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The Combined Effects of Acetic Acid, Formic Acid, and Hydroquinone on *Debaryomyces hansenii* Physiology

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Abstract

The combined effects of inhibitors present in lignocellulosic hydrolysates was studied using a multivariate statistical approach. Acetic acid (0–6 g/L), formic acid (0–4.6 g/L), and hydroquinone (0–3 g/L) were tested as model inhibitors in synthetic media containing a mixture of glucose, xylose, and arabinose simulating concentrated hemicellulosic hydrolysates. Inhibitors were consumed sequentially (acetic acid, formic acid, and hydroquinone), alongside to the monosaccharides (glucose, xylose, and arabinose). Xylitol was always the main metabolic product. Additionally, glycerol, ethanol, and arabitol were also obtained.

The inhibitory action of acetic acid on growth, on glucose consumption and on all product formation rates was found to be significant ($p \leq 0.05$), as well as formic acid inhibition on xylose consumption and biomass production. Hydroquinone negatively affected biomass productivity and yield, but it significantly increased xylose consumption and xylitol productivity. Hydroquinone interactions, either with acetic or formic acid or with both, are also statistically significant. Hydroquinone seems to partially lessen the acetic acid and amplify formic acid effects. The results clearly indicate that the interaction effects play an important role on the xylitol bioprocess.

Index Entries: Lignocellulosic byproducts; xylitol; interaction effects; *Debaryomyces hansenii*; inhibition.

Introduction

The xylitol bioproduction is a possible valuable alternative for upgrading the pentose-rich hemicellulosic hydrolysate stream arising from lignocellulosic materials pretreatments. However, the xylitol yield and productivity by yeasts (most noteworthy, some *Candida* spp. *[1–4]* and *Debaryomyces hansenii [5–7]*) are affected by several factors, for example, oxygen availability, and the initial concentrations of biomass, hexoses, pentoses, and inhibitors in the hydrolysates *(8)*. Among those factors,

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the inhibitors influence is still the less understood, conversely to what happens for the bioethanol process, for which the inhibitor effects are currently under extensive study both in bacteria, for example, *Escherichia coli* (9–11), *Klebsiella oxytoca* (11), or *Zymomonas mobilis* (12) and yeasts, for example, *Saccharomyces cerevisiae* (13–15), *Pichia stipitis* (16), or *Kluyveromyces marxianus* (17,18). Unfortunately, much of the data concerning the effect of hydrolysis byproducts on bioethanol production do not fully apply to xylitol bioproduction, not only because different microorganisms are involved and tolerance is strain specific, but also owing to both the hydrolysates and operational conditions are relatively different between the two processes.

The potential inhibitors present in hemicellulosic hydrolysates can be divided into three major categories, aliphatic acids (e.g., acetic and formic acid), phenolic compounds (mostly with low molecular weight) and furan derivatives (furfural and HMF). The xylitol bioprocess requires concentrated hydrolysates because xylitol production is favored at high xylose concentrations. Therefore, a concentration (e.g., evaporation) step before the fermentation is usually performed (8). This step may also act as a detoxification process and it is particularly effective in the reduction of furan derivatives levels (19,20). Although phenolic compounds and, at some extent, aliphatic acids may also be partially evaporated, they usually have their levels increased thus becoming, in quantitative terms, the most important inhibitors present in concentrated hydrolysates.

The study of the inhibitors impact in the xylitol production is usually achieved using two approaches, experiments carried out in hydrolysate media, comparing between hydrolysates with different degrees/processes of detoxification (19,21–23) and experiments carried out in synthetic media using single inhibitors selected from the mentioned categories (4,24–26). The first approach is by far the most used and gives relevant information relating the technological impact of the inhibitors. The second approach is more useful to quantify and understand the specific impact of the inhibitors on the microbial physiology. However, it is generally accepted the assumption that the toxicity also depends on the interaction of the inhibitors (8,20), as it has been established for ethanol production (10,15,17,18). Nevertheless, this has not yet been clearly shown for the xylitol bioproduction.

To identify the possible combination effects, a multivariate statistical approach is the best strategy because it enables to easily estimate both the main and the interaction effects (27). Among the many possibilities, we applied a modified central composite design, that, in spite of some drawbacks (e.g., it is a nonorthogonal and nonrotatable design), has already been successfully applied to study the inhibitor interaction problem for the bioethanol process.

