

# Effects of environmental conditions on production of xylitol by *Candida boidinii*

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*Candida boidinii* NRRL Y-17213 produced more xylitol than *C. magnolia* (NRRL Y-4226 and NRRL Y-7621), *Debaryomyces hansenii* (C-98 M-21, C-56 M-9 and NRRL Y-7425), or *Pichia (Hansenula) anomala* (NRRL Y-366). With *C. boidinii*, highest xylitol productivity was at pH 7 but highest yield was at pH 8, using 5 g urea and 5 g Casamino acids/l. Decreasing the aeration rate decreased xylose consumption and cell growth but increased the xylitol yield. When an initial cell density of 5.1 g/l was used instead of 1.3 g/l, xylitol yield and the specific xylitol production rate doubled. Substrate concentration had the greatest effect on xylitol production: increasing xylose concentration 7.5-fold (to 150 g/l) gave a 71-fold increase in xylitol production (53 g/l) and a 10-fold increase in xylitol/ethanol ratio. The highest xylitol yield (0.47 g/g), corresponding to 52% of the theoretical yield, was obtained with 150 g xylose/l after 14 days. Xylose at 200 g/l inhibited xylitol production.

**Key words:** *Candida boidinii*, xylitol production, xylose fermentation.

Primary interest in xylitol, a naturally occurring, five-carbon polyalcohol, centres on its properties and potential use as a sugar substitute. It has a sweetness equivalent to that of sucrose but its diminished metabolism makes it useful as a 'low caloric' sweetener (Aminoff *et al.* 1978). It could also help decrease the incidence of dental caries as it is non-cariogenic (Mäkinen 1989).

Xylitol is currently produced by chemical reduction of xylose, which is derived mainly from wood hydrolysates (Aminoff *et al.* 1978). However, its microbial production has lately attracted a lot of attention because such a process would not require a pure xylose syrup. Several yeast strains, including *Pachysolen tannophilus* (Jeffries 1983), *Candida* sp. (Gong *et al.* 1981; Barbosa *et al.* 1988), *Kluyveromyces fragilis* (Gong *et al.* 1981; Barbosa *et al.* 1988) and *Debaryomyces hansenii* (Roseiro *et al.* 1991), various bacteria (Yoshitake *et al.* 1971, 1976) and mycelial fungi (Dahiya 1991) can produce xylitol.

Among the yeasts, the best yields of xylitol are from *C. guilliermondii* (Barbosa *et al.* 1988; Meyriai *et al.* 1991) and a

mutant strain of *C. tropicalis* (Gong *et al.* 1981). However, a strain of *Saccharomyces cerevisiae*, transformed with a gene encoding the xylose reductase of *Pichia stipitis* CBS 6054 and capable of producing xylitol close to theoretical yield, has been reported (Hallborn *et al.* 1991).

The objective of the present study was to screen a number of yeasts for even higher xylitol production: *C. boidinii* NRRL Y-17213 was identified as a promising xylitol producer and therefore selected for investigation of the effects of environmental conditions on its xylitol production.

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## Materials and Methods

### Yeasts and Growth

*Candida boidinii* NRRL Y-17213, *C. magnolia* NRRL Y-4226 and NRRL Y-7621, *Debaryomyces hansenii* NRRL Y-7426, and *Pichia (Hansenula) anomala* NRRL Y-366 were obtained from C.P. Kurtzman at the National Center for Agricultural Utilization Research, Peoria, IL. *Debaryomyces hansenii* C-98 M-21 and C-56 M-9 were from the culture collection of the Institute of Microbial and Biochemical Technology in the Forest Products Laboratory, Madison, WI. The yeasts were maintained on YPG, containing (g/l): yeast extract, 10; Bacto-peptone, 20; glucose, 20; and agar, 20.

Two media were employed for screening the yeasts for xylitol production. The first medium, (A), contained (g/l): yeast nitrogen base (Difco), 6.7; yeast extract, 10, and xylose, 50. The second medium, (B), contained (g/l): yeast nitrogen base without amino

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Table 1. Growth and product formation by various yeasts in two D-xylose-containing media.

