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# Characterization of a new xylitol-producer *Candida tropicalis* strain

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**Abstract**

A xylitol-producer yeast isolated from corn silage and designated as ASM III was selected based on its outstanding biotechnological potential. When cultivated in batch culture mode and keeping the dissolved oxygen at 40% saturation, xylitol production was as high as 130 g $l^{-1}$ with a yield of 0.93 g xylitol $g^{-1}$ xylose consumed. A preliminary identification of the yeast was performed according to conventional fermentation and assimilation physiological tests. These studies were complemented by using molecular approaches based on PCR amplification, restriction-fragment length polymorphism analysis and sequencing of the rDNA segments: intergenic transcribed spacer (ITS) 1- 5.8S rDNA – ITS 2, and D1/D2 domain of the 26S rRNA gene. Results from both the conventional protocols and the molecular characterization, and proper comparisons with the reference strains *Candida tropicalis* ATCC 20311 and NRRL Y-1367, led to the identification of the isolate as a new strain of *C. tropicalis*.

## Introduction

Xylitol is a naturally occurring five-carbon pentahydroxy alcohol with high sweetening power. Its use as a food sweetener, anti-cariogenic and sugar substitute in the treatment of diabetes is widespread and markedly increasing. Xylitol solutions exhibit low viscosity and it has a negative heating when dissolved in a solution. Because of these properties and other physiological functions, xylitol has received much attention (Vandeska et al. 1995; Kim et al. 1997).

The synthesis of xylitol by chemical reduction of xylose from natural sources is a high cost process: first, because of the low initial sugar availability and second, due to the extensive purification and separation steps required in order to remove the by-products formed during the reaction. In contrast, the feasibility for xylitol to be fermentatively produced as an intermediate in the d-xylose metabolism of many yeasts and bacteria is nowadays becoming a more attractive alternative (Parajó et al. 1997). The advantage of not requiring pure xylose syrup at the starting point for microbiological production, as the chemical synthesis does, could be of high economical interest since low-cost hemicellulosic hydrolysates may become potential substrates (Roseiro et al. 1991).

Xylose-utilising yeasts, such as *Debaryomyces hansenii, Candida guilliermondii* and *Candida parapsilosis*, produce high amounts of xylitol as the major product of xylose metabolism (Girio et al. 1996). Accumulation of xylitol in culture media varies according to the yeast and the culture conditions used (Saha and Bothast 1999). The ability to produce xylitol as a normal metabolic product has been frequently observed for diverse yeasts, and particularly for *Candida* species (Latif and Rajoka 2001; Silva and Roberto 2001; Walther et al. 2001; Faria et al. 2002).

Procedures conventionally used for yeast identification include cellular morphology and distinctive reactions based on standardized fermentation and assimilation tests. These assays are laborious and they sometimes lead to ambiguous results because of strain phenotypic variability. Given these drawbacks and the impracticability for identifying most species from genetic crosses, molecular approaches including sequencing, restriction-fragment length polymorphism (RFLP; Belloch et al. 1998; Bruns et al. 1991), pulsed field gel electrophoresis (PFGE; Bignell and Evans 1990) and random amplified polymorphic DNA-PCR (RAPD; Oda et al. 1999) are consistently used for yeast identification.

Nowadays, sequencing appears to be the most systematically used molecular technique for identification, probably because strain comparisons can be readily undertaken against the available dataset. Comparison of ribosomal RNA (rRNA) gene sequences offers a powerful means for estimating phylogenetic relationships. Nevertheless, the determination of complete sequences is laborious and, to date, only a few hundred are available for either the large (26S) or the small (18S) rDNA subunits (Kurtzman 2001). Partial sequences can lead to essentially the same phylogenetic affiliations as the complete sequences do (McCarroll et al. 1983; Lane et al. 1985) and complementation with further information provided by short non-coding sequences such as the internal ribosomal transcribed spacers (ITS; James et al. 1996; Turenne et al. 1999) is often used to derive more precise taxonomic inferences.

The present work is focused on the systematic characterization of the selected high xylitol-producer yeast strain ASM III isolated from nature by means of both traditional and molecular methods.

