

Xylitol Formation and Key Enzyme Activities in *Candida boidinii* under Different Oxygen Transfer Rates

ELEONORA VANDESKA,§* SLOBODANKA KUZMANOV § AND THOMAS W. JEFFRIES
Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI 53703, USA

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Under oxygen transfer rates (OTR), from 10 to 30 mmol. l⁻¹h⁻¹, *Candida boidinii* NRRL Y-17213 exhibited both NADH and NADPH linked D-xylulose reductase activities with the former being higher. Xylitol dehydrogenase was mainly NAD dependent. Maximum xylitol production was attained at OTR of 14 mmol. l⁻¹h⁻¹. Ethanol, glycerol and ribitol were also produced. A correlation between xylitol accumulation, oxygen availability and key enzyme activities was viewed.

[Key words: xylitol, oxygen transfer rate, *Candida boidinii*, D-xylulose reductase, xylitol dehydrogenase]

The availability of oxygen has a significant influence on D-xylulose fermentation by yeasts and therefore has been a subject of many investigations (1-5). Yet, most of these investigations are related to ethanol as a major product, and only as of lately, to xylitol (6-9).

Several yeast strains belonging to *Candida* sp. (10-12), *Debaryomyces hansenii* (7) and *Pachysolen tannophilus* (3), have been reported to produce xylitol from D-xylulose. These yeasts possess the first two enzymes needed for metabolism of D-xylulose; D-xylulose reductase which, using either NADH or NADPH, reduces D-xylulose to xylitol, and predominantly NAD-linked xylitol dehydrogenase which deoxidizes xylitol to D-xylulose. Bruinenberg *et al.* (13) reported that the catabolism of D-xylulose by yeasts resulted in an accumulation of NADH, the extent of which depended on the degree of aerobiosis. Under anaerobic conditions or at very low oxygen transfer rate, NAD linked xylitol dehydrogenase was considerably inhibited, thus leading to xylitol accumulation rather than efficient conversion of D-xylulose to D-xylulose.

Our first studies on the influence of aeration on xylitol formation by *Candida boidinii*, when cultivated in shake flasks, showed that oxygen plays an important role in the conversion of D-xylulose to xylitol by the investigated yeast (14). There was a critical level of oxygenation at which xylitol yield was high while cell mass yield low. Therefore, we made an attempt to determine at which oxygen transfer rate xylitol production in *C. boidinii* is maximized when cultivated in a lab fermentor, and to investigate the relationship between the key enzyme activities in said yeast and the level of oxygenation.

C. boidinii NRRL Y-17213, was maintained on agar slants at 4°C. The slant medium, (YPG), contained (g. l⁻¹): yeast extract, 10; Bactopeptone, 20; glucose, 20 and agar 20. The fermentation medium contained (g. l⁻¹): yeast nitrogen base w/o amino acids and ammonium sulfate (Difco), 1.7; urea, 5; Casamino acids (Difco, Mich., USA), 5 and D-xylulose, 130. The medium was filter sterilized without D-xylulose. The sugar solution was sterilized separately by autoclaving and added aseptically to the medium.

The inoculum was prepared by transferring a loopful

of cells from a 3-day-old YPG slant into 50 ml of medium in a 125 ml Erlenmeyer flask plugged with foam and cultivated with shaking at 150 rev·min⁻¹ for 48 h at 30°C. After a subsequent preculturing in flasks with larger volumes (500 and 2,000 ml), the cultures were centrifuged, washed with distilled water twice and used as an inoculum at an initial cell density of approximately 5.0 g · l⁻¹.

The fermentor used was a 2- l bench top fermentor (New Brunswick) with a working volume of 1.4 l. Temperature (30°C), pH (5.5) and agitation (150 rev·min⁻¹) were adjusted and controlled during the experiments. Different oxygen transfer rates (OTR) were obtained by controlling air supply by a flowmeter. OTR was estimated by sodium sulfite oxidation method (15).

The harvested yeast cells (4-5 g), were centrifuged, washed twice and suspended in 0.1 M MOPS (3-(N-morpholino) propanesulfonic acid) buffer (pH 6.8), quickly frozen in a dry ice acetone bath, and stored at -80°C. After thawing, the cell free extract was prepared as described by Alexander *et al.* (16). D-xylulose reductase (EC 1.1.1.21) (alditol: NADP/NAD 1-oxidoreductase) activity was determined by following the oxidation of NADPH or NADH according to the method of Chiang and Knight (17). Xylitol dehydrogenase (EC 1.1.1.9) (xylitol: NAD 2-oxidoreductase) activity was measured by following the reduction of NAD or NADP according to the method of Chakravorty *et al.* (18).