In this work, we studied the interaction effects induced by selected hydrolysis byproducts in *D. hansenii* semi-aerobic growth and metabolism. The compounds were chosen to be representative of the potential microbial

inhibitors usually present in concentrated hydrolysates, namely, from the aliphatic acids, acetic, and formic acid, and from the phenolic compounds, hydroquinone. Their maximal concentrations were chosen based on the range of concentrations previously found in hemicellulosic hydrolysates.

Methods

Microorganism and Growth Conditions

Microorganism

D. hansenii CCMI 941 *(28)* obtained from Colecção de Culturas de Microrganismos Industriais (CCMI, Portugal), was used in all experiments. To increase inocula reproducibility a yeast stock culture was prepared as follows: a 24 h grown slant was inoculated in a synthetic medium *(26)* containing 10 g/L of xylose as sole carbon and energy source and incubated in the same conditions as described as follows. At late exponential growth phase sterile glycerol was added to a final concentration of 15% (vol/vol). 5 mL aliquots were then preserved in sterile vials at –70°C.

Media

Complete chemically defined media were used in all experiments. Inoculum medium contained 5 g/L D-glucose, 15 g/L D-xylose, and 5 g/L L-arabinose, as carbon sources and no inhibitors, as described elsewhere *(26)*.

In order to determine the main and interaction effects induced by acetic acid, hydroquinone, and formic acid, a central composite design *(15,18)* was used. The codified values for the inhibitor concentrations used are shown in Table 1. The experimental domain range between 0 and 6 g/L, 3 g/L and 4.6 g/L for acetic acid (Merck, Darmstadt, Germany), hydroquinone (Sigma Chemical Co., St. Louis, MO), and formic acid (Riedel-de Haën, Seelze, Germany), respectively.

This design was extended to incorporate replicates for the reference fermentations (fermentations without inhibitors) in each block, and also replicates of the central condition.

The growth media sugar composition aims to simulate the composition of concentrated pentoses-rich hemicellulosic acid hydrolysates. The reference medium contained per liter: 20 g D-glucose, 60 g D-xylose, 20 g L-arabinose, 24 g $(NH_4)H_2PO_4$, 8 g $(NH_4)_2HPO_4$, 4 g KH_2PO_4, and trace elements and vitamins in a double concentration of that described previously *(26)*. The phosphate sources were slightly modified *(26)* in order to increase the medium buffering capacity. Media pH was set to 5.5 before filter sterilization with 0.22 μm Gelman membrane filters (Ann Arbor, MI). To obtain the initial concentrations of inhibitors established by the experimental design, three similar media, supplemented either with 15 g/L acetic acid, 7.5 g/L hydroquinone, or 17 g/L sodium formate (11.5 g/L formic acid), freshly

Table 1
Codified Levels for the Modified Central Composite Design for the Factors Acetic Acid (HAc), Hydroquinone (HQ), and Formic Acid (HF)

		Factors		
Block	Experiment	HAc	HQ	HF
	1	–1	–1	–1
	2	–1	–1	–1
	3	–0.6	–0.6	–0.6
	4	0.6	–0.6	–0.6
	5	–0.6	–0.6	0.6
I	6	0.6	–0.6	0.6
	7	–0.6	0.6	–0.6
	8	0.6	0.6	–0.6
	9	–0.6	0.6	0.6
	10	0.6	0.6	0.6
	11	0	0	0
	1	–1	–1	–1
	2	–1	–1	–1
	3	–1	0	0
	4	1	0	0
	5	0	0	–1
II	6	0	0	1
	7	0	–1	0
	8	0	1	0
	9	0	0	0
	10	0	0	0
	11	0	0	0

prepared, were mixed with the reference medium in appropriated volumes under sterile conditions.

Growth Conditions

The inoculum was prepared in 1000-mL baffled Erlenmeyer flasks capped with cotton wool stoppers containing 100 mL of inoculum medium and incubated in an Infors® Unitron (Bottmingen, Switzerland) orbital incubator set at 30°C and 150 rpm. Each flask was seeded with 0.5 mL of yeast stock suspension. After 17 h the culture was centrifuged at 8600*g* under sterile conditions in a Sartorius Sigma 2-16K centrifuge (Götingen, Germany). The cell mass was used to inoculate 55 mL of the different media to an initial cell dry weight of about 2.5 g/L. The experiments were carried out aerobically in 500-mL Erlenmeyer flasks also capped with cotton wool stoppers and incubated in the same conditions as the inoculum. The yeast growth was followed for 168 h for each condition established by the modified central composite design. Samples were taken at regular intervals. Experiments within the blocks were carried out simultaneously.

