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RESEARCH ARTICLE

Effects of pH and Aeration Conditions on Xylitol Production by Candida and Hansenula Yeasts

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Abstract

During the fermentative production of xylitol the following environmental parameters have a controlling effect on the xylitol yield: concentration of monosaccharides, temperature, aeration and pH. The purpose of the present work was to evaluate xylitol production by four yeast strains at different pH values and oxygen transfer rates (OTRs). The highest xylitol yields were obtained under the following conditions: Candida parapsilosis: pH 5.0, OTR 6.1 mmol L^{-1} h^{-1}; Candida guilliermondii: pH 4.5, OTR 5.7 mmol L^{-1} h^{-1}; Candida boidinii: pH 6.0, OTR 5.7 mmol L^{-1} h^{-1}; Hansenula anomala: pH 4.5, OTR 2.8 mmol L^{-1} h^{-1} using 50 g L^{-1} initial xylose concentration.

Keywords

xylitol fermentation, Candida and Hansenula yeasts, aeration, shake flask fermentation

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1 Background

Xylitol, a natural five-carbon sugaralcohol, has attracted attention because of its wide variety in use and its good biological impacts [1,2]. Its sweetness is equal to sucrose, hence xylitol is used increasingly as a sweetener and it has anticariogenic attribute [3]. Xylitol raises the blood sugar level less than sucrose, and it can be used as an insulin-independent carbohydrate source for diabetics [4-6]. Xylitol is also used in chewing gums and toothpastes, because it appears to have caries-preventive effect on root surfaces [6-9].

Xylitol is naturally present in some fruits and vegetables (e.g. cauliflower or cabbage lettuce) [10], however its low concentration in plants makes production by extraction difficult and uneconomical [1]. On an industrial scale, xylitol has been manufactured by catalytic hydrogenation of D-xylose using e.g. Raney nickel catalysts, and this requires expensive purification, in which D-xylose is separated from the other sugars obtained in the acidic hydrolysis of plant hemicelluloses [11,12].

The bioconversion of xylose by microorganisms is an alternative xylitol production route, a technology with great economic potential. In yeasts D-xylose is converted into D-xylulose by two enzymatic steps [13]. The first phase involves the xylose reductase (XR, E.C.1.1.1.21), which reduces D-xylose into D-xylitol, and it depends on either NADH or NADPH cofactors [14]. The subsequent conversion of D-xylitol into D-xylulose is catalyzed by a NAD^+-dependent xylitol dehydrogenase (XDH, E.C.1.1.1.9). After a phosphorylation step, D-xylulose can enter the pentose phosphate pathway [14,15].

The dissolved oxygen concentration is one of the most important environmental parameter in the production of xylitol by yeasts [1,14,15]. The oxygen concentration directly affects the yield and the volumetric productivity of xylitol, because the generation rate of the necessary cofactors depends on it [16,17]. It has been reported that the xylose bioconversion to xylitol was strongly affected by the oxygen supply: microaerobic condition is necessary for sufficient xylitol production [15,18,19]. When it occurs, the terminal oxidation step is unable to generate enough NAD^+ from the NADH produced. As a consequence, an increase in intracellular NADH levels

occurs, suppressing the reaction catalysed by XDH and allowing xylitol to accumulate [1,12,20].

The concentration of different monosaccharides and vitamins also play a major role in the production of xylitol [1,11,20]. An ideal strategy employed in xylitol biotechnological production is that glucose and arabinose can be used to supply energy for the cell growth, while xylose can be used for xylitol production. However, xylose and arabinose are not metabolized by the most microorganisms, until glucose is present in the medium [11]. This regulation called carbon catabolite repression prevents the uptake and catabolism of less favourable carbon sources [11,15,20,21]. The pH value is also important for the optimal production of xylitol. For yeast growth and xylitol production the appropriate pH values are between 4 and 6 [15,22,23].

The purpose of the present work was to evaluate the xylitol and cell production during the xylitol fermentation by strains *Candida guilliermondii (Pichia guilliermondii)*, *Candida boidinii*, *Candida parapsilosis* and *Hansenula anomala (Pichia anomala)* at various pH values and under different aeration conditions. These different aeration conditions were obtained by varying the liquid volumes (filling of the flasks) in the 100 mL Erlenmeyer flasks.