Yeast	Medium	Xylose consumption (g/l)*		Cell mass (g/l)		Xylitol (g/l)		Ethanol (g/l)	
		72 h	96 h	72 h	96 h	72 h	96 h	72 h	96 h
<i>Candida boidinii</i> NRRL Y-17213	A	9.44	15.95	4.30	5.88	0.08	0.15	0.86	1.76
	B	15.66	27.75	4.54	6.25	1.30	2.94	3.08	3.98
<i>Candida magnolia</i> NRRL Y-4226	A	7.31	8.28	1.12	1.38	ND	ND	ND	ND
	B	0.56	1.10	0.07	0.14	0.27	0.21	ND	ND
<i>Candida magnolia</i> NRRL Y-7621	A	2.85	4.56	0.41	0.51	ND	ND	ND	ND
	B	0.54	1.31	0.05	0.06	0.11	0.10	ND	ND
<i>Debaryomyces hansenii</i> C-98 M-21	A	3.54	4.15	0.17	0.51	0.49	0.75	ND	ND
	B	0.00	0.00	0.05	0.05	ND	ND	ND	ND
<i>Debaryomyces hansenii</i> C-56 M-9	A	7.77	9.04	0.40	1.02	0.13	0.18	ND	ND
	B	0.00	0.00	0.05	0.05	ND	ND	ND	ND
<i>Debaryomyces hansenii</i> NRRL Y-7426	A	1.66	1.79	0.05	0.06	ND	ND	ND	ND
	B	0.00	0.00	0.06	0.06	ND	ND	ND	ND
<i>Pichia (Hansenula) anomala</i> NRRL Y-366	A	17.70	30.73	4.31	5.02	0.02	0.09	ND	ND
	B	10.21	12.85	3.39	4.01	1.05	2.04	ND	ND

\*Initial xylose concentration was 50 g/l.

ND—Not detected.

acids and  $(\text{NH}_4)_2\text{SO}_4$  (Difco), 1.7; urea, 2.27; Casamino acids (Difco), 1.0; and xylose, 50. Both media were filter-sterilized without xylose. The sugar solution was sterilized separately by autoclaving and added aseptically to the medium. The initial pH of both media was 5.

One loopful of cells from a 3-day-old, YPG slant was inoculated into 50 ml of medium in a 125 ml Erlenmeyer flask and cultivated with shaking at 150 rev/min for 48 h at 30°C. The cultures were centrifuged, washed twice with distilled water and used as an inoculum at an initial cell density of 0.05 g/l. Cultivation conditions for screening the yeasts were the same as those used for inoculum preparation, except that cultures were shaken at 125 rev/min.

#### Cultivation of *Candida boidinii* for Xylitol Production

*Candida boidinii* was grown on a medium containing (g/l): yeast nitrogen base without amino acids and  $(\text{NH}_4)_2\text{SO}_4$ , 1.7; urea, 5; Casamino acids, 5; and xylose, 50. Cultures were usually inoculated to an initial dry cell mass of 1.3 g/l. To study the effect of aeration, cultures of 25, 50 and 75 ml were shaken in 125 ml Erlenmeyer flasks at 125 rev/min. Anoxic and fully anaerobic conditions were achieved using 50 ml medium in a 125 ml sealed vial or a sealed vial flushed with  $\text{N}_2$ .

#### Analytical Methods

Cell concentrations were estimated either turbidometrically at 525 nm, after diluting the samples within the range of 0.05 to 0.5 units, or by drying the biomass at 102°C to a constant cell mass. One O.D. unit corresponded to 0.27 mg dry cell mass/ml. Xylose, xylitol and ethanol were determined as described by Sreenath *et al.* (1986).

## Results and Discussion

### Selection of Yeasts for Production of Xylitol

All the yeasts tested for xylitol production were classified taxonomically as being able to assimilate D-xylose (Barnett *et al.* 1990) and, moreover, able to produce xylitol (Barbosa *et al.* 1988). However, *D. hansenii* NRRL Y-7426 did not produce xylitol (Table 1). *Candida magnolia* strains were poor xylitol producers; when cultivated on medium B they formed 0.10 to 0.27 g xylitol/l but they did not produce any detectable xylitol in medium A. In contrast, *D. hansenii* strains C-98 M-21 and C-56 M-9 produced xylitol (0.13 to 0.75 g/l) when cultivated on medium A but not on medium B.

*Pichia (Hansenula) anomala* NRRL Y-366 and *C. boidinii* NRRL Y-17213 were better xylitol producers than the other yeasts when cultivated on medium B, accumulating, respectively, 2.04 and 2.94 g xylitol/l in 96 h. They grew on both media and produced more biomass than the other yeast strains tested. The maximum biomasses for *P. anomala* (5.02 g/l) and *C. boidinii* (6.25 g/l) were both medium B.

