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Influence of Inhibitory Compounds and Minor Sugars on Xylitol Production by *Debaryomyces hansenii*

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Abstract

To obtain in-depth information on the overall metabolic behavior of the new good xylitol producer *Debaryomyces hansenii* UFV-170, batch bioconversions were carried out using semisynthetic media with compositions simulating those of typical acidic hemicellulose hydrolysates of sugarcane bagasse. For this purpose, we used media containing glucose (4.3-6.5 g/L), xylose (60.1-92.1 g/L), or arabinose (5.9-9.2 g/L), or binary or ternary mixtures of them in either the presence or absence of typical inhibitors of acidic hydrolysates, such as furfural (1.0-5.0 g/L), hydroxymethylfurfural (0.01-0.30 g/L), acetic acid (0.5-3.0 g/L), and vanillin (0.5-3.0 g/L). *D. hansenii* exhibited a good tolerance to high sugar concentrations as well as to the presence of inhibiting compounds in the fermentation media. It was able to produce xylitol only from xylose, arabitol from arabinose, and no glucitol from glucose. Arabinose metabolization was incomplete, while ethanol was mainly produced from glucose and, to a lesser less extent, from xylose and arabinose. The results suggest potential application of this strain in xylose-to-xylitol bioconversion from complex xylose media from lignocellulosic materials.

Index Entries: Xylitol; *Debaryomyces hansenii*; sugar mixtures.

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Introduction

Lignocellulosic residues are mainly constituted by cellulose, hemicellulose, and lignin *(1)*. Because of its heterogeneous structure and low degree of polymerization *(2)*, xylan, one of the hemicellulose components, can be easily hydrolyzed to give mainly D-xylose, a pentose that can be used to produce ethanol *(3)*, propionic acid *(4)*, acetone/butanol *(5)*, butanediol/acetoin *(6)*, lactic acid *(7)*, or xylitol *(1,8)*.

Xylitol is a five-carbon polyalcohol (pentitol) that, because of a large number of possible applications, is gaining increasing acceptance in the food *(2)*, odontologic *(9)*, and medical-pharmaceutical industries *(10,11)*. Its industrial production is based on the acid hydrolysis of xylan-rich lignocellulosic raw materials, followed by several expensive steps of purification of the released D-xylose and its subsequent catalytic hydrogenation to xylitol using nickel as a catalyst *(2,12)*. However, bioprocesses using microorganisms for the bioconversion of synthetic xylose solutions *(8,13)* or lignocellulosic hydrolysates *(1,14–16)* appear to be very promising in view of possible reduction of costs.

In addition to pentoses (xylose, arabinose) and hexoses (glucose, galactose, and mannose), hemicellulose hydrolysates usually contain inhibitors formed during acid hydrolysis, among which furfural, hydroxymethylfurfural (HMF), acetic acid, and lignin are the derived products *(1)*. Vacuum evaporation of hydrolysates, by increasing the concentration of xylose and diminishing those of volatile inhibitors *(17)*, is expected to improve xylose-to-xylitol bioconversion and to make product recovery cheaper. However, the simultaneous concentration of minor sugars and, mainly, of nonvolatile inhibitors was shown to decelerate microbial growth *(18)*. High monosaccharide levels can in fact lead to osmotic stress to the cell *(19)*; repress the synthesis of xylose reductase *(20)*, the enzyme allowing xylose to enter the pentose phosphate shunt; and produce ethanol up to inhibitory levels *(21)*. In addition, the sugar level can influence their transfer into the cell or the enzyme kinetics when the same transport system is utilized for more than one sugar and/or in the case of simultaneous metabolization *(22)*.

The presence of inhibitory compounds in the hydrolysate requires its purification before use as a cultivation medium *(18,23–26)*, or high inoculum level *(27)*, and/or microorganism adaptation *(28,29)*. Among the different types of treatment, impurity precipitation by pH variation; adsorption on charcoal; extraction with solvents; treatment with ion-exchange resins; and, in some instances, a combination of them were performed with success *(1,12,23,24)*.

Sugarcane bagasse is an agricultural residue from the industrial sugar extraction process. Although utilized in sugar factories as fuel for the boilers, large quantities are accumulated in the mills, creating environmental problems *(30,31)*. Therefore, there is an increasing trend toward the use of alternative and environmentally friendlier methods of its utilization,

among which is xylitol production *(17,23,26,27)*. In view of the possible industrial application of this process, batch xylose-to-xylitol bioconversions were carried out in the present study using semisynthetic media that had compositions simulating those of typical acid hemicellulose hydrolysates of this raw material and that were adequately concentrated and detoxified before use. The effects of the various components of the hydrolysate on xylose-to-xylitol bioconversion by the promising yeast Debaryomyces hansenii UFV-170 *(32,33)* were studied to obtain sufficient information on its overall metabolic behavior.

Materials and Methods

Microorganism and Maintenance

The new strain *D. hansenii* UFV-170, previously selected as a good xylitol producer *(32,33)*, was utilized. Cells were maintained at 4°C on Petri plates containing YPD-agar (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of D-glucose, 15 g/L of agar), weekly transferred to the same medium, incubated at 30°C for 48 h, and then kept again at 4°C.

Preparation of Inoculum

A loopful of cells was transferred to 250-mL Erlenmeyer flasks containing 100 mL of a medium consisting of 3.4 g/L of KH_2PO_4, 6.8 g/L of K_2HPO_4, 1.0 g/L of $(NH4)_2SO_4$, 1.1 g/L of $MgSO_4$, 5.0 g/L of yeast extract, and 20 g/L of D-xylose (pH 6.0). The flasks were maintained at 30°C on a rotary shaker at 200 rpm. After incubation for 20-24 h, cells were recovered by centrifuging at 8000 rpm for 10 min (model PK 131, ALC, Cologno Monzese-MI, Italy), washed twice with sterile water, and then used for the inoculum. All tests were carried out using an initial biomass concentration of 1.4 g/L dry wt.