2 Materials and methods

2.1 Strains and inoculum culture conditions

The experiments were carried out with the following strains: *C. boidinii* (NCAIM Y.01308); *C. guilliermondii* (NCAIM Y.01050); *C. parapsilosis* (NCAIM Y.01011); and *H. anomala* (NCAIM Y.01499), obtained from National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary). The cultures were maintained on glucose agar slant at 4-8°C. The cells, aged for 6-10 days, were transferred to 750 mL Erlenmeyer flasks containing 100 mL of the medium: 30 g L^{-1} D-glucose; 10 g L^{-1} yeast extract; 15 g L^{-1} KH_2PO_4; 3 g L^{-1} $(NH_4)_2HPO_4$; and 1 g L^{-1} $MgSO_4$ x $7H_2O$. All the chemicals used in this study were purchased from Sigma-Aldrich (Sigma-Aldrich Ltd., Budapest, Hungary). The flasks were incubated in a rotary shaker (CERTOMAT® IS, B. Braun Biotech International, Berlin, Germany) at 30°C and 220 rpm for 24 hours.

2.2 Fermentation conditions

In those cases, when the aim of the experiment was the examination of the effect of pH, the fermentations were carried out in 100 mL Erlenmeyer flasks with 50 mL medium containing: 50 g L^{-1} D-xylose; 10 g L^{-1} yeast extract; 15 g L^{-1} KH_2PO_4; 3 g L^{-1} $(NH_4)_2HPO_4$ and 1 g L^{-1} $MgSO_4$ x $7H_2O$. The pH value of the fermentation media was adjusted to 4.5; 5.0; 5.5; 6.0 at each strain. The pH was monitored and controlled during the fermentations with sterile 10% NaOH and 10% HCl. The agitation speed was set to 125 rpm.

After these experiments aeration-condition-oriented fermentations were performed at 220 rpm on that pH value, which resulted in the highest xylitol yield before. These experiments were carried out in 100 mL Erlenmeyer flasks with 30/40/50/60 mL medium (Filling ratio 0.3/0.4/0.5/0.6).

To determine the aeration capacities of the shake flasks, the volumetric mass-transfer coefficient of oxygen (K_La) was measured with non-fermentative gassing-out method. The dissolved oxygen concentration of the solution (C) is lowered by gassing the liquid out with nitrogen gas [24]. The deoxygenated liquid is then agitated and the increase of dissolved oxygen concentration due to the shaking of the flask was measured until a constant level (C*). The measurements were performed at 30°C by using different levels of medium volume (30, 40, 50 and 60 mL) and rotation speed (125 and 220 rpm). The values of the expression ln[1-(C/C*)] was plotted as a function of time. The slope of the fitted linear curve gave the value of K_La (h^{-1}). Maximum possible oxygen transfer rate (OTR) was calculated by multiplying K_La (h^{-1}) and C* (mmol L^{-1}). The oxygen concentration was monitored using Hamilton Bonaduz AG (Switzerland) VisiFerm® DO (ECS) 120 online dissolved oxygen sensor.

The initial cell mass was 4.0 g L^{-1} during all shake flask fermentations. Flasks were closed with cotton wool plugs and paper caps. All experiments were performed with daily sampling at 30°C for 96 hours.

2.3 Analytical methods

The fermentations were monitored through periodic sampling to determine xylose uptake, xylitol production and cell growth. Samples were prepared by filtration through 0.45 μm regenerated cellulose membrane for chromatography analysis. Xylose and xylitol were analysed by high-performance liquid chromatography (LC-VP, Shimadzu, Kyoto, Japan) using refractive index detector, a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) at 65°C and degasified 5 mM sulfuric acid solution as mobile phase at a flow rate of 0.5 mL min^{-1}.The xylitol yield was calculated from the highest obtained xylitol concentration [g L^{-1}] and the theoretical maximum based on the initial xylose concentration [g L^{-1}] see Eq. (1).

$$Xylitol\ yield = \frac{highest\ xylitol\ concentration\left[\frac{g}{L}\right]}{initial\ xylose\ concentration\left[\frac{g}{L}\right] \times \frac{152\left[\frac{g}{mol}\right]}{150\left[\frac{g}{mol}\right]}} \times 100[\%] \quad (1)$$

Cell growth was estimated by optical density measurement at 600 nm [25]. The cell concentration was determined using standard curves of absorbance vs. cell mass concentration. The measurement accuracies of the temperature, the pH, the rotary speed, the oxygen, xylose, xylitol, cell mass concentrations were 0.1°C, 0.01 pH unit, 1 rpm, 0.05 mmol L^{-1}, 0.01 g L^{-1}, 0.01 g L^{-1}, 0.05 g L^{-1}, respectively.