*Candida boidinii* was the only yeast strain that produced higher ethanol concentrations than xylitol (3.98 g/l in 96 h). This, at first sight, does not favour the selection of *C. boidinii* for xylitol production. However, its high rate of

Table 2. Effect of nitrogen source on xylitol yield and dry cell mass formed by *Candida boidinii*.\*

Parameter	Nitrogen source					
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	KNO <sub>3</sub>	NH <sub>4</sub> NO <sub>3</sub>	NH <sub>4</sub> Cl	Urea	Urea and Casamino acids (5 g/l each)
C <sub>cm</sub> (g/l)	5.88	4.96	4.31	5.07	6.25	9.03
Y <sub>xs</sub> (g/g)	0.06	0.01	0.05	0.06	0.10	0.12

\*Fermentation time was 4 days and each source yielded 1.06 g nitrogen/l. The medium contained (g/l): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.00; KNO<sub>3</sub>, 7.65; NH<sub>4</sub>NO<sub>3</sub>, 3.03; NH<sub>4</sub>Cl, 4.05; and urea, 2.27.

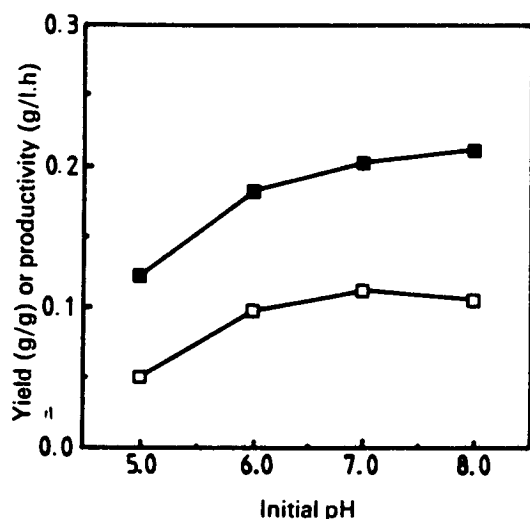


Figure 1. Effect of initial pH on xylitol yield (□) and productivity (■) after 4 days' fermentation.

xylose consumption and rapid growth rate indicated that this organism could be adapted further. *Pichia anomala* had a higher specific xylitol yield but produced 44% less xylitol than *C. boidinii*. *Candida* strains are, in general, better xylitol producers than *Pichia* strains (Gong *et al.* 1981; Barbosa *et al.* 1988; Meyrial *et al.* 1991). Therefore, *C. boidinii* NRRL Y-17213 was chosen for further study.

#### Xylitol Production by *Candida boidinii*: Effect of Environmental Parameters

**Nitrogen Source.** Of all nitrogen sources tested singly, urea favoured xylitol production the most. Use of both urea and Casamino acids resulted in a small increase in xylitol yield and a significant increase in biomass (Table 2).

**Initial pH.** The highest xylitol productivity (0.11 g/lh) was obtained at pH 7, but the yield was slightly higher at pH 8 (0.21 g/g) (Figure 1). The results are similar to those of Vongsuvanlert & Tani (1989), who reported that *C. boidinii* grew best at an initial pH of 6.5 but gave a maximum yield (0.13 g/g) at pH 7. Although initial pH ranged from 5 to 8,

the final pH in all experiments only ranged between 5.45 and 5.65.

**Aeration.** The aeration level was important in determining both growth and product formation. Decreasing aeration decreased xylose consumption and cell growth but increased the specific yield of xylitol. Fully anaerobic conditions resulted in a virtual cessation of growth and low xylitol production (Figures 2 and 3).

