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Isolation and Identification of Xylitol-Producing Yeasts from Agricultural Residues

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ABSTRACT. Selected yeast strains isolated from corn silage and viticulture residues were screened for their capacities to convert D-xylose into xylitol. A conventional TLC was adapted for easy determination of xylose and xylitol in the culture supernatant solutions. This technique is suitable for the first steps of a screening program to select xylitol-producing yeasts from natural environments. *Candida tropicalis* ASM III (NRRL Y-27290), isolated from corn silage, appears to be a promising strain for xylitol production with a high yield (0.88 g xylitol per g of xylose consumed).

Xylitol is a naturally occurring five-carbon pentahydroxy alcohol with a high sweetening power. It is increasingly used as food sweetener, dental caries reducer and sugar substitute in the treatment of diabetes. It is a normal intermediate of saccharide metabolism in humans and animals. It is also widely distributed in the plant kingdom, particularly in certain fruits and vegetables (da Silva and Afschar 1994).

Xylitol is currently produced by chemical reduction of D-xylose. Therefore, the biological production of xylitol could be of economic interest since it does not require a pure xylose syrup as chemical synthesis does. Hence, low-cost hemicellulosic hydrolyzates might be potential substrates (Roseiro *et al.* 1991).

Xylose-utilizing yeasts, such as *Debaryomyces hansenii*, *Candida quilliermondii* and *Candida parapsilosis*, produce high amounts of xylitol as the major product of xylose metabolism (Gírio *et al.* 1996).

Current literature includes several studies of optimization or production from various substrates, but less attention has been paid to isolation of novel yeast strains capable of producing xylitol in a high yield (Rodrigues *et al.* 1998; Silva *et al.* 1998, 2000; Saha and Bothast 1999).

In the present work the capacity to produce xylitol of several yeast strains, isolated from different natural sources, was first evaluated by TLC and thereafter the best xylitol producers were analyzed by HPLC. A strain of *Candida tropicalis* was found to be the most efficient.

MATERIALS AND METHODS

Microorganisms. Debaryomyces hansenii NRRL Y-7426 and *Candida utilis* NRRL Y-1084 served as control strains; 13 yeast strains were isolated from natural sources. These yeast strains were named ASM (isolated from corn silage) and SJV (isolated from viticulture residues), followed by serial numbers.

Culture media and maintenance. Isolation medium: YM broth (*Difco*, USA), pH 5.0, and the same YM broth acidified to pH 3.5. *Isolation medium for xylitol production* (g/L): yeast nitrogen base (YNB) 6.7; yeast extract 5; D-xylose 20; rose bengal 0.03; agar 20 (pH 3.5). *Shaken-flask assay medium* (g/L): D-xylose 30; yeast extract 2; YNB 6.7 (pH 5.0) (Barbosa *et al.* 1988). *Fermentor assay medium* (g/L): D-xylose 127; yeast extract 2; YNB 6.7 (pH 5.0) (Vandeska *et al.* 1995).

Yeast cultures were maintained on YEPD agar slants containing (g/L): glucose 20; peptone 20; yeast extract 10; agar 20. Cultures were incubated at 30 °C for 1 d, stored at 4 °C and subcultured at regular intervals.

Isolation and identification. Yeast strains were isolated from corn silage and viticulture residues. Samples of 5–10 g were obtained and transported in sterile plastic bags. The samples were suspended in 50 mL of YM medium diluted 1 : 10 with sterile water containing 1 g/L Tween 80 (Middelhoven 1997). After shaking (1 h, 25 °C) the suspension was poured into sterile culture tubes and stored overnight at 4 °C. Most of the supernatant was discarded and the remaining (about 2 mL) was shaken in order to resuspend the settled cells. This suspension was streaked on acidified YM agar. The inoculated plates were incubated at 25 °C and after 3, 5 and 12 d, well isolated colonies were transferred on YM agar until growth was observed. Colonies were restreaked and picked on selective medium with xylose as sole carbon source.

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Yeast strains were identified according to their saccharide and nitrogen assimilation patterns, usi the keys and description of Kurtzman and Fell (1998) and the computerized yeast identification progra devised by Barnett, Payne and Yarrow, obtained from Dr. J.A. Barnett (*University of East Anglia*, Norwic UK) (Spencer *et al.* 1996).

Fermentations. Shaken-flask assays were performed in triplicate, in 250-mL Erlenmeyer flasks co taining 100 mL medium, at 30 °C. Inocula were prepared by growing a loopful of cells from a stock cultu in 50-mL Erlenmeyer flasks containing 15 mL fermentation medium, and incubated (2 d, 30 °C) in a rota shaker at 2.5 Hz. Fermentation flasks were inoculated to a final cell concentration of 10/nL and incubat (rotary shaker, 2.5 Hz; 30 °C). Samples were withdrawn after 2 d. For fermentor assays three separa experiments were performed using a 4-L LH-210 (*Inceltech*) fermentor containing 2.5 L medium, inoculat to a final concentration of 0.2 g/L (dry matter) cells, at 30 °C and 5 Hz. The aeration rate was 0.4 VV Samples were withdrawn periodically according to the experimental protocol.