Table 1 Maximum xylitol concentrations, required cultivation times and volumetric productivities during the pH experiments (30°C, initial xylose concentration: 50 g L^{-1}; filling: 50 mL / 100 mL; rotary speed: 125 rpm

	Candida parapsilosis			*Candida guilliermondii*			*Candida boidinii*			*Hansenula anomala*		
pH	**Xylitol conc. [g L^{-1}]**	**Time [h]**	**Productivity [g $L^{-1}h^{-1}$]**	**Xylitol conc. [g L^{-1}]**	**Time [h]**	**Productivity [g $L^{-1}h^{-1}$]**	**Xylitol conc. [g L^{-1}]**	**Time [h]**	**Productivity [g $L^{-1}h^{-1}$]**	**Xylitol conc. [g L^{-1}]**	**Time [h]**	**Productivity [g $L^{-1}h^{-1}$]**
4.5	13.7	96	0.14	11.2	96	0.12	12.2	96	0.13	21.7	96	0.23
5.0	16.7	96	0.17	7.8	96	0.08	13.8	96	0.14	19.0	96	0.20
5.5	15.5	96	0.16	7.5	96	0.08	16.4	72	0.23	19.3	96	0.21
6.0	11.1	96	0.12	6.7	96	0.07	17.5	72	0.24	17.4	96	0.18

3 Results and discussion

3.1 pH experiments

Figure 1 shows the xylitol yield obtained during the pH experiments, where the OTR was 2.8 mmol $L^{-1}h^{-1}$.

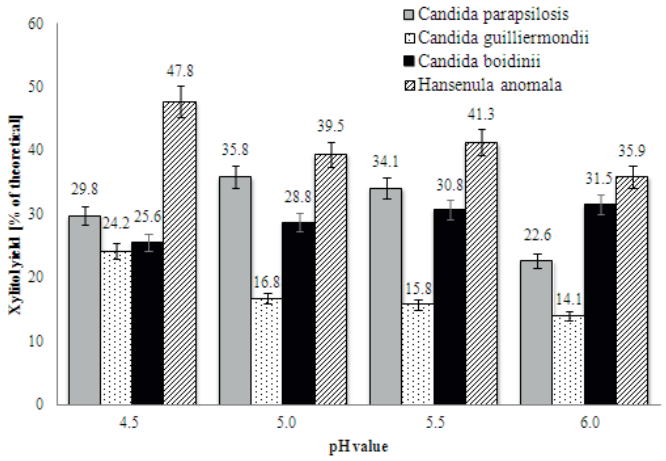

Fig. 1 Xylitol yield (% of theoretical) during the pH experiments [30°C, filling: 50 mL / 100 mL; rotary speed: 125 rpm; OTR: 2.8 mmol L^{-1} h^{-1}]. The standard deviations were calculated from triplicates.

H. anomala showed the best performance under these conditions among the four strains. Its xylitol yields were higher than those of the other strains at all pH values, and its maximum yield (47.8%) was obtained at pH 4.5. Tao et al. [26] evaluated the optimal pH range for *H. anomala* and, it was observed that pH 4.5 was the appropriate pH value for this strain. The xylitol yield was 24.2% in the case of *C. guilliermondii* at pH 4.5, and this yield decreased at the other pH values. Felipe et al. [27] also reports that pH 4.5 is appropriate for xylitol production by *C. guilliermondii*. However, other studies demonstrated that the proper pH for this strain was 5.0 [28] and 5.5 [15]. As shown in Fig. 1, the xylitol yield by *C. boidinii* increased by increasing the pH, the highest final xylitol yield (31.5%) was observed at pH 6.0. Sakai et al. [29] also found that pH 6.0 was appropriate for *C. boidinii*. In the case of *C. parapsilosis* the maximum xylitol yield (35.8%) was obtained at pH 5.0, however there is no significant difference between the xylitol yields at pH 5.0 and 5.5. The yield decreased at pH 4.5 and 6.0 in the case of this strain (Fig. 1). Kim et al. [30] also reported that pH 5.0-5.5 was appropriate for *C. parapsilosis*. However, Nolleau et al. [31] demonstrated that the eligible pH for this strain was 4.75. Results in Table 1 indicated that in the case of *H. anomala* the highest xylitol concentrations (21.7 g L^{-1}; 19.0 g L^{-1} and 19.3 g L^{-1}) were obtained at 96 hours at pH 4.5; pH 5.0 and pH 5.5, respectively. In these cases the calculated productivity was 0.23, 0.20 and 0.21 g $L^{-1}h^{-1}$, respectively. However, at 72 hours higher productivities, 0.23 and 0.24 g $L^{-1}h^{-1}$ were observed in the case of *C. boidinii* at pH 5.5 and 6.0, respectively (Table 1). This strain obtained the maximum xylitol concentration (17.5 g L^{-1}) 24 hours earlier than *H. anomala*, hence the productivity of *C. boidinii* is the highest (Table 1). The results show that the productivity decreased from 0.12 g $L^{-1}h^{-1}$ to 0.07 g $L^{-1}h^{-1}$ in the case of *C. guilliermondii*, as the pH value increased.