Cultivation Media and Fermentations

Bioconversions were performed in duplicate on the medium given in the previous section plus yeast extract but containing the same levels of xylose, glucose, arabinose, and inhibitors as the threefold (*H3*), fourfold (*H4*), and fivefold (*H5*) concentrated and detoxified sugarcane bagasse hemicellulose hydrolysate produced by Carvalho et al. *(26)* (Table 1). In particular, to investigate the effects of inhibitory compounds, furfural, HMF, acetic acid, and vanillin were added at variable concentrations in a medium having almost the same sugar composition as H3 after detoxification. Vanillin was used to simulate the acid-soluble lignin (ASL), because it was found to be the most abundant lignin byproduct in lignocellulose hydrolysates and because other more toxic byproducts such as syringaldheyde are present only in traces *(1)*. In addition, boiling the hydrolysate was found to effectively reduce the acetic acid concentration *(34)*, and, thus, is a fundamental step of the detoxification procedure for an industrial process. Conse-

Table 1
Average Composition of Three- (*H3*), Four- (*H4*), and Fivefold (*H5*) Concentrated Sugarcane Bagasse Hemicellulosic Hydrolysate After Detoxification *(26)*

Concentration (g/L)			
Compound	H3	H4	H5
Glucose	4.3	5.8	6.5
Xylose	60.1	77.8	92.1
Arabinose	5.9	8.3	9.2
Acetic acid	3.9	4.4	3.9
Furfural	0.00	0.00	0.01
HMF	0.00	0.01	0.01
ASL	0.8	1.5	1.8

quently, acetic acid concentrations (0.5–3.0 g/L) lower than those reported in Table 1 (3.9–4.4 g/L) were selected for the present study.

The effect of different sugars as well as their simultaneous presence was investigated on media containing the inhibitors at the *H3* levels and single sugars or binary or ternary mixtures of them at the same concentrations as in *H3*, *H4*, and *H5*.

Concentrated solutions of yeast extract, $MgSO_4$, and the aforementioned components were prepared and autoclaved separately at 121°C for 20 min. All the experiments were performed in 250-mL Erlenmeyer flasks containing 50 mL of the medium at 30°C on a rotary shaker at 200 rpm.

Analytical Methods

Aliquots of the fermented broths were centrifuged at 8000 rpm for 10 min. Concentrations of sugars and pentitols in the supernatants were determined by a high-performance liquid chromatograph (model 1100; Hewlett Packard, Palo Alto, CA), equipped with a refractive index detector (model HP 1047A), and a 300 × 7.8 mm column (model Supelcogel C610H; Supelco, Bellefonte, PA). A 0.01 N H_2SO_4 solution was used as mobile phase at a flow rate of 0.5 mL/min while the column was kept at 50°C. Ethanol concentration was determined by a gas chromatograph (model Sigma 3; Perkin-Elmer, Norwalk, CT) equipped with a thermal conductivity detector and a 25% Carbowax 600 80/100 Chromosorb WHP 10 in. × 1/8 in. column (Supelco) with helium as carrier gas at 3.9 bars. Temperatures of the column and injector were 90 and 120°C, respectively. The same analysis was used to detect possible formation of volatile byproducts.

Biomass concentration either in the fermentation broths or in the inoculum suspensions was determined by optical density (OD) measurements at 600 nm. A calibration curve was used to relate OD with cell dry weight (1 OD = 0.247 g of dry matter/L).

Kinetic Parameters and Yields

Final concentrations of xylitol, arabitol, and ethanol were defined as P_{Xyt}, P_{Art}, and P_{Eth}, respectively. Their respective average volumetric productivities (Q_{Xyt}, Q_{Art}, and Q_{Eth}) were calculated at the start of the stationary phase by dividing their concentrations by the time required to reach this phase. These values were then divided by the cell concentration at the same time to obtain the corresponding specific productivities (q_{Xyt}, q_{Art}, and q_{Eth}). The yields of xylitol ($Y_{Xyt/S}$), arabitol ($Y_{Art/S}$), ethanol ($Y_{Eth/S}$), and biomass ($Y_{X/S}$) were calculated as the ratios of the maximum concentrations of these products to the consumed substrate subtracting for the last parameter the initial value of biomass concentration.

Results and Discussion

Effect of Toxic Compounds

As is well known, HMF, furfural, and acetic acid present in hemicellulosic hydrolysates are produced from the decomposition of carbohydrates, whereas vanillin is the most representative of the aromatic compounds released by partial lignin degradation *(35)*. To assess the influence of these compounds on xylose-to-xylitol bioconversion by D. hansenii UFV-170, a set of experiments was performed on semisynthetic media containing variable levels of the compounds and initial sugar concentrations as the threefold concentrated and detoxified hydrolysate of sugarcane bagasse (H3). The main results of these experiments are compared in Table 2 with those of a reference test performed in the absence of inhibitors.