## Materials and methods

### *Yeast strains and culture conditions*

*Candida tropicalis* ATCC 20311, *Candida tropicalis* NRRL-Y 1367 and *Saccharomyces cerevisiae* ATCC 32052 were used as reference strains. The new isolate, ASM III, is maintained in the Agricultural Research Culture Collection as NRRL Y-27290 (Altamirano et al. 2000).

Cultures were maintained on YEPD agar slants containing (in g-$l^{-1}$): glucose, 20; peptone, 20; yeast extract, 10 and agar, 20. They were incubated at 30 °C for 24 h, subsequently stored at 4 °C and subcultured at regular intervals.

### *Fermentation assays*

Shaken flask assay medium, in g $l^{-1}$: d-xylose, in the range from 30 to 250; peptone, 20; yeast extract, 10; pH 5.0.

Experiments were performed in triplicate in 250-ml Erlenmeyer flasks containing 100 ml of medium, inoculated to a final concentration of $10^7$ cells $ml^{-1}$ with an active overnight pre-inoculum and incubated at 30 °C in an orbital shaker at 200 rpm. Samples were withdrawn after 48 h.

Fermentation medium, in g $l^{-1}$: d-xylose, 200; $KH_2PO_4$, 3; $(NH_4)_2HPO_4$, 1; $MgSO_4 \cdot 7H_2O$, 1; yeast extract, 10, pH 5.0.

Batch cultures were performed in a 4-litre LH-210 (Inceltech) fermenter with a working volume of 2.5 l, equipped with automatic control of dissolved oxygen, pH, foam and temperature. It was inoculated to a final concentration of 0.2 g $l^{-1}$ cells (dry weight). The pH was maintained at 5.0 by the addition of either 0.1N NaOH or 0.1N $H_2SO_4$. The agitation rate was kept at 300 rpm and temperature at 30 °C. Dissolved oxygen was controlled at 40% saturation by automated air supply via a proportional integrative and derivative (PID) controller, with maximal fluctuations lower than 5%. Samples were periodically withdrawn according to the experimental protocol. Results represent the average of three independent experiments.

### *Analytical methods*

Cell concentration was determined by drying washed biomass at 105 °C to constant weight. Residual d-xylose and xylitol concentrations (g $l^{-1}$) were determined by using a Gilson (France) HPLC, equipped with a 305 pump, a differential refractometer 2142 LKB (Sweden) and a recorder/integrator chromatopac CR 301 (Shimadzu, Japan). Concentration of each substance was determined with a refractive index (RI) detector, under the following conditions: Rezex Organic Acid ROA (Phenomenex) column (300 × 7.8 mm); temperature, 55 °C; eluent, 0.02 N $H_2SO_4$; flow rate, 0.6 ml $min^{-1}$; sample volume, 20 μl. Samples (0.7 ml) were pre-treated with an equal volume of 6% trichloroacetic acid, centrifuged and the supernatant used for HPLC determinations.

*Conventional characterization of the isolate*

Cellular and colony morphology, growth temperature, carbon assimilation, nitrogen source, urease activity, vitamin requirements and sugar fermentation were analysed as described by Yarrow (1998). Urease activity was assayed after growth on Bacto Urea Broth (Difco, Detroit, Michigan, USA) under optimal culture conditions.

*Molecular characterization of the isolate*

Chromosomal DNA was isolated from 35 ml of overnight cultures using the procedure described by Philippsen et al. (1991).

Universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) were used to amplify a fragment comprising the internal transcribed spacer 1 (ITS1), the 5.8S rDNA and the internal transcribed spacer 2 (ITS2), according to Lott et al. (1993). Another set of primers, NL-1 (5´-GCATATCAAAAGCGGAGGAAAAG-3´) and NL-4 (5´-GGTCCGTGTTTCAAGACGG-3´), were used to amplify the 26S rDNA D1/D2 domain, as described by Kurtzman et al. (2001). PCR assays were performed in a GeneAmp PCR 9700 System (Applied Biosystems). Reactions were carried out in a 25-µl final volume containing 100 ng of DNA template, 100 nM of each primer, 2.5 µl of 10 × STR buffer, and 1 U of *Pfu* DNA Polymerase (Promega). In order to check amplifications, 10 µl of each amplicon were electrophoresed on 1% (w/v) agarose gels (Sambrook et al. 1989).