Analytical methods. Samples were centrifuged and biomass dry matter was determined by washi the cells twice with distilled water and drying to constant mass at 105 °C.

Thin-layer chromatography was performed using Silicagel F_{254} plates (*Merck*). After being spott with 4 μL of samples and standards, the plates were developed using the double-ascending method in a so vent system consisting of ethyl acetate–2-propanol–water (130 : 57 : 23), at 30 °C, during 35 min. After dr ing with hot air, the plates were sprayed with bromocresol green–boric acid. Xylose and xylitol we visualized as yellow spots on blue background. Solutions of xylitol (ranging from 5 to 30 g/L) and xylo (20 g/L) were used as standards.

High-pressure liquid chromatography (HPLC). Residual D-xylose and xylitol production by t strains selected by TLC were analyzed in a *Gilson* equipment having a 305 pump, a 2142 LKB differenti refractometer and a chromatopac CR601 (*Shimadzu*) recorder–integrator. Concentration of sugars was dete mined with a refractive index detector, under the following conditions: Rezex ROA (*Phenomenex*) colum (300 × 7.8 mm); temperature 55 °C; 10 mmol/L sulfuric acid as eluent; flow rate 0.6 mL/min; samp volume 20 μL.

RESULTS AND DISCUSSION

Thirty-six yeast strains were isolated from corn silage and viticulture residues, and 13 of the (36 %) grew in a medium with xylose as sole carbon source. These yeasts were identified as *Candida mem branifaciens* (ASM I), *Candida tropicalis* (ASM III), *C. membranifaciens* (ASM IV), *Candida guilliermond* (ASM VI), *Candida shehatae* (ASM VII), *Pichia capsulata* (ASM VIII), *Candida utilis* (SJV I), *C. shehat* (SJV II), *C. utilis* (SJV III), *C. shehatae* (SJV VI), *Candida* sp. (SJV 32), *Pichia angusta* (SJV 33) a *C. shehatae* (SJV 35). The identification of *C. tropicalis* ASM III was confirmed by C.P. Kurtzma *National Center for Agricultural Utilization Research*, Peoria (IL, USA), and accessioned into the *ARS Cu ture Collection* (NRRL) as NRRL Y-27290.

Shaken-flask fermentation assays were done to detect xylitol production by these strains. TLC wa used as a rapid preselection step. Supernatant samples showing spots of at least the same size as th corresponding to 5 g/L xylitol standard, were selected to be further analyzed by HPLC.

It should be pointed out that with the TLC technique used it was possible to separate xylose fro xylitol. *D. hansenii* was used as positive control for xylitol production (Gírio *et al.* 1996), and *C. utilis* (Ba bosa *et al.* 1988) as negative control.

The TLC method allowed a simple and rapid detection of yeast isolates with the ability to produc xylitol (Fig. 1), visualized as yellow spots with the same R_F value as that of the xylitol standard. In this wa the following yeast strains were selected for further studies: *C. membranifaciens* ASM I (lane *9*), *C. trop calis* NRRL Y-27290 (*10*), *C. membranifaciens* ASM IV (*11*), *C. guilliermondii* ASM VI (*12*), *P. capsu lata* ASM VIII (*14*), *Candida* sp. SJV 32 (*15*), *C. shehatae* SJV 35 (*16*), and *C. shehatae* SVJ VI (*21*). Th methodology allows both the evaluation of numerous samples and the detection of xylitol production to b made in a very simple and rapid way.

Xylitol concentration produced by these strains was quantified by HPLC. Table I shows that th best xylitol producer was *C. tropicalis* NRRL Y-27290 (20.7 g/L xylitol with a yield of 0.69, expressed a g xylitol per g xylose consumed). It was also found that after 2 d of cultivation the available xylose wa exhausted. Strains isolated from corn silage, *i.e. C. tropicalis* NRRL Y-27290, *C. membranifaciens* ASM *C. membranifaciens* ASM IV, *C. guilliermondii* ASM VI and *P. capsulata* ASM VIII, and those obtaine from viticulture residues, *i.e.*, *Candida* sp. SJV 32, *C. shehatae* SJV VI and *C. shehatae* SJV 35 (Table I showed similar xylitol production as those reported by Barbosa *et al.* (1988).