A low HMF level (0.01 g/L) inhibited xylitol formation with respect to the reference test, but, surprisingly, a progressive increase up to 0.30 g/L stimulated it (P_{Xyt} = 40.9 g/L). Similar effects are evident in Table 2 for almost all the kinetic parameters. On the contrary, although remaining very effective in terms of pentitol yield on consumed sugar ($Y_{Art/S}$ = 0.78–1.08 g/g), arabitol concentration decreased from 4.19 to 2.33–3.11 g/L, the consumption of arabinose was incomplete (37–72%), and arabitol productivities were lower (Q_{Art} = 0.03–0.04 g/[L·h]; q_{Art} <0.01 g/[g·h]). Glucose was quickly consumed under all the conditions tested, with only some variations in its consumption rate (results not shown), whereas ethanol production was not appreciably influenced ú◊ the presence of HMF within the concentration range investigated. Because pentitol accumulation is the combined result of high xylose reductase (XR) and low xylitol dehydrogenase activities, the increased xylitol formation with an increase in the HMF level in the medium could have been owing to, among other possible causes, inhibiting effects of different extents on the activities of these enzymes. Furans such as HMF and furfural are in fact reported to act as strong inhibitors of many soluble enzymes *(36)*, with those involved in glycolysis and most dehydrogenases among the most sensitive *(37)*.

Table 2
Results of Batch Bioconversions of Xylose/Glucose/Arabinose Mixtures Simulating Detoxified Hydrolysate *H3 (26)* in Presence of Different Concentrations of HMF, Furfural, Acetic Acid, and Vanillin

		HMF (g/L)			Furfural (g/L)			Acetic acid (g/L)			Vanillin (g/L)		
Parameter	Reference[a]	0.01	0.16	0.30	1.00	3.00	5.00	0.50	1.73	3.00	0.50	1.75	3.00
Time (h)b	83	70	70	78	70	78	70	78	70	78	70	92	120
$Y_{X/S}$ (g/g)	0.10	0.10	0.10	0.08	0.09	0.09	0.08	0.11	0.10	0.08	0.09	0.07	0.07
P_{Xyt} (g/L)	38.5	34.9	36.2	40.9	33.2	31.9	32.5	38.4	37.2	38.1	35.6	30.3	25.0
P_{Art} (g/L)	4.19	2.33	2.48	3.11	2.25	3.36	2.53	4.75	3.55	4.12	3.25	5.05	0.90
P_{Eth} (g/L)	3.73	3.84	3.85	3.34	3.11	3.14	4.69	1.59	2.95	3.34	3.58	3.05	2.29
$Y_{Xyt/S}$ (g/g)	0.74	0.64	0.66	0.70	0.63	0.62	0.64	0.77	0.75	0.71	0.66	0.56	0.69
$Y_{Art/S}$ (g/g)	0.95	1.00	1.08	0.78	0.93	0.81	0.93	0.73	0.89	0.74	1.02	0.96	1.12
$Y_{Eth/S}$ (g/g)	0.06	0.06	0.06	0.05	0.05	0.05	0.08	0.03	0.20	0.22	0.06	0.06	0.03
Q_{Xyt} (g/[L·h])[c]	0.54	0.50	0.52	0.52	0.47	0.41	0.46	0.49	0.53	0.49	0.51	0.33	0.21
Q_{Art} (g/lL·h])[c]	0.05	0.03	0.04	0.04	0.03	0.04	0.04	0.06	0.05	0.05	0.05	0.05	0.01
Q_{Eth} (g/[L·h])[c]	0.04	0.05	0.06	0.04	0.04	0.04	0.07	0.02	0.04	0.04	0.05	0.03	0.02
q_{Xyt} (g/[g·h])[d]	0.14	0.12	0.12	0.14	0.12	0.11	0.12	0.16	0.17	0.15	0.12	0.10	0.06
q_{Art} (g/[g·h])[d]	0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	<0.01
q_{Eth} (g/[g·h])[d]	<0.01	0.01	0.01	0.01	<0.01	<0.01	0.02	0.02	0.03	0.04	0.01	<0.01	0.01

[a]Xylose/glucose/arabinose mixture without inhibitors.
[b]Time of total xylose consumption.
[c]Average volumetric productivity calculated at the start of the stationary phase.
[d]Average specific productivity calculated at the start of the stationary phase.

Xylitol and arabitol productions were affected by furfural, with no appreciable dependence on its concentration. At the highest level of this inhibitor, which is considered to be stronger than HMF *(38)*, final xylitol concentration (P_{Xyt} = 32.5 g/L) was about 15% lower than that of the reference test, and all the related kinetic parameters and yields appreciably decreased. At the same time, the uptake of initial arabinose was only 44% (results not shown), thus affecting arabitol production, whereas ethanol formation increased by about 25%. According to Wahlbom and Hahn-Hägerdal *(39)*, furfural could have acted as an electron acceptor, hence affecting xylitol excretion and increasing ethanol yield. These results partially agree with those reported for *Candida parapsilosis* ATCC 28474, whose xylose consumption was remarkably decelerated by furfural *(35)*. Finally, in accordance with the results obtained with other yeasts *(1,40)*, the yield of growth fell in the presence of both furans, likely owing to inhibitions of respiration, oxidative phosphorylation *(41)*, or cell replication *(42)*.

When acetic acid was added to the medium at concentrations up to 3.0 g/L, it was metabolized by the yeast as a cosubstrate, without appreciably influencing the final concentrations and productivities of xylitol, arabitol, and ethanol. Similar consumption of acetic acid was associated in *Candida guilliermondii* FTI 20037 with slight improvement of growth *(43)* and in *C. parapsilosis* ATCC 28474 with stimulation of xylitol production at the expense of growth *(35)*. Using carbon material balances, Carvalho et al. *(44)* proposed that the respiration of low levels of secondary carbon sources (including acetic acid) could have stimulated either the growth or the xylitol production from sugarcane bagasse acid hydrolysate by the former microorganism. None of these effects was observed in our study with *D. hansenii* UFV-170, thus confirming the marked difference in the metabolic behaviors of pentose-fermenting yeasts.