*RFLP analysis*

PCR fragments obtained with the ITS1/NL-4 primer pair from the yeasts *C. tropicalis* ATCC 20311, *S. cerevisiae* ATCC 32052 and ASM III were comparatively digested with 10 U of *Bspd*I, *Ssp*I, *Eco*RI, *Bam*HI and *Hae*III restriction enzymes (Promega) according to the supplier's instructions. The restriction fragments were electrophoresed in a 3% agarose gel.

*rDNA sequence analyses*

DNA sequencing on both strands was performed by the dideoxy chain termination method with an ABI Prism 3100 DNA Analyser, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reactions kit (PE Biosystems) according to the manufacturer's protocol.

The sequences were registered in the GenBank Data Library under accession numbers AF268095 and AF267497 for the ITS 1-5.8S rDNA-ITS2 fragment and for the D1/D2 26S rDNA domain, respectively. Sequences belonging to the same species or closely related ones, available through the public databases, were aligned. Only unambiguously aligned positions from all sequences were used to calculate a similarity matrix, and gaps were not included in the match/mismatch count. *S. cerevisiae* (GenBank accession number AB018043) was used as the outgroup strain.

## Results and discussion

*Xylitol production*

Shaken flask fermentation assays were performed in order to determine the best initial xylose concentration for xylitol production by the ASM III strain. Initial xylose concentration was varied in the range from 30 to 250 g $l^{-1}$, with 200 g $l^{-1}$ being the optimum set point leading to a maximal xylitol production of 98 g $l^{-1}$ (data not shown).

The isolate was thereafter assessed for its ability to produce xylitol in a fermentation medium with 200 g $l^{-1}$ of xylose when grown in batch culture mode under controlled conditions. Since oxygen limitation influences xylitol production by *Candida* species (Vandeska et al. 1995; Kim et al. 1997; Altamirano et al. 2000; Walther et al. 2001) the dissolved oxygen was kept at 40% saturation. The time courses of biomass and xylitol production as well as residual xylose concentration were determined.

As depicted in Figure 1, xylitol production accompanied xylose consumption during yeast growth, traces of ethanol being also detected (data not shown). A final xylitol concentration of 130 g $l^{-1}$ was obtained in association with a yield of 0.93 g xylitol $g^{-1}$ xylose consumed. Biomass production was rather low (3.67 g $l^{-1}$), very likely as a consequence of the selected fermentation conditions. These results were similar to those previously reported for *C. parapsilosis* (Kim et al. 1997) and for *C. tropicalis* (Horitsu et al. 1992), and higher than those obtained by Vandeska et al. (1995) for *Candida boidinii*.

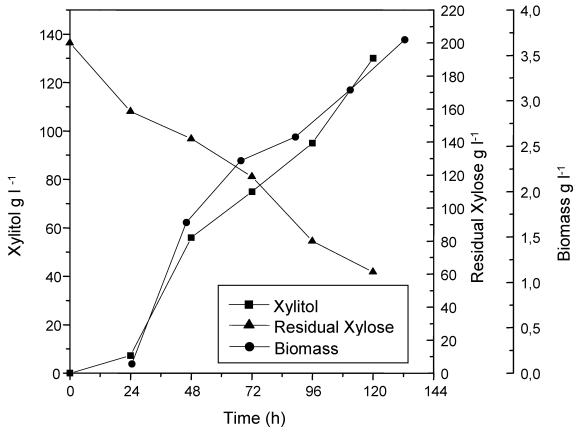


*Figure 1.* Time course of xylitol production during a batch culture of the ASM III yeast strain using a fermentation medium with 200 g $l^{-1}$ initial xylose concentration. Cultivation conditions: 30 °C, 300 rpm and dissolved oxygen at 40% saturation.