Samples were taken every day and centrifuged. After washing the yeast cells with distilled water, twice, they were dried for cell mass determination at 102°C. D-Xylulose, xylitol, and polyols (ribitol, glycerol, arabitol etc.) were determined by high-performance liquid chromatography while ethanol was analyzed by gas chromatography as described elsewhere (19). Protein determination in cell free extracts was carried out by the Bradford method (20).

For studying the influence of aeration rate on xylitol formation by *C. boidinii*, we have reported that decreasing the aeration rate decreased D-xylulose consumption and cell growth, but increased xylitol yield. Fully anaerobic condition resulted in a virtual cessation of growth and low xylitol production (14). Obviously, the oxygen level which favors xylitol production is in the range of oxygen limitation. In order to find the level of oxygenation which will maximize xylitol production, we used the

* Corresponding author.

§ Present address: Faculty of Technology and Metallurgy, Rudjer Boskovic 16, 91000 Skopje, Macedonia.

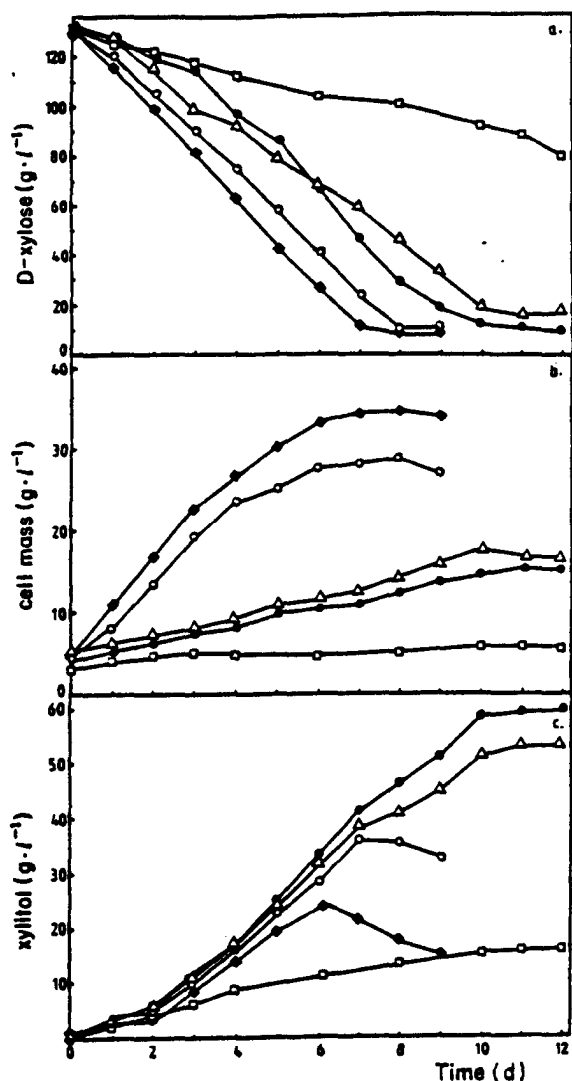


FIG. 1. D-Xylose fermentation by *C. boidinii* under different oxygen transfer rates: (□) 10 mmol·l⁻¹h⁻¹, (●) 14 mmol·l⁻¹h⁻¹, (Δ) 18 mmol·l⁻¹h⁻¹, (○) 24 mmol·l⁻¹h⁻¹ and (◆) 30 mmol·l⁻¹h⁻¹.

oxygen transfer rate to define the level of oxygen in the fermentor.

D-Xylose, cell mass and xylitol concentration profiles for different oxygen transfer rates are depicted in Fig. 1. The consumption of D-xylose increased from 38.38% (12th day) for the OTR of 10 mmol·l⁻¹h⁻¹ to 92.53% (9th day) for the OTR of 30 mmol·l⁻¹h⁻¹ (Fig. 1a). Increasing the OTR from 10 to 30 mmol·l⁻¹h⁻¹ resulted in a 6-fold increase in cell mass, from 5.50 to 34.53 g·l⁻¹ (Fig. 1b). However, xylitol production did not follow the same pattern (Fig. 1c). For oxygen transfer rates of from 10 to 18 mmol·l⁻¹h⁻¹, xylitol concentration was continuously increasing and then leveled off after 10 d, while for the higher OTRs, 24 and 30 mmol·l⁻¹h⁻¹, xylitol concentration peaked at 6th and 7th day, respectively, and then declined as consumption exceeded production. Xylitol concentration was the highest, 58.90 g·l⁻¹ for OTR of 14 mmol·l⁻¹h⁻¹ on the twelfth day.