On the other hand, the addition of vanillin at concentrations up to 3.0 g/L led to 40 and 36% decreases in xylitol volumetric and specific productivities, respectively. The rates of formation and the final concentrations of ethanol and arabitol were also strongly affected by the presence of this inhibitor, whereas no appreciable effect was evident on final xylitol concentration. In general, vanillin concentrations higher than 0.5 g/L had a marked inhibiting effect on yeast metabolism, thereby negatively influencing the fermentation parameters. Although vanillin is considered one of the strongest hydrolysate inhibitors *(45)*, its addition at 0.1 g/L did not appreciably alter the bioconversion pattern of *C. parapsilosis* ATCC 28474 *(35)*, which suggests that efforts should be made to reduce the concentration of this powerful inhibitor in hemicellulosic hydrolysate below its inhibiting threshold (0.1–0.5 g/L).

Comparison of the results of all these bioconversions reveals that xylose was almost totally consumed after 70–78 h, except for tests performed in the presence of 1.75–3.0 g/L of vanillin that took a longer time (92–120 h). As a general rule, a decrease in the biomass yield was observed with respect to the reference test without inhibitors (0.10 g/g), but biomass

growth was strongly inhibited only at the highest levels of these byproducts ($Y_{X/S}$ = 0.07–0.08 g/g with 0.30 g/L of HMF, 5.0 g/L of furfural, 3.0 g/L of acetic acid, or 1.75–3.0 g/L of vanillin).

An additional effect of difficult evaluation is the possible total substrate inhibition affecting product formation as a whole. Transportation of these sugars can in fact limit their individual uptake rate, thus resulting in longer adaptation, especially in the simultaneous presence of inhibitors.

Because the inhibiting effect of a compound can be synergistically modulated by the simultaneous presence of other substances and their concentrations *(1,26)*, an additional set of experiments was performed to investigate the combined effect of inhibiting compounds on the metabolism of *D. hansenii* UFV-170 utilizing single sugars as carbon sources as well as a specific combination of them.

Batch Cultivations on Single Sugars

Table 3 presents the results of *D. hansenii* UFV-170 cultivations carried out on semisynthetic media either with or without inhibitors containing single sugars (xylose, glucose or arabinose) at the levels detected for these components by Carvalho et al. *(26)* in three-, four- and fivefold concentrated sugarcane hemicellulose hydrolysates.

As expected, an increased initial xylose concentration (Fig. 1A) without inhibitors was responsible for the longer time necessary to complete xylose-to-xylitol bioconversion (up to 83 h). The presence of inhibitors (Fig. 1B) further decelerated the bioconversion (93-104 h) only in media with initial xylose concentrations greater than 60 g/L, which suggests that these byproducts when simultaneously present in the medium exerted their negative action beyond given concentration thresholds. In particular, the presence of inhibitors reduced the yield of xylitol on xylose, the final concentration of xylitol in the medium, the xylitol volumetric productivity, and the final concentration of biomass (results not shown), thus favoring ethanol accumulation. It should be noted that the specific growth rate of biomass in the absence of inhibitors followed a general trend in this work that was opposite that of specific xylitol productivity, because conditions favoring growth decreased the amount of substrate available for xylitol formation. As an example, whereas this parameter decreased from 0.023 to 0.018 h^{-1} when xylose concentration in the medium was raised from 60.1 to 92.1 g/L, q_{Xyt} increased from 0.18 to 0.23 g/(g·h). On the other hand, no certain correlation was found in the presence of inhibitors, because of possible inhibition of the overall metabolism.

Arabinose was metabolized only in the absence of inhibitors, but the time of its depletion dramatically increased with its concentration (up to 96 h) (Table 3). Increasing arabinose concentration, arabitol yield ($Y_{Art/S}$ = 0.65–0.69 g/g), and specific productivity (q_{Art} = 0.04 g/[g·h]) were not significantly influenced. Only a small amount of ethanol (P_{Eth} = 0.02–0.05 g/L) was produced, likely because of a carbon flux shift from the pentose-phosphate

Table 3
Fermentation Parameters of Batch D. hansenii UFV-170 Cultivations Performed on Xylose, Glucose, and Arabinose Solutions WithSame Sugar and Inhibitor Levels as Three-, Four-, and Fivefold Concentrated and Detoxified Sugarcane Bagasse Hemicellulose Hydrolyzates (*H3*, H4, and *H5*) *(26)*

	Without inhibitors									With inhibitors								
	Xylose			Glucose			Arabinose			Xylose			Glucose			Arabinose		
Parameter	*X3*	*X4*	*X5*	*G3*	*G4*	*G5*	*A3*	*A4*	*A5*	*x3*	*x4*	*x5*	*g3*	*g4*	*g5*	*a3*	*a4*	*a5*
Time (h)[a]	59	72	83	6	6	6	28	59	96	56	93	104	13	21	29	—	—	—
$Y_{X/S}$ (g/g)	0.09	0.08	0.07	0.59	0.64	0.66	0.30	0.25	0.08	0.06	0.09	0.06	0.42	0.36	0.30	—	—	—
P_{Xyt} (g/L)	43.5	61.2	64.2	—	—	—	—	—	—	39.1	51.4	60.9	—	—	—	—	—	—
P_{Art} (g/L)	—	—	—	—	—	—	3.67	5.92	6.35	—	—	—	—	—	—	—	—	—
P_{Eth} (g/L)	0.29	0.28	0.19	0.54	0.42	0.37	0.02	0.02	0.05	0.60	1.70	2.20	1.02	1.78	2.22	—	—	—
$Y_{Xyt/S}$ (g/g)	0.78	0.79	0.79	—	—	—	—	—	—	0.68	0.69	0.67	—	—	—	—	—	—
$Y_{Art/S}$ (g/g)	—	—	—	—	—	—	0.66	0.69	0.65	—	—	—	—	—	—	—	—	—
$Y_{Eth/S}$ (g/g)	<0.01	<0.01	<0.01	0.15	0.11	0.01	<0.01	<0.01	<0.01	0.02	0.04	0.04	0.25	0.29	0.32	—	—	—
Q_{Xyt} (g/[L·h[)[b]	0.74	0.85	0.89	—	—	—	—	—	—	0.70	0.55	0.58	—	—	—	—	—	—
Q_{Art} (g/[L·h])[b]	—	—	—	—	—	—	0.16	0.10	0.06	—	—	—	—	—	—	—	—	—
Q_{Eth} (g/[L·h])[b]	<0.01	<0.01	<0.01	0.12	0.09	0.08	<0.01	<0.01	<0.01	0.02	0.03	0.03	0.05	0.08	0.07	—	—	—
q_{Xyt} (g/[g·h])[c]	0.18	0.20	0.21	—	—	—	—	—	—	0.23	0.13	0.16	—	—	—	—	—	—
q_{Art} (g/[g·h])[c]	—	—	—	—	—	—	0.04	0.04	0.04	—	—	—	—	—	—	—	—	—
q_{Eth} (g/[g·h])[c]	<0.01	<0.01	<0.01	0.03	0.02	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01	0.02	0.02	—	—	—