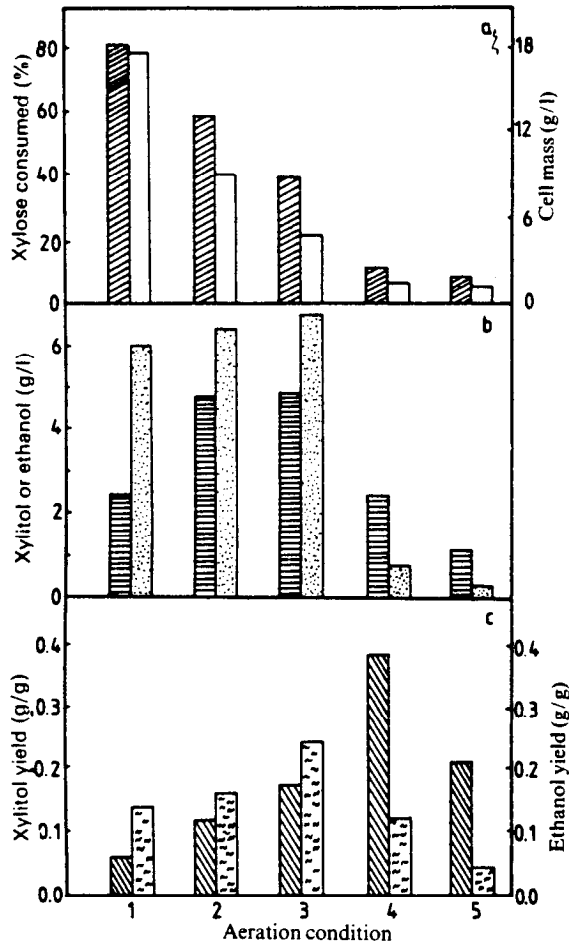
The growth of *C. boidinii* was obligately O<sub>2</sub> dependent. This was expected, since O<sub>2</sub> is necessary for growth in xylose-assimilating yeasts (Slininger *et al.* 1987). At the highest aeration rate, the yeast grew fastest, accumulating the largest biomass of 18 g/l (condition 1; Figure 2a).

*Candida boidinii* produced xylitol and ethanol over the investigated range of aeration conditions; both at higher amounts as aeration increased (conditions 1 to 3; Figure 2b). However, the lower aeration rates (conditions 2 and 3) gave 90% more xylitol than the highest aeration rate (condition 1). At the same time, ethanol production was also **enhanced**, but only by 13%.

Unlike xylitol concentration, xylitol yield was highest (0.38 g/g) under condition 4. Nevertheless, considering other fermentation parameters, this anoxic condition could not be optimal.

In order to clarify which aerated condition favoured xylitol production, the yeast was grown as described in Figure 3. Condition 3 gave the slightly better results.

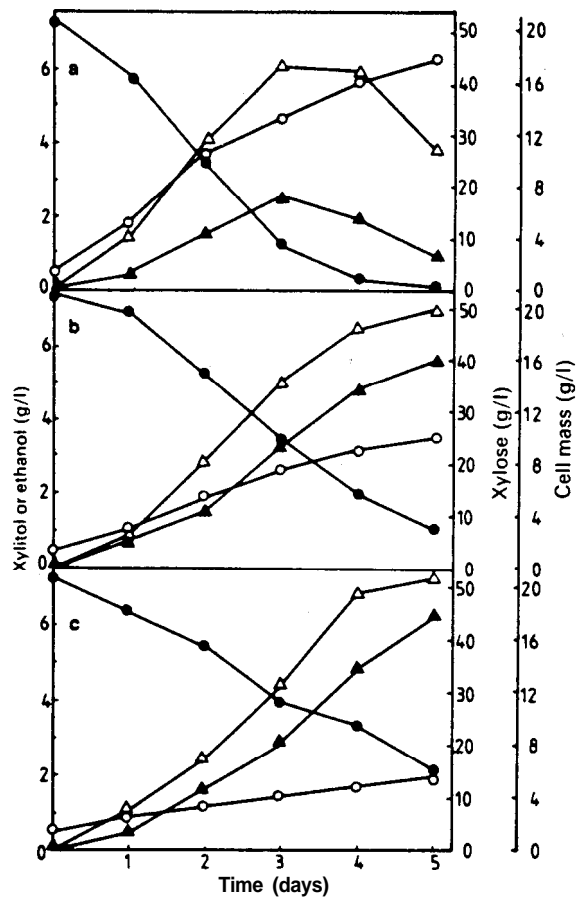
In addition, the effect of aeration was tested in 50 ml cultures by agitating at 125, 150 or 180 rev/min. Both xylitol yield (0.15 g/g), and productivity (0.07 g/lh) were highest at 150 rev/min. Consequently, for xylitol formation by this yeast, O<sub>2</sub> has to be controlled because the O<sub>2</sub> uptake rate is a key parameter that determines whether xylose is fermented or respired. Barbosa *et al.* (1988), working with *C. guilliermondii* FTI 20037, and Roseiro *et al.* (1991), working with *D. hansenii* DTIA-77, also found that xylitol production increased but ethanol formation decreased as the aeration rate was decreased. In general, this is in agreement with our results, but the optimal oxygenation levels appear to be specific for each yeast strain.