The cell mass concentrations are presented in Table 2. We observed that the yeasts reached the highest cell mass concentrations at those pH values where the highest final xylitol concentrations were obtained. There is a relation between the maximum xylitol yield and final cell mass concentration: the higher the cell mass concentration the higher the xylitol concentration is (Table 1 and Table 2).

The final cell mass concentration results show that the *C. boidinii* achieved lower cell mass, as the pH decreased; the cell concentration was 5.0 g L^{-1} at pH 4.5. We observed that *C. guilliermondii* achieved the highest final cell mass (8.6 g L^{-1} at pH 5.0).

Table 2 Final cell mass concentrations during the pH experiments at the maximum xylitol concentrations (30°C, initial xylose concentration: 50 g L^{-1}; filling: 50 mL / 100 mL; rotary speed: 125 rpm)

pH	**Cell mass concentrations [g L^{-1}]**			
	C. parapsilosis	***C. guilliermondii***	***C. boidinii***	***H. anomala***
4.5	8.0	8.5	5.0	8.2
5.0	8.0	8.6	5.8	7.4
5.5	7.3	7.8	6.4	7.5
6.0	7.1	7.6	7.0	7.2

Table 3 Maximum xylitol concentrations, required cultivation times and volumetric productivities during the aeration-oriented fermentations (30°C, initial xylose concentration: 50 g L^{-1}; Rotary speed: 220 rpm)

Filling [mL / 100 mL]	*Candida parapsilosis*			*Candida guilliermondii*			*Candida boidinii*			*Hansenula anomala*		
	Xylitol conc. [g L^{-1}]	Time [h]	Productivity [g $L^{-1}h^{-1}$]	Xylitol conc. [g L^{-1}]	Time [h]	Productivity [g $L^{-1}h^{-1}$]	Xylitol conc. [g L^{-1}]	Time [h]	Productivity [g $L^{-1}h^{-1}$]	Xylitol conc. [g L^{-1}]	Time [h]	Productivity [g $L^{-1}h^{-1}$]
30	5.5	72	0.08	6.3	96	0.07	21.5	48	0.45	6.4	72	0.09
40	6.9	96	0.07	6.3	96	0.07	20.3	48	0.42	5.3	96	0.06
50	25.2	96	0.26	23.0	96	0.24	23.6	48	0.49	6.0	96	0.06
60	23.1	96	0.24	23.2	96	0.24	23.7	48	0.49	8.7	96	0.09

3.2 Aeration-condition-oriented experiments

Figure 2 shows the xylitol yields obtained during the aeration-oriented fermentations. These experiments were performed on that pH value, which resulted in the highest xylitol yield before. At filling ratios of 0.3 and 0.4 the OTR was 6.5 and 6.3 mmol $L^{-1}h^{-1}$ respectively. The four investigated strains achieved low xylitol yields under these aeration conditions (Fig. 2). In the case of *C. boidinii*, *C. guilliermondii* and *C. parapsilosis* the xylitol yields varied between 43.2 and 46.9% at filling ratios of 0.5 and 0.6 with oxygen transfer rate 6.1 and 5.7 mmol $L^{-1}h^{-1}$, respectively.

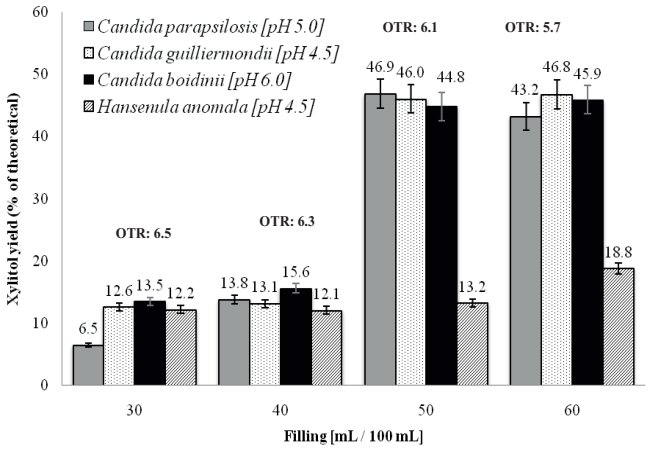

Fig. 2 Xylitol yield (% of theoretical) during the during aeration-condition-oriented fermentations [30°C, rotary speed: 220 rpm]. The standard deviations were calculated from triplicates.