[a]Time of total xylose consumption.
[b]Average volumetric productivity calculated at the start of the stationary phase.
[c]Average specific productivity calculated at the start of the stationary phase.

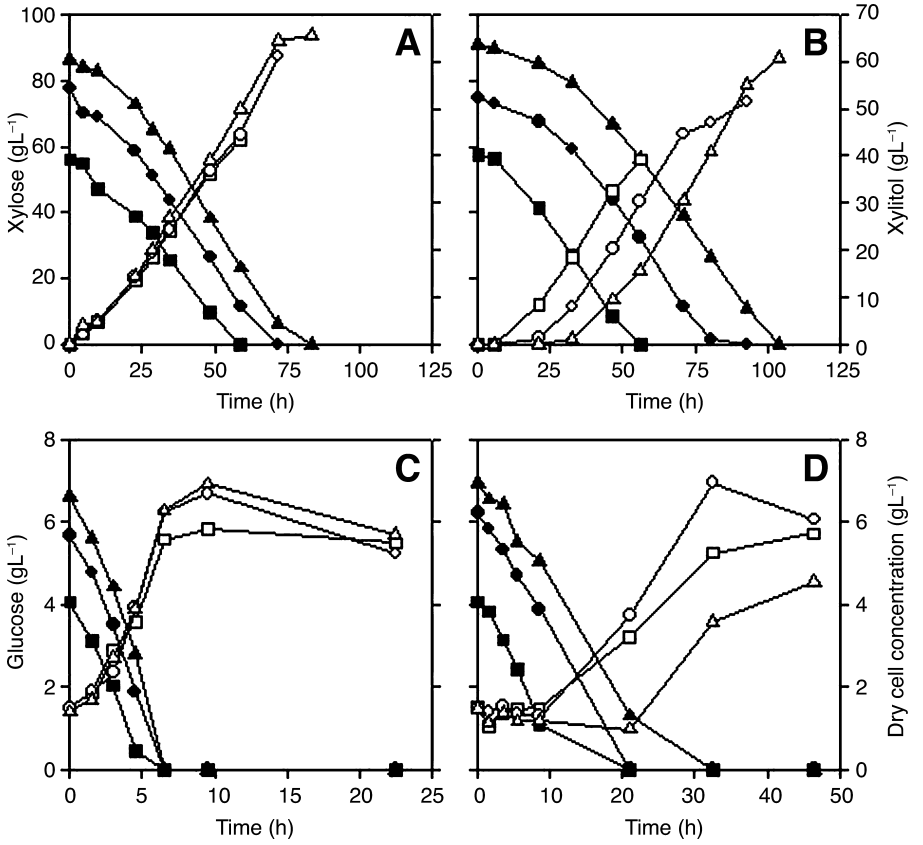

Fig. 1. Batch profiles of *D. hansenii* UFV-170 cultivations in shake flasks on xylose **(A)** without or **(B)** with inhibitors and on glucose **(C)** without or **(D)** with inhibitors. Solid symbols represent substrates (xylose and glucose) and open symbols represent products (xylitol and biomass). *X3*, *x3*, *G3*, *g3*: (■,□); *X4*, *x4*, *G4*, *g4* (●,○); *X5*, *x5*, *G5*, *g5* (▲,△).

shunt to the Embden-Meyeroff pathway *(32)*. On the contrary, in the presence of inhibitors, there was no uptake of arabinose, thus pointing out a toxic effect to the cell as the result of synergism of these inhibiting factors. Meyrial et al. *(46)* observed that a longer time was required for complete arabinose consumption by *C. guilliermondii*, leading to the production of only biomass and arabitol, whereas *Candida tropicalis* ATCC 96745 was unable to metabolize arabinose as a carbon and energy source *(22)*.

In media containing glucose as the only carbon source without inhibitors, this sugar was completely consumed within only 6 h at all the concentrations tested (Fig. 1C), producing only biomass and ethanol, likewise *C. guilliermondii* NRC 5578 *(46,47)*. This result agrees with the observation that ethanol was produced essentially from glucose when this carbon source was present in sugar mixtures *(22,35)*. In the presence of inhibitors, total glucose consumption took two- to fivefold longer, and all these activi-

ties were strongly affected (Fig. 1D). Biomass production on this sugar was considerably higher than on arabinose or xylose and remarkably decreased either with increasing glucose concentration or in the presence of inhibitors. In the latter situation, biomass grew even after complete consumption of glucose at the expense of ethanol (results not shown), thus exhibiting particularly high biomass yield. Finally, no pentitol or glucitol accumulation was observed from glucose, confirming the results obtained with *C. tropicalis* ATCC 96745 (22).