From these data, it is obvious that some finite oxygen supply stimulates xylitol formation in *C. boidinii*. Considering that under these experimental conditions, OTR can not be controlled more precisely, with smaller incre-

TABLE 1. The growth and maximum fermentation parameters of *C. boidinii* under different oxygen transfer rates

Parameter	Oxygen transfer rate (mmol·l ⁻¹ h ⁻¹)				
	10	14	18	24	30
$Y_{w/s}$ (g·g ⁻¹)	0.38	0.48	0.45	0.34	0.23
$Y_{w/t}$ (%)	41.76	52.75	49.45	37.36	25.28
q_x (g·g ⁻¹ h ⁻¹)	0.014	0.026	0.019	0.013	0.009
Q_x (g·l ⁻¹ h ⁻¹)	0.06	0.24	0.21	0.22	0.17
C_x (g·l ⁻¹)	9.24	14.19	17.94	13.21	10.12
$Y_{w/s}$ (g·g ⁻¹)	0.22	0.12	0.14	0.12	0.10
$R_{w/s}$ (g·g ⁻¹)	1.72	4.10	2.87	2.76	2.37
C_p (g·l ⁻¹)	3.81	6.52	5.77	2.60	2.40
S_x (%)	31.81	90.20	85.88	82.16	79.34
$C_{w/m}$ (g·l ⁻¹)	5.5	14.6	17.56	28.45	33.16
$Y_{w/t}$ (g·g ⁻¹)	0.06	0.09	0.11	0.22	0.27
t (d)	11	10	10	7	6

* Theoretical xylitol yield is 0.91 g xylitol per g D-xylose consumed.

ments, this OTR of 14 mmol·l⁻¹h⁻¹ should be taken as an optimal.

The growth and maximum fermentation parameters are given in Table 1. The maximum xylitol yield of 0.48 g·g⁻¹ (52.75% of the theoretical yield) was reached at an OTR of 14 mmol·l⁻¹h⁻¹. Further decreases in oxygen transfer rate declined sharply xylitol concentration. The xylitol yield of 0.38 g·g⁻¹, obtained for OTR of 10 mmol·l⁻¹h⁻¹, which was relatively high, was not due to the high xylitol accumulation but to the low D-xylose consumption (31.81%).

Investigating the effect of aeration on xylitol production in *C. parapsilosis*, Furlan *et al.* (6) reported that global xylitol yield dropped from 0.27 g·g⁻¹ to 0.02 g·g⁻¹ with the increase of the aeration from 0.25 to 2 vvm. But, *C. shehatae* and *P. stipitis* have shown inverse relationship between oxygen supply and extracellular xylitol accumulation (4). These findings about inverse relationship between oxygen supply and xylitol formation in D-xylose fermenting yeasts are ascribed to the role of oxygen as terminal electron acceptor, thus relieving the redox imbalance of the initial two steps of D-xylose metabolism under anaerobic conditions or very low oxygen transfer rates (5).

Concurrently to xylitol production, *C. boidinii* accumulated ethanol, glycerol and ribitol (Table 1). The most favorable OTR for ethanol formation was higher than the one for xylitol formation. At OTR of 18 mmol·l⁻¹h⁻¹, the highest ethanol concentration was reached, 17.94 g·l⁻¹, giving a xylitol/ethanol ratio of 2.87. On the other hand, for the OTR of 14 mmol·l⁻¹h⁻¹, which was favorable for xylitol production, the same ratio was 4.10. Due to the relatively small concentrations of the glycerol and ribitol, they are represented cumulatively as polyols. Their yield increased with the oxygen limitation, from 0.02 to 0.09 g·g⁻¹ inferring that only small fraction of carbon source is converted to these polyols.

To determine an eventual relation between xylitol accumulation under different oxygen transfer rates and the activities of the key enzymes for D-xylose fermentation, the activities of D-xylose reductase and xylitol dehydrogenase were measured under the investigated oxygen transfer rates. The activities of NADH and NADPH linked D-xylose reductase and NAD and NADP linked xylitol dehydrogenase were measured for cells harvested in the late exponential growth phase.