*Morphological and physiological characterization*

Cell and colony morphological analyses were performed for the isolated yeast under study, ASM III (Altamirano et al. 2000), and the other different yeasts included for comparison purposes, that is to say *C. tropicalis* ATCC 20311 and NRRL-Y1367. After 7 days of incubation at 25 °C on different culture media (corn meal agar, potato dextrose agar and YNB without aminoacids plus D-ribose or starch as C-source), the macro-morphological characteristics of the colony were first assessed by naked eye observation and corroborated with the aid of magnification (×40). The ASM III isolate exhibited similar colony morphology to the *C. tropicalis* reference strains ATCC 20311 and NRRL-Y1367, presenting white, smooth, butyrous colonies. This was in accordance to previous observations by Meyer et al. (1998) for *C. tropicalis*. However, ASM III showed poorer pseudohyphae production after 7 days of incubation.

Growth at 40 °C in YPD culture medium at 200 rpm became positive after overnight incubation and growth on YNB without aminoacids plus either D-ribose or starch as C-source was positive after 7 days of incubation at 30 °C. Both ASM III and the reference strains showed the same behaviour. As another criterion for identification, urease activity was also tested. Phenol-red containing media confirmed the absence of urease activity for ASM III and *C. tropicalis* ATCC 20311 and NRRL-Y1367, in contrast to the positive result for a control strain *S. cerevisiae* ATCC 32052. These results were in agreement to those previously reported by Meyer et al. (1998) for *C. tropicalis*.

*RFLP analysis*

For the endonucleases assayed, the RFLP analysis of the ITS1/NL-4 amplified fragments from *C. tropicalis* ATCC 20311 and ASM III (1,200 bp-long) showed identical profiles, while the fragment from *S. cerevisiae* ATCC 32052 (1,500 bp-long) led to a different pattern (Figure 2).

*rDNA sequence analysis*

In order to confirm the RFLP results, sequence analysis of the conserved rDNA regions was performed. According to Lott et al. (1993), *C. tropicalis* and *C. parapsilosis* species constitute a complex at the 5.8S rDNA level. The 5.8S rDNA sequence from ASM III showed around 98 and 99% similarity with both species, differing only at nt 58, 63 and 100. Thus, the highly conserved nature of this gene hindered the establishment of a more accurate phylogenetic affiliation for the ASM III strain.

On the other hand, when ITS1 and ITS2 sequences were compared, ASM III displayed 98% identity with those ITS sequences from *C. tropicalis* (L47112). In contrast, ASM III showed only 70% identity with *C. parapsilosis* ITS sequences (L47109). On this basis *C. parapsilosis* was included in a different cluster to that defined for ASM III and *C. tropicalis* strains.

The 26S rDNA D1/D2 domain sequence analysis (approximately 600 bp), revealed a highly conserved relationship at the species level, consistent with the molecular identification of different yeasts (Kurtzman et al. 2001) of this sequence. In the present case, the ASM III D1/D2 domain showed a 99% similarity with available sequences from *C. tropicalis* and a 95% similarity when compared to the same region from *C. parapsilosis*.

The phylogenetic tree constructed on the basis of the ITSs and D1/D2 domain sequences (Figure 3) showed a close relationship between *C. tropicalis* and ASM III strains; *C. parapsilosis* strains were located in a different cluster.

Summarizing, conventional biochemical and physiological tests allowed us to assign the xylitol-producing yeast ASM III to the *C. tropicalis* species and the use of PCR-based procedures (ITS1-5.8S rDNA-ITS2 and 26S rDNA D1/D2 domain sequencing) confirmed this phylogenetic affiliation. The use-