To better understand the metabolic behavior of the yeast *D. hansenii* UVF-170, additional bioconversions were performed in the simultaneous presence of different combinations of these sugars and inhibitors.

Batch Cultivations on Binary and Ternary Sugar Mixtures

Table 4 gives the results of cultivations on xylose/glucose, xylose/arabinose, and xylose/glucose/arabinose solutions simulating the compositions of hydrolysates increasingly concentrated (*H3*, *H4*, and *H5*) in either the presence or absence of inhibitors. Comparison of these results with those presented in the previous section dealing with cultivations on one-sugar solutions (Table 3) reveals that more factors affected xylitol formation during the mixed sugars fermentation, such as the specific rate of oxygen uptake and the regeneration of cofactors (NADPH via pentose cycle, NAD^+ via respiratory chain). These factors influence directly the yield of xylitol and its volumetric productivity.

The fermentation behavior on xylose/glucose mixture in the presence of inhibitors (*xg3*) is depicted in Fig. 2A,B as an example. In accordance with previous studies performed with other yeasts either in synthetic solutions or in hydrolysates *(14,22,47)*, glucose was taken up as the first carbon source, and xylose started to be consumed only after its depletion (Fig. 2A). Without inhibitors, all the parameters of xylitol production from xylose remarkably decreased when compared with the bioconversions performed on media containing only xylose (*X3-5*) (Table 3). These results, which agree with the negative effect exerted by this sugar on xylitol production by *C. tropicalis (22)*, are consistent with the repression of XR synthesis by glucose *(20)*. The highest values of xylitol concentration ($P_{Xyt} = 42.6$ g/L) and yield ($Y^{Xyt/S} = 0.68$ g/g) were obtained on *XG4* only after glucose depletion, whereas ethanol was overproduced (up to 12.6 g/L) on the most concentrated binary mixture (*XG5*). Such a production of ethanol was higher than in controls *X3-5* and *G3-5* and was detected also in the presence of inhibitors (Fig. 2B). Both ethanol overproduction and reduced xylitol formation could have also been the result of the simultaneous action of excess glucose and NADH accumulated under oxygen-limited conditions, as has recently been observed for *Candida mogii (48)*.

The presence of inhibitors in both *xg4* and *xg5* limited the growth and likely led to an imbalance in the respiratory NAD^+/NADH regeneration, thus increasing xylitol accumulation in the cells. Owing to the resulting

Table 4
Fermentation Parameters of Batch *D. hansenii* UFV-170 Cultivations Performed on Sugar Mixtures Simulating Compositions of Three-, Four-, and Fivefold Concentrated and Detoxified Sugarcane Bagasse Hemicellulose Hydrolysates (*H3*, *H4*, and *H5*) *(26)*

	Without inhibitors									With inhibitors								
	Xylose/glucose			Xylose/arabinose			Xylose/glucose/arabinose			Xylose/glucose			Xylose/arabinose			Xylose/glucose/arabinose		
Parameter	*XG3*	*XG4*	*XG5*	*XA3*	*XA4*	*XA5*	*XGA3*	*XGA4*	*XGA5*	*xg3*	*xg4*	*xg5*	*xa3*	*xa4*	*xa5*	*xga3*	*xga4*	*xga5*
Time (h)[a]	83	120	168	59	72	83	83	107	146	71	93	117	71	93	104	71	104	117
$Y_{X/S}$ (g/g)	0.09	0.09	0.08	0.10	0.08	0.09	0.10	0.08	0.07	0.08	0.05	0.03	0.12	0.07	0.07	0.10	0.06	0.05
P_{Xyt} (g/L)	38.0	42.6	38.9	42.8	58.4	71.1	38.5	52.8	49.8	38.8	50.4	54.7	37.3	47.9	54.2	37.9	48.5	53.6
P_{Art} (g/L)	—	—	—	6.55	4.00	5.75	4.19	3.50	6.62	—	—	—	4.18	3.93	4.66	3.57	5.69	4.89
P_{Eth} (g/L)	4.96	9.02	12.6	0.01	0.18	0.14	3.73	6.74	11.8	2.48	4.36	3.54	0.52	1.32	2.53	2.39	4.01	3.80
$Y_{Xyt/S}$ (g/g)	0.60	0.68	0.56	0.77	0.70	0.79	0.74	0.76	0.69	0.66	0.63	0.58	0.64	0.63	0.64	0.64	0.66	0.62
$Y_{Art/S}$ (g/g)	—	—	—	0.95	0.69	0.97	0.95	1.10	0.94	—	—	—	0.77	0.85	1.02	1.05	1.00	1.03
$Y_{Eth/S}$ (g/g)	0.21	0.13	0.18	<0.01	0.01	<0.01	0.06	0.08	0.14	0.06	0.07	0.05	0.16	0.05	0.14	0.04	0.07	0.07
Q_{Xyt} (g/[L·h])[b]	0.46	0.44	0.32	0.72	0.70	0.85	0.54	0.49	0.46	0.55	0.54	0.53	0.52	0.52	0.49	0.53	0.52	0.52
Q_{Art} (g/[L·h])[b]	—	—	—	0.11	0.06	0.07	0.05	0.03	0.04	—	—	—	0.06	0.04	0.04	0.05	0.05	0.04
Q_{Eth} (g/[L·h])[b]	0.14	0.08	0.11	<0.01	0.02	0.02	0.04	0.06	0.10	0.04	0.06	0.04	0.09	0.03	0.05	0.03	0.06	0.05
q_{Xyt} (g/[g·h])[c]	0.10	0.10	0.07	0.17	0.15	0.17	0.14	0.11	0.10	0.14	0.15	0.16	0.12	0.13	0.15	0.132	0.16	0.14
q_{Art} (g/[g·h])[c]	—	—	—	0.02	0.01	0.01	0.01	<0.01	<0.01	—	—	—	0.01	0.01	c.01	0.01	0.01	0.01
q_{Eth} (g/[g·h])[c]	0.03	0.02	0.02	<0.01	<0.01	<0.01	<0.01	0.01	0.02	0.01	0.02	0.01	0.06	0.01	0.03	0.01	0.03	0.02

[a]Time of total xylose consumption.
[b]Average volumetric productivity calculated at the start of the stationary phase.
[c]Average specific productivity calculated at the start of the stationary phase.