Analytical Methods

D-xylose, D-glucose, L-arabinose, formic and acetic acids, ethanol, and hydroquinone were analyzed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column from Bio-Rad (Hercules, CA) in the same conditions and equipment described previously *(26)*. Owing to the partial overlap of arabinose, xylitol, and arabitol, samples were also analyzed by HPLC using a Waters Sugar Pak 1 column (Millfort, MA) as described earlier *(26)*. All samples were filtered by 0.45 μm Gelman membrane filters before analysis.

Biomass dry weight was quantified gravimetrically by centrifugation of 2 mL of culture broth at 20,600*g* in a Sartorius Sigma 2-16K centrifuge (Göttingen, Germany), using predried Eppendorf tubes. The resulting cell pellet was washed with an equal amount of filtered deionized water and dried overnight at 100°C to constant weight.

Calculations and Statistical Analysis

The volumetric substrate consumption rates (g/[Lh]), were based on grams of substrate consumed per liter of culture medium/h. The biomass and product productivities (g/[Lh]), were based on grams of biomass or product produced/liter of culture medium/h. The biomass and product yield (g/g), were calculated as the ratio between the productivity and the volumetric consumption rate for the relevant substrates. The values were calculated for the duration of maximum relevant monosaccharide consumption rate, i.e., parameters related to glycerol, ethanol, and biomass were calculated for the sample with higher glucose consumption rate. For xylitol and arabitol parameters the calculations were performed for the sample with higher xylose and arabitol consumption rate, respectively. The specific growth rate (μ, h^{-1}) was calculated by linear regression of the $\ln(OD/OD_i)$ vs time for the exponential growth phase.

In order to describe the influence of the factors on the dependent variables (the response variables), a second order polynomial model was used.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2 + \beta_{123} x_1 x_2 x_3 + \varepsilon \quad (1)$$

where Y is the response, the subscripts 1, 2, and 3 are referred to acetic acid, hydroquinone, and formic acid, respectively), β_0 is the specific intercept; x_1, x_2, and x_3 are the concentrations (g/L) of the factors 1, 2, and 3, respectively; β_1, β_2, and β_3 are the coefficients of the factors 1, 2, and 3, respectively; β_{12}, β_{13}, and β_{23} are the coefficients for the interactions between the factors 1 and 2; 1 and 3; and 2 and 3, respectively; β_{11}, β_{22}, and β_{33} are the coefficients for the quadratic effects of the factors 1, 2, and 3, respectively; and β_{123} is the coefficient for the interaction between the factors 1, 2, and 3; ε are independent random errors, assumed to be normally and independently distributed with expectation 0 and variance σ^2. The model was built on the assumption

that there is no interaction between the regressor variables and the block effects. This means that all parameters are common to both blocks. Because the design used is not orthogonal, the regression model was fitted by the forward stepwise procedure implemented in STATISTICA (data analysis software system, version 6) from StatSoft, Inc. (2002).

Results and Discussion

Fermentation Kinetics of D. hansenii

Hemicellulosic hydrolysates used for xylitol production contain a mixture of hexoses and pentoses, which induce quite different microbial performances as in comparison with single substrate cultures *(29–32)*. Therefore, a synthetic culture medium that simulates the hydrolysate sugars composition must be used to investigate the effect of the inhibitor compounds. Otherwise, the true inhibitors impact on the monosaccharides metabolism might be unnoticed or misapprehended.

As examples of the fermentation kinetics obtained, the biomass, pH, monosaccharides, and metabolic product profiles during the fermentation time course for the reference medium and the medium containing all inhibitors in their central concentrations are presented in Figs. 1 and 2, respectively. Because no significant differences between the replicates were found, only the results for one of the assays is presented.