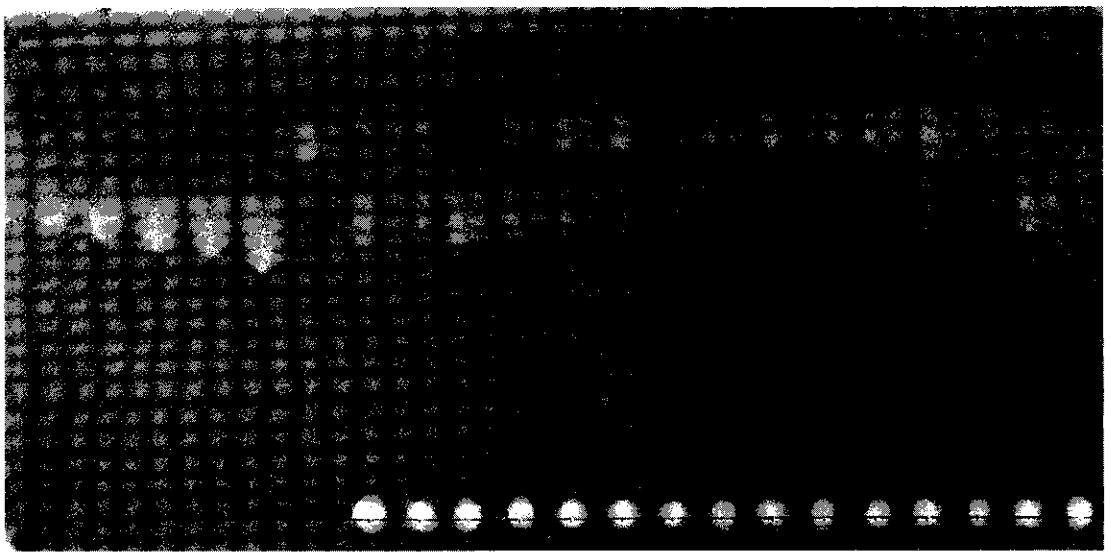

Fig. 1. Detection of xylose and xylitol by TLC; *1–6* – xylitol standards of 5, 10, 15, 20, 25 and 30 g/L, respectively, with R_F of 0.69; *7* – xylose standard 20 g/L, with R_F of 0.83; *8* – *D. hansenii*; *9* – ASM I; *10* – ASM III; *11* – ASM IV; *12* – ASM VI; *13* – ASM VII; *14* – ASM VIII; *15* – SJV 32; *16* – SJV 35; *17* – SJV III; *18* – SJV II; *19* – SJV I; *20* – SJV 33; *21* – SJV VI; *22* – *C. utilis*; for further explanation of strain symbols *see* text.

Table 1. Xylitol production after 2 d of fermentation determined by HPLC

Strain	HPLC xylitol, g/L	Residual xylose, g/L	Biomass g/L	Xylitol yield[a]
D. hansenii[b]	15.5	4.3	3.0	0.60
C. guilliermondii ASM VI	10.6	10.7	2.2	0.55
C. membranifaciens ASM I	14.6	3.3	3.8	0.55
C. membranifaciens ASM IV	14.0	4.9	2.5	0.56
C. shehatae SJV 35	6.2	11.1	2.3	0.33
C. shehatae SJV VI	14.6	3.8	2.7	0.56
C. tropicalis NRRL Y-27290	20.7	0.0	3.1	0.69
Candida sp. SJV 32	7.0	9.4	3.2	0.34
P. capsulata ASM VIII	15.1	5.6	3.5	0.62
C. utilis[c]	0	4.9	5.1	0

[a]g xylitol per g consumed. [b]Positive control. [c]Negative control.

C. tropicalis NRRL Y-27290 was selected for further studies in fermenter assays. The basic medium contained YNB and 127 g/L of D-xylose (Vandesca *et al.* 1995). It was found that the main products obtained were xylitol and biomass. Traces of ethanol were also detected under these fermentation conditions. Fig. 2 shows that xylose was completely consumed during fermentation. The final xylitol production was 103 g/L, with a yield of 0.88 g xylitol per g xylose consumed. Biomass production was rather low (5.4 g/L), probably as a consequence of the fermentation conditions.

The results obtained in the present work are better than previous studies performed with *Candida* species. Barbosa *et al.* (1988) obtained a yield of 0.74 g xylitol per g xylose consumed with a *C. guilliermondii* strain using 104 g/L of initial xylose concentration, and Vandeska *et al.* (1995) reported a yield of 0.47 g xylitol per g xylose consumed with a *C. boidinii* strain using 150 g/L of initial xylose concentration.

C. tropicalis NRRL Y-27290 is thus a promising yeast strain for xylitol production. From an industrial point of view, further investigations are being carried out to study the feasibility of using hemicellulosic hydrolyzates as carbon source.

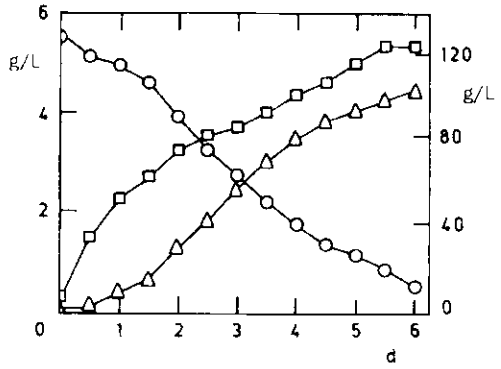

Fig. 2. Time course of xylitol production during fermentor assays in batch cultures of *C. tropicalis* NRRL Y-27290. *left axis*. □ – biomass; *right axis*: △ – xylitol production, ○ – D-xylose consumption

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