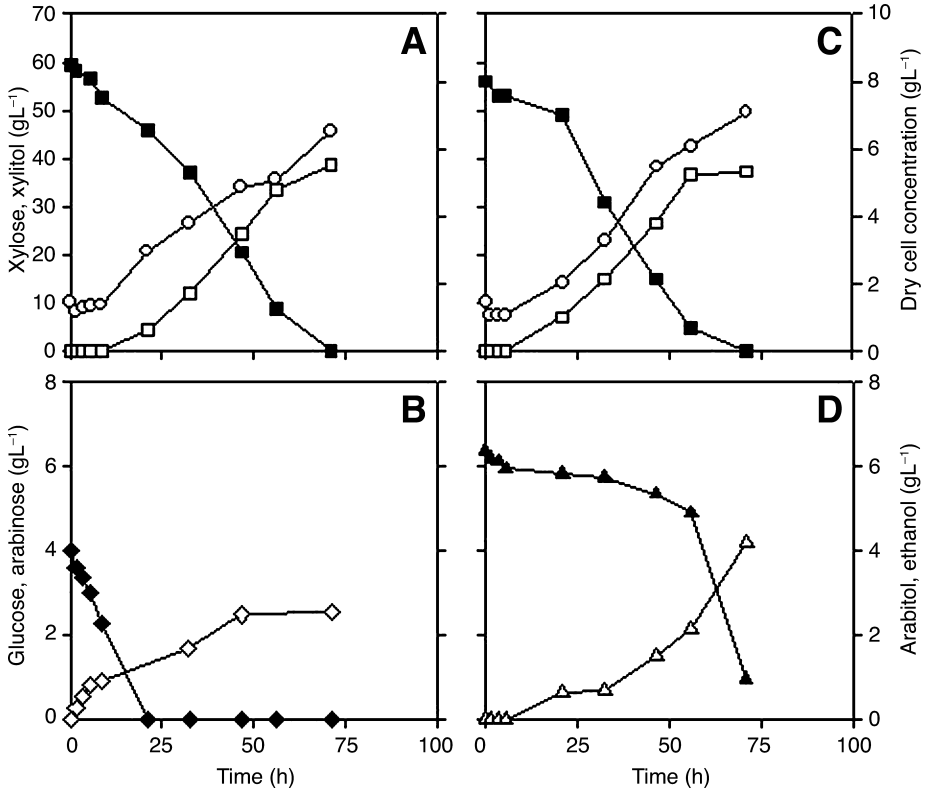

Fig. 2. Batch cultivation profile of *D. hansenii* UFV-170 grown in shake flasks on **(A,B)** xylose/glucose (*xg3*) and **(C,D)** xylose/arabinose (*xa3*) mixtures with inhibitors: (■) xylose; (◆) glucose; (▲) arabinose; (□) xylitol; (○) biomass; (◇) ethanol; (△) arabitol.

adverse energetic situation, ethanol formation and biomass production slowed. However, xylose was more quickly taken up with respect to the medium without inhibitors (*XG3–5*), likely because more substrate was utilized as energy source for cell maintenance, thus leading to lower yields of biomass on consumed substrate.

When using arabinose as a cosubstrate instead of glucose, in the absence of inhibitors ethanol formation was almost suppressed (P_{Eth} = 0.01–0.14 g/L) and xylose was consumed even more quickly (Fig. 2C,D), thus remarkably improving xylitol production and yield. Xylitol production was comparable with that obtained from xylose alone (*X3–5*) (Table 3) and, as expected, it increased with xylose concentration in the medium (P_{Xyt} = 42.8 g/L in *XA3* and 71.1 g/L in *XA5*). Because arabinose was shown to stimulate xylose-to-xylitol bioconversion by *C. tropicalis*, likely acting as an inducer of XR *(22)*, while affecting *D. hansenii* cultivation *(49)*, its action appears to be strain dependent like that of glucose. Meanwhile, arabinose was taken up very slowly and simultaneously to xylose, even when its maximum consumption and bioconversion to arabitol took place after xylose shortage. Its con-

sumption was incomplete, varying from 50% in *XA3* to 78% in *XA5* (results not shown), compared with that observed in the presence of arabinose alone (*A3-5*). Different behaviors are reported in the literature for different yeasts. For example, arabinose was similarly taken up by *C. guilliermondii* FTI 20037 grown on rice straw hemicellulose hydrolysate *(43)*, whereas no arabinose consumption by *Candida sp.* 11-2 was observed after 72 h of cultivation on sugarcane hemicellulose hydrolysate *(18)*, and simultaneous uptake of arabinose and glucose took place in synthetic medium with *D. hansenii (49)*.

