SWA2 gene fused to the *ADH1* promoter was constitutively expressed using complete media containing maltose (Fig. 2). The maximal α-amylase activity (approximately 7 U) was maintained for at least 140 h of growth (data not shown). Similar time courses of growth and extracellular amylase levels were obtained using glucose (data not shown). Intracellular amylase was determined as well, although it was not detected. To study the capacity of *S. cerevisiae* SAS2-1 to hydrolyze starch, the transformant was grown in medium containing soluble starch as carbon source (Fig. 3). Cell growth and enzyme expression ki-
netics (data not shown) were similar to those observed using previously studied saccharides, reaching almost maximal values after 25 h of growth. Transformant cells were able to grow to a density of \(A_{660\text{nm}} = 6.5\). Starch was efficiently hydrolyzed (Fig. 3A), with almost 35%, 90% and 95% of iodine valuable starch being hydrolyzed in 5 h, 25 h and 35 h of culture growth, respectively. The parental wild-type strain could grow to a density of \(A_{660\text{nm}} = 2.4\) in this medium. Although no amylase activity (data not shown) or starch hydrolysis was observed, addition of maltose to the starch medium increased the growth of the control strain to a level close to that of the transformant (Fig. 3B).

Growth of the parental wild-type strain, as observed in soluble starch medium (Fig. 3A), probably occurred as a consequence of the small saccharides present in the original starch preparation. Growth of control wild-type strains in soluble starch medium has also been reported by Ma et al. [18].

The growth profile and the \(\alpha\)-amylase activity produced in molasses minimal medium was also evaluated in the SAS2-1 strain. As expected, no differences in the amount of yeast biomass were observed with respect to the control wild-type strain, but the SAS2-1 strain secreted \(\alpha\)-amylase into the medium in a constitutive manner (data not shown). The SAS2-1 yeast strain has the \(SWA2\) amylase gene inserted at a non-essential genomic region. Therefore, it should retain the industrially favorable characteristics of the parental strain. Consequently, no effect on its growth was observed using maltose (Fig. 2) or molasses (results not shown) as carbon sources compared with the parental yeast strain. Amylolytic capacity is the only phenotypic change acquired by the SAS2-1 strain.

The new industrial yeast strain obtained in this work hydrolyzed 95% of starch within 35 h of growth and only residual starch levels were detected over longer periods. This strain showed a clear improvement on starch hydrolysis efficiency as related to other recombinant yeasts previously reported. For example, the \(\alpha\)-amylase genes from different \(Bacillus\) spp. were able to promote a substantial growth of a laboratory yeast strain on soluble starch, only when they were coexpressed with different microorganism glucoamylase [2,19–21] or pullulanase [4] genes. Similar results were obtained by coexpressing an \(Aspergillus\) awamori glucoamylase gene and a \(Pseudomonas\) amyloderamosa isoamylase gene [18]. In none of these cases the amylolytic capacity of the new organism reached the level reported here. Construction of a stable industrial baker’s yeast strain expressing \(A.\) oryzae \(\alpha\)-amylase in a...
high copy number was reported by Nieto et al. [14], but no data concerning hydrolysis of soluble starch were provided.

3.3. Fermentative capacity

The yeast gassing rates are critical in baking technology. The yeast produces CO$_2$ that results in dough leavening and contributes to the flavor and crumb structure of bread. Therefore, the ability of the SAS2-1 strain containing the $SWA2$ amylase gene to ferment starch was evaluated. Inocula from both SAS2-1 and wild-type baker's yeast strains were grown in YEPSM (0.05% maltose) medium, at 28°C for 12 h without shaking. Produced CO$_2$ was estimated in μl using Durham tubes in accordance with the insert tube capacity. Gassing activity of the novel amylolytic strain increased 3.6 times relative to the wild-type parental yeast (from 160 μl to 580 μl) (data not shown). The α-amylase activity was approximately 7 U ml$^{-1}$, similar to the value obtained in the different used media. As predicted, no amylase activity was obtained using the parental wild-type strain.

As the food industry continues to move toward developing new amylolytic enzymes and novel starch processes to provide products that meet the high expectations of consumers, genetically engineered strains of $S$. cerevisiae secreting heterologous starch-degrading enzymes will be in continuous development. Strain SAS2-1 is a very efficient amylolytic organism despite the fact that it only contains an α-amylase. In any case, to obtain a higher level of amylolytic capacity strain, a multicopy integration of the transformation cassette containing $SWA2$ gene and/or co-expression of this gene with that of a glucoamylase one will obviously be required. Progress on both approaches has been initiated.

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