Under all investigated oxygen transfer rates, *C. boidinii* exhibited both NADH and NADPH linked D-xy-

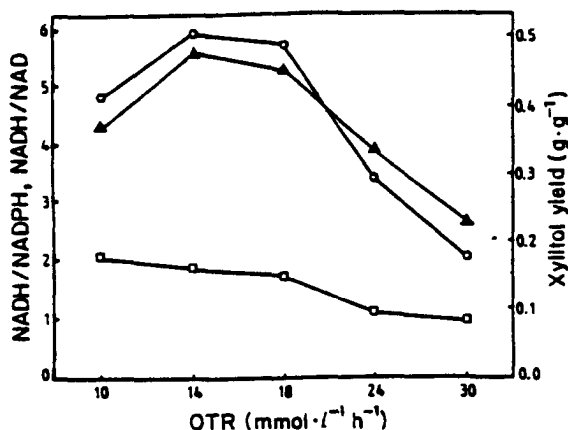


FIG. 2. Effect of oxygen transfer rate on (○) NADH and NADPH linked D-xylose reductase activity ratio, (□) NADH linked D-xylose reductase and NAD linked dehydrogenase activity ratio and (▲) xylitol yield.

lose reductase activities with NADH D-xylose reductase activity being higher. The maximum NADH linked D-xylose reductase activity resulted in the highest NADH/NADPH ratio, 5.88. It was reached at an OTR of 14 mmol · l⁻¹ h⁻¹ when the yeast exhibited maximum xylitol yield of 0.48 g · g⁻¹. Almost the same ratio existed for an OTR of 18 mmol · l⁻¹ h⁻¹ ($Y_{x/s} = 0.45$ g · g⁻¹). Further increase in the OTR, up to 30 mmol · l⁻¹ h⁻¹ sharply decreased NADH/NADPH ratio to 2.05 (Fig. 2).

It is noteworthy that this yeasts, under oxygen limitation, in contrast to all other D-xylose fermenting yeasts (4, 8, 16), exhibited a NADH/NADPH ratio higher than 1. Vongsuvalert and Tani (11), working with the same yeast, observed a similar NADH/NADPH ratio.

Xylitol dehydrogenase in *C. boidinii* was mainly NAD dependent with a very low NADP linked activity. At the most favorable OTR for xylitol production, 14 mmol · l⁻¹ h⁻¹, NADPH and NADH linked D-xylose reductases exhibited specific enzyme activities of 0.019 and 0.112 U · (mg protein)⁻¹, respectively. At the same time NAD xylitol dehydrogenase exhibited specific activity of 0.060 U · (mg protein)⁻¹ while NADP xylitol dehydrogenase, only 0.003 U · (mg protein)⁻¹.

Oxygen may lower the NADH to NAD ratio and minimize xylitol accumulation in D-xylose fermenting yeasts (3). This was observed in *C. boidinii*. The ratio NADH linked D-xylose reductase activity to NAD xylitol dehydrogenase activity decreased with the increasing of the oxygen availability by 2 fold in the investigated range of OTR (from 2.08 for OTR 10 mmol · l⁻¹ h⁻¹ to 0.98 for OTR 30 mmol · l⁻¹ h⁻¹).

Comparing the enzymatic data with the fermentation kinetic parameters, a correlation between xylitol formation, oxygen availability and key enzyme activities in *C. boidinii* was viewed.

NOMENCLATURE

- C_{cm} : dry cell mass, g · l⁻¹
 C_e : ethanol concentration, g · l⁻¹
 C_p : polyol (glycerol and ribitol) concentration, g · l⁻¹
 OTR : oxygen transfer rate, mmol · l⁻¹ h⁻¹
 q_x : specific xylitol production rate, g · g⁻¹ h⁻¹
 Q_x : volumetric xylitol production rate, g · l⁻¹ h⁻¹
 $R_{x/e}$: ratio xylitol/ethanol, g · g⁻¹

- S_c : D-xylose consumed, %
 t : time, h, d
 $Y_{cm/s}$: cell yield coefficient, g dry cell mass per g D-xylose consumed, g · g⁻¹
 $Y_{e/s}$: ethanol yield coefficient, g ethanol per g D-xylose consumed, g · g⁻¹
 $Y_{x/s}$: xylitol yield coefficient, g xylitol per g D-xylose consumed, g · g⁻¹
 $Y_{x/t}$: percentage of the xylitol yield from the theoretical value, %

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