In the reference condition (Fig. 1), glucose was the preferred monosaccharide, being depleted from the culture medium in less than 24 h. Biomass productivity is high during glucose metabolism and after its depletion significantly decreases. As a consequence of glucose metabolism, ethanol, and some glycerol were produced, together with residual amounts (up to 0.4 g/L) of acetic acid. These products are typical for sub-optimum glucose metabolism and after glucose depletion, they were consumed by the yeast together with xylose *(33)*. Xylose utilization began after a short lag phase and it is consumed simultaneously with glucose, albeit at a slower rate. Xylose consumption induced the production of xylitol, arabitol, and residual amounts of acetate. Xylitol was the main product, presenting an higher production rate, and its accumulation occurs up to xylose depletion. Arabinose consumption slowly starts after xylose concentration decreases to ca. 10 g/L but increased substantially after xylose depletion. During late arabinose consumption, xylitol was also consumed. During this period, arabitol production rate increased being accumulated up to arabinose depletion, after which it is also metabolized.

In the medium containing inhibitors (Fig. 2), the overall monosaccharides consumption and products formation profiles were similar to the reference fermentations. Nevertheless, some specific changes occurred. Glucose and xylose consumption rates decreased, but arabinose consumption rate increased. The biomass and the broth pH profiles were considerably different to the reference ones. Growth rate was slower, although growth

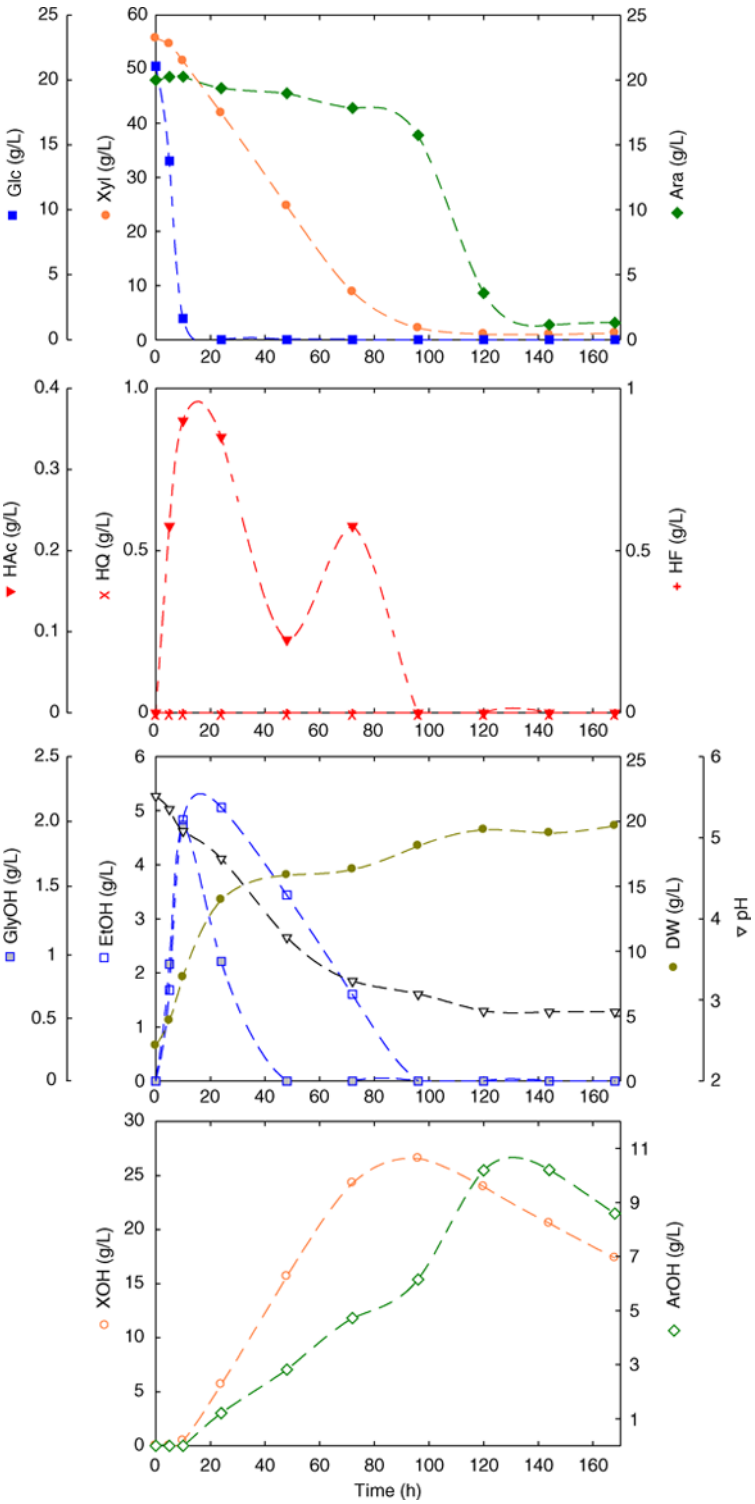

Fig. 1. Time-course of pH, biomass, monosaccharides, and metabolic products during the growth of *D. hansenii* CCMI 941 in the reference medium (Exp. I.1).