Opposite to the effect observed for xylose/glucose mixture, the presence of inhibitors remarkably decelerated the bioconversion, likely owing to the longer time of adaptation to the new medium, and was responsible for an appreciable decrease in both xylitol concentration (from 71.10 to 51.41 g/L) and conversion yield (from 0.79 to 0.64 g/g). However, as stressed earlier, when utilizing arabinose as the only sugar (a3-5), D. hansenii UFV-170 was not able to metabolize it at all, whereas it was consumed in a mixture with xylose (*xa*). The presence of inhibitors in xylose/arabinose medium (*xa5*) favored ethanol production (from 0.14 to 2.53 g/L) but did not exert any appreciable effect on arabinose consumption. Contrary to the observations made with *D. hansenii*, no xylitol formation was observed from arabinose *(49)*.

When fermentation was performed with the three carbon sources, either in the absence (*XGA*) or in the presence (*xga*) of inhibitors, it followed the same order of sugar consumption as those observed earlier for binary mixtures, i.e., glucose, xylose, and arabinose (Fig. 3 and Table 4). Glucose consumption was slower in the presence of inhibitors, and this effect was more evident in the most concentrated media simulating *H4* and *H5*, likely owing to substrate inhibition and then to longer adaptation time. On the other hand, contrary to binary mixtures, for which no appreciable effect was detected, xylose consumption sped up.

In the absence of inhibiting compounds (*XGA3–5*), final xylitol concentration (P_{Xyt} = 38.5–52.8 g/L), volumetric productivity (Q_{Xyt} = 0.46–0.54 g/[L·h]), and specific productivity (q_{Xyt} = 0.10–0.14 g/[g·h]) were substantially lower than those obtained with single xylose solutions (*X3–5*) or binary xylose/arabinose mixtures (*XA3–5*) but higher than those of xylose/glucose mixtures (*XG3–5*); therefore, it is reasonable to ascribe the negative influence of glucose on xylose-to-xylitol bioconversion to the indirect inhibition exerted by ethanol. Arabitol production in the ternary mixture was almost the same as in the medium containing only arabinose, exhibiting yields close to the theoretical value ($Y_{Art/S}$ = 0.94–1.10 g/g), which suggests that the microorganism utilized preferentially the most easily assimilable carbon sources (glucose and xylose) for its primary metabolism. However, arabinose metabolization was incomplete (37–65%) and slower than that observed with arabinose alone, thus showing much lower arabitol productivities. Ethanol production (P_{Eth} = 3.73–11.8 g/L) was even higher

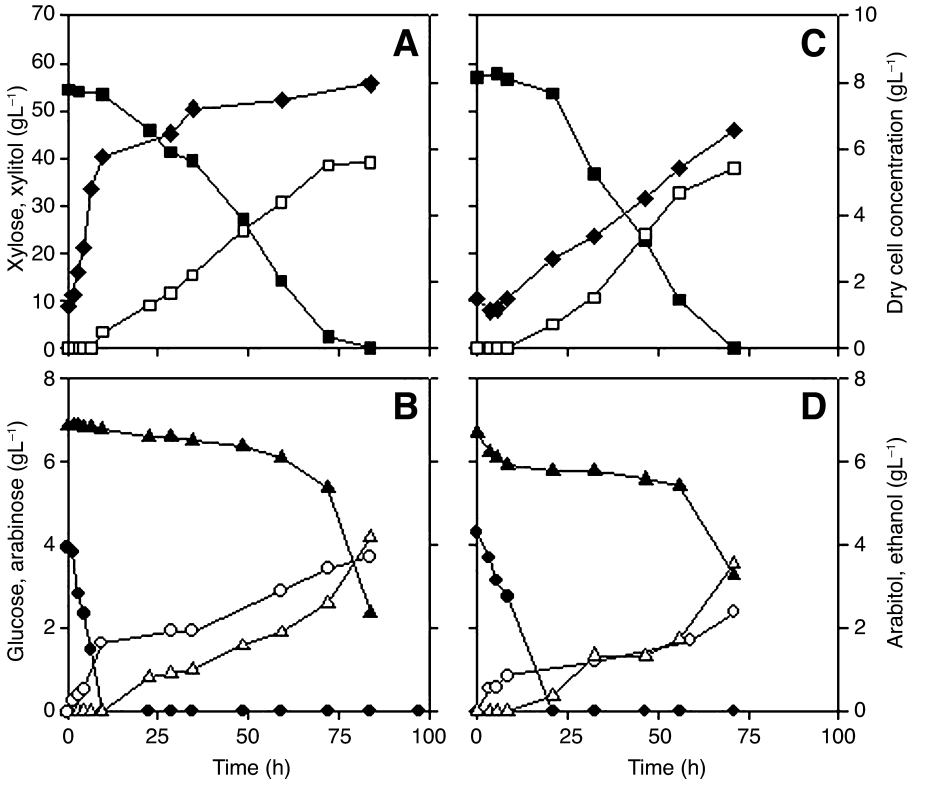

Fig. 3. Batch profiles of *D. hansenii* UFV-170 cultivations in shake flasks on xylose/glucose/arabinose mixtures **(A,B)** without and **(C,D)** with inhibitors: (■) xylose; (●) glucose; (▲) arabinose; (□) xylitol; (◆) biomass; (△) arabitol; (○) ethanol.

than the sum of those from the three single sugars alone, but lower than from the xylose/glucose mixture and higher than from the xylose/arabinose mixture, which points out a possible inhibition of arabinose on the Embden-Meyeroff pathway.

Although speeding up the bioconversion, the presence of hydrolysis byproducts in the media led to worsening of most bioconversion parameters. On the other hand, xylitol volumetric and specific productivity as well as all the arabitol production parameters were not appreciably influenced. Ethanol formation was comparable with that obtained using xylose/glucose mixtures (*xg3–5*), confirming that this product was mainly the result of the fermentative metabolism of only these sugars.

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