**Figure 2.** Effect of aeration on (a) xylose consumption (▨) and cell mass (□), (b) xylitol (▤) and ethanol (▥) concentrations and (c) xylitol (▧) and ethanol (▩) yield. Flask or vial size was 125 ml and medium volumes were 25 ml (1), 50 ml (2), 75 ml (3) in aerobic conditions, 50 ml with sealed vial (4) and 50 ml with sealed vial flushed with N<sub>2</sub> (5). Fermentation time for all conditions was 4 days, except for the condition 1, when it was 3 days.

**Cell Density.** The yeast accumulated the highest cell mass (9.96 g/l) when an inoculum of 2.6 g/l was used (Table 3). Inoculum size did not affect the specific rates of ethanol production and xylose consumption. However, xylitol yield and its specific production rate at the highest cell density were double that at the lowest.

**Substrate Concentration.** Increasing the initial xylose concentration from 20 to 150 g/l favoured xylitol production at the expense of ethanol (Table 4). Ethanol was always produced as a by-product, but as xylose concentration was increased the xylitol/ethanol ratio increased from 0.33 to 3.61. Furthermore, increasing xylose concentration 7.5-fold (from 20 to 150 g/l) resulted in a 71-fold increase in xylitol production (from 0.75 to 53.14 g/l).



**Figure 3.** Time course of xylose fermentation by *C. boydii* NRRL Y-17213 under aeration conditions, 1(a), 2(b) and 3(c), showing xylitol (▲), ethanol (Δ), xylose (●) and cell mass (○). The conditions were the same as the first three conditions given in Figure 2.

**Table 3.** Effect of initial cell density on growth, xylose consumption and product formation by *Candida boydii*.

Parameter	Initial cell density (g/l)			
	1.3	2.6	3.6	5.1
C <sub>cm</sub> (g/l)	9.03	9.96	9.28	6.61
Y <sub>cs</sub> (g/g)	0.19	0.20	0.19	0.16
q <sub>s</sub> (g/g.h)	0.05	0.05	0.06	0.06
Y <sub>xs</sub> (g/g)	0.12	0.12	0.13	0.24
q <sub>x</sub> (g/g.h)	0.01	0.01	0.01	0.02
Y <sub>es</sub> (g/g)	0.16	0.19	0.20	0.18
q <sub>e</sub> (g/g.h)	0.02	0.02	0.02	0.02

\*Fermentation time was 4 days.

Substrate concentration had the greatest effect on xylitol production of any parameter tested.

High xylose concentrations also increased xylitol yields. The highest xylitol yield (0.47 g/g, or 52% of the theoreti-

Table 4. Effect of initial substrate concentration on the fermentation parameters of *Candida boidinii*.

Parameter	initial xylose concentration (g/l)					
	20	50	100	130	150	200
$C_x$ (g/l)	0.75	4.81	25.21	41.72	53.14	9.98
$Y_{x/s}$ (g/g)	0.05	0.12	0.29	0.39	0.47	0.22
$Q_x$ (g/l.h)	0.02	0.05	0.13	0.17	0.16	0.04
$q_x$ (g/g.h)	0.00	0.01	0.02	0.02	0.02	0.01
$Y_{x/t}^{(a)}$ (%)*	5.49	13.19	31.87	42.86	51.65	24.18
$C_e$ (g/l)	2.38	6.43	11.59	12.50	15.11	8.98
$Y_{e/s}$ (g/g)	0.15	0.16	0.14	0.12	0.13	0.20
$R_{x/e}$ (g/g)	0.33	0.75	2.07	3.25	3.61	1.10
$S_c$ (%)	79.22	77.58	85.55	82.73	74.71	22.20
$C_{cm}$ (g/l)	4.79	9.03	11.15	12.62	10.96	7.00
$Y_{c/s}$ (g/g)	0.18	0.20	0.11	0.10	0.07	0.09
$t$ (days)	2	4	8	10	14	8

\*Theoretical maximum xylitol yield is 0.91 g xylitol/g xylose used.

Table 5. A survey of xylitol production by various yeasts.