A significant difference was observed between the theoretical xylitol yields obtained at filling ratios of 0.3-0.4 and 0.5-0.6. The most probable reason of this result is the difference between the oxygen transfer rates. The assessed yeasts obtained lower xylitol yields, when the OTR was 6.5 or 6.3 mmol $L^{-1}h^{-1}$. However, when the OTR was 6.1 or 5.7 mmol $L^{-1}h^{-1}$, it resulted in higher xylitol yields in all the cases. The step-wise raising of the xylitol yield as a function of OTR (Fig. 2) can be due to the sensitive behaviour of the xylose-xylitol-xylulose pathway, however, to prove this hypothesis further experiments are required. Higher xylitol yields were obtained by *C. boidinii*, *C. guilliermondii* and *C. parapsilosis* with 50 mL fermentation bulk volume at 220 rpm (at an OTR of 6.1 mmol $L^{-1}h^{-1}$) than at 125 rpm during the pH experiments, when the OTR was 2.8 mmol $L^{-1}h^{-1}$ (Figs. 1 and 2). However, *H. anomala* obtained 13.2% xylitol yield at a filling ratio of 0.5 with 220 rpm (OTR 6.1 mmol $L^{-1}h^{-1}$). This yield is significantly lower than during the pH experiments, when this strain obtained 46.9% xylitol yield at a filling ratio of 0.5 and with 125 rpm rotary speed and OTR 2.8 mmol $L^{-1}h^{-1}$.

The appropriate aeration condition for the *H. anomala* yeast was obtained with OTR 2.8 mmol $L^{-1}h^{-1}$, however the strains *C. parapsilosis*, *C. boidinii* and *C. guilliermondii* achieved the highest xylitol yields with OTRs of 5.7 and 6.1 mmol $L^{-1}h^{-1}$.

In the case of *C. boidinii* maximum xylitol concentrations were obtained at all volumes at 48 hours, and the productivity was 0.49 g $L^{-1}h^{-1}$ at filling ratios of 0.5 and 0.6 (Table 3). Although similar maximum xylitol concentrations were obtained by the other strains at filling ratios of 0.5 and 0.6, except *H. anomala*, the productivities were lower, since longer fermentation times were required (Table 3).

Table 4 Final cell mass concentrations during the aeration-oriented fermentations at the maximum xylitol concentrations (30°C, initial xylose concentration: 50 g L^{-1}; Rotary speed: 220 rpm)

Filling [mL / 100mL]	Cell mass concentrations [g L^{-1}]			
	C. parapsilosis	*C. guilliermondii*	*C. boidinii*	*H. anomala*
30	10.6	11.0	7.8	11.2
40	8.6	10.3	8.8	11.5
50	12.0	14.0	7.9	10.2
60	8.9	11.4	7.8	10.5

In the case of the four strains there is not any tendency in the obtained final cell mass concentrations under different aeration conditions (Table 4).

4 Conclusion

The highest xylitol yields obtained for the four strains investigated in this study were between 45 and 48% of the theoretical maximum, which were comparable to the yields reported elsewhere [16]. For the four yeast strains the maximum xylitol yields were achieved at the following pH values and aeration conditions: *H.anomala*: pH 4.5, 125 rpm with filling ratio 0.5 (OTR 2.8 mmol L^{-1} h^{-1}); *C.parapsilosis*: pH 5.0, 220 rpm with filling ratio 0.5 (OTR 6.1 mmol L^{-1} h^{-1}); *C. guilliermondii*: pH 4.5, 220 rpm with filling ratio 0.6 (OTR 5.7 mmol L^{-1} h^{-1}) and *C. boidinii*: pH 6.0, 220 rpm with filling ratio 0.6 (OTR 5.7 mmol L^{-1} h^{-1}) using 50 g/L initial xylose concentration. On the contrary, Vandeska et al. [32] reported that the highest xylitol yield was obtained at an OTR of 14 mmol $L^{-1}h^{-1}$ for *C. boidinii* strain using 130 g L^{-1} initial xylose concentration. Feher et al. [33] found that the appropriate OTR for *C. boidinii* was 2.8 mmol $L^{-1}h^{-1}$ using 30 g L^{-1} initial xylose in the xylitol fermentations. Based on these observations it can be presumed that using this yeast the higher the initial xylose concentration the higher the required OTR is.

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