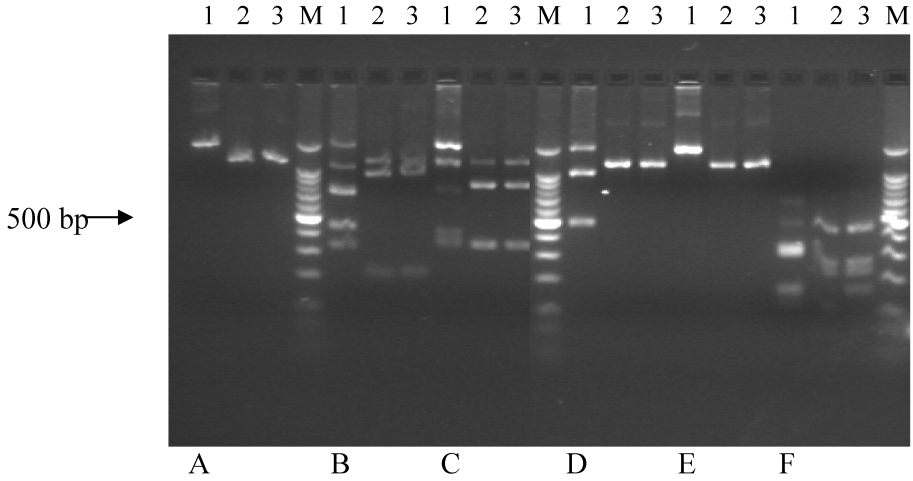


*Figure 2.* RFLP of ITS1-NL4 regions. Lanes: 1, *S. cerevisiae* ATCC 32052; 2, ASM III; 3, *C. tropicalis* ATCC 20311. A, undigested PCR products; B, *Bspd*I digested PCR products; C, *Ssp*I digested PCR products; D, *Eco*RI digested PCR products; E, *Bam*HI digested PCR products; F, *Hae*III digested PCR products; M, molecular weight marker 100 bp DNA Ladder.

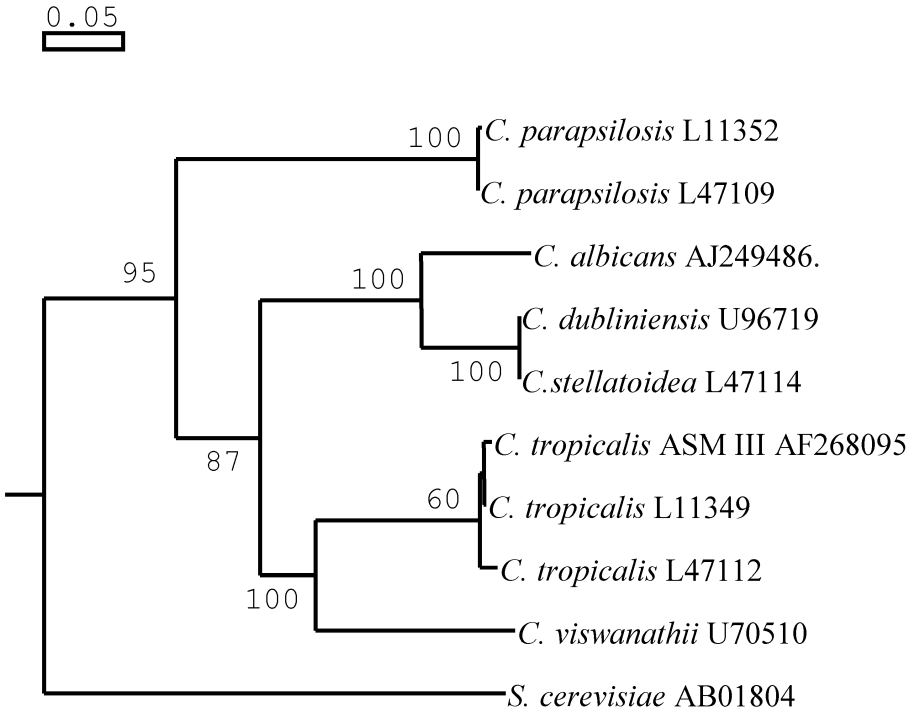


*Figure 3.* Phylogenetic placement of ASM III derived from maximum parsimony analysis of 26S rDNA D1/D2 domain sequences from related species. *S. cerevisiae* (AB01804) was included as outgroup and 5,000 bootstraps were performed.

fulness of the herein applied molecular approaches to overcome several difficulties and the lack of practicability of traditional yeast identification procedures has been once more demonstrated and it also allowed a proper identification of the ASM III isolate.

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