Microorganism	Xylose (g/l)	$S_c$ (%)	$C_x$ (g/l)	$Y_{x/s}$ (g/g)	$Y_{x/t}$ (%)	$C_e$ (g/l)	$t$ (h)	Vessel volume (ml)	Culture volume (ml)	Shaking speed (rev/min)	Reference
<i>C. boidinii</i> ( <i>Kloeckera</i> sp.) No. 2201*	100	82	36.0	0.44	48.35		144	500	100	100	Vongsuvanlert & Tani (1989)
	150	51	17.0	0.22	24.18		144	500	100	100	Vongsuvanlert & Tani (1989)
<i>C. tropicalis</i> HXP2	10	100	3.5	0.35	38.46		17	250	100		Gong <i>et al.</i> (1981)
	50	100	38.5	0.77	84.62		24	250	100		Gong <i>et al.</i> (1981)
<i>D. hansenii</i> DTIA-77	90	76	36.0	0.53	58.24	8	32	250	40	200	Roseiro <i>et al.</i> (1991)
<i>C. guilliermondii</i> FTI 20037	104	100	77.2	0.74	81.32	2	96	250	100		Barbosa <i>et al.</i> (1988)
<i>C. guilliermondii</i> NRC 5578	300	100	221.0	0.75	82.42		406	500	400	150	Meyrial <i>et al.</i> (1991)
<i>C. boidinii</i> NRRL Y-17213*	150	75	53.1	0.47	51.65	15	336	125	50	125	Present study

\*According to the National Center for Agricultural Utilization Research, Peoria, IL, these are the same yeast strain.

cal maximum) was obtained with 150 g xylose/l and was the highest yield achieved in any experiment. A somewhat lower concentration, of 130 g/l, decreased xylitol yield to 0.39 g/g but productivity (0.17 g/l. h), xylose consumption (83%) and cell mass (12.62 g/l) were higher. Increasing xylose concentration to 200 g/l reduced xylitol concentration to 9.89 g/l (Table 4).

Some of the present results are compared with several found in the literature in Table 5. Vongsuvanlert & Tani (1989) reported that *C. boidinii* produced 0.44 g xylitol/g xylose within 6 days, when 100 g xylose/l was initially present. With 150 g/l, the yield was 0.22 g/g. In the present study, an initial xylose concentration of 150 g/l was found to be a better xylitol inducer than 100 g/l. Gong *et al.* (1981) found that, at initial xylose concentra-

tions of 10 and 50 g/l, *C. tropicalis* HXP2 exhibited xylitol yields of 0.35 and 0.77 g/g, respectively. *Debaromyces hansenii* DTIA-77 achieved a maximum xylitol yield of 0.53 g/g when cultivated on 90 g xylose/l (Roseiro *et al.* 1991).

The comparison in Table 5 shows that *C. guilliermondii* is the best xylitol-producing yeast reported to date, giving maximum yields of 0.74 (Barbosa *et al.* 1988) to 0.75 g/g (Meyrial *et al.* 1991).

## Conclusions

Of the tested N sources, urea and Casamino acids gave the highest yield. Highest xylitol productivity was observed at pH 7, but highest yield was at pH 8. As the aeration rate decreased, xylitol yield increased. Under anaerobic condi-

tions, however, xylitol production was negligible. A high initial cell density (5.1 g/l) gave the best xylitol yield. Substrate concentration had the greatest effect on xylitol production, the highest xylitol yield observed (0.47 g/g) being produced with 150 g xylose/l.

It appears that *C. boidinii* NRRL Y-17213 is a promising yeast for xylitol production but, for commercial feasibility, its xylitol yield and productivity have to be improved. Therefore, further investigations should focus on additional optimization of the culture conditions. Because xylitol yield increases but growth rate decreases with increasing xylose concentration, this fermentation would probably be optimized by using immobilized cells in semi-continuous fed-batch mode.

## Nomenclature

$C_{cm}$	dry cell mass (g/l)
$C_e$	ethanol concentration (g/l)
$C_x$	xylitol concentration (g/l)
$q_e$	specific ethanol production rate (g/gh)
$q_x$	specific xylitol production rate (g/gh)
$q_s$	specific xylose uptake rate (g/gh)
$Q_x$	volumetric xylitol production rate (g/lh)
$R_{x/e}$	ratio xylitol/ethanol (g/l)
$S_c$	xylose consumed (%)
$t$	time (h or day)
$Y_{c/s}$	cell yield coefficient (g dry cell mass per g xylose used)
$Y_{e/s}$	ethanol yield coefficient (g ethanol per g xylose used)
$Y_{x/s}$	xylitol yield coefficient (g xylitol per g xylose used)
$Y_{x/t}$	percentage of the xylitol yield from the theoretical value

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