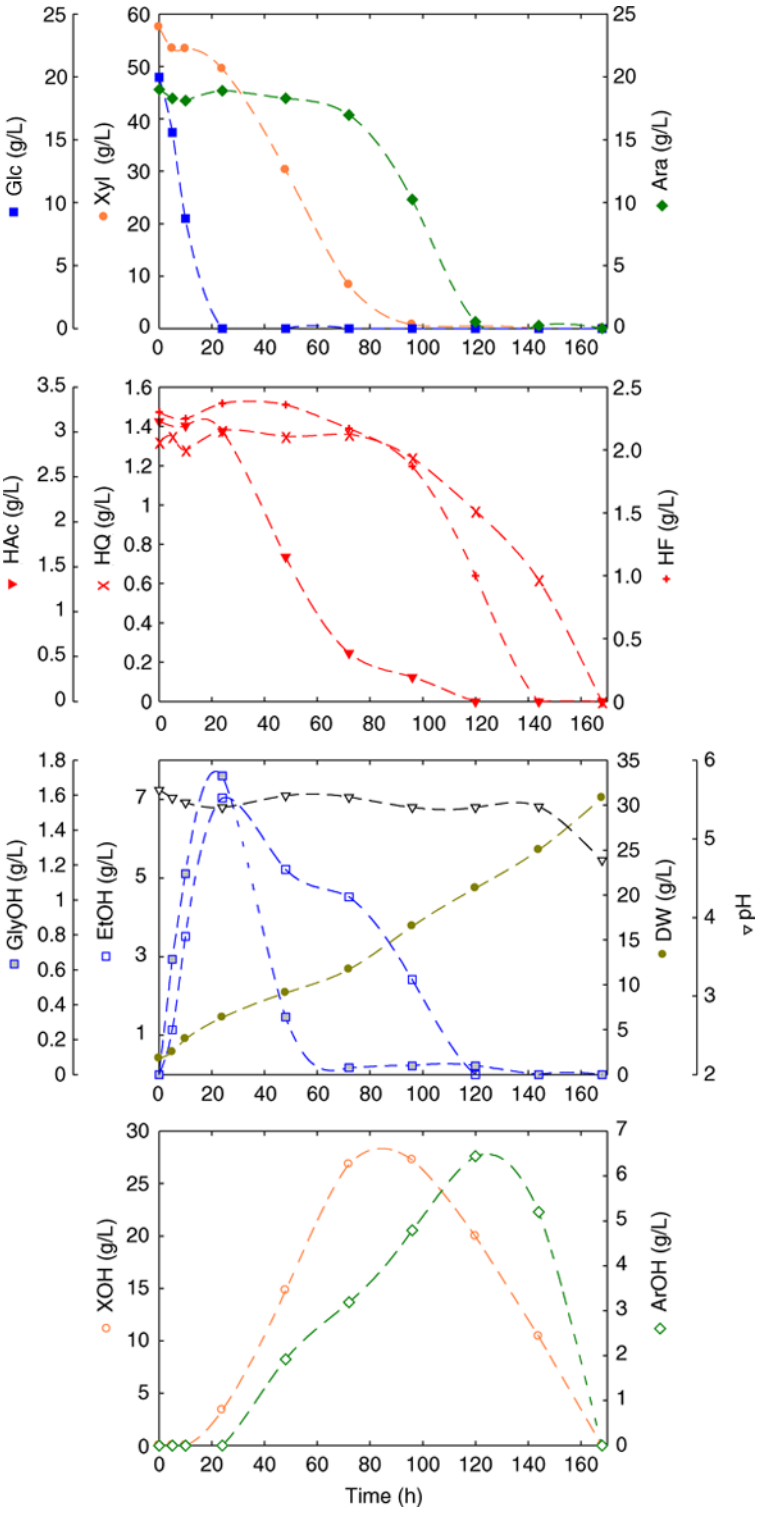
Glc (g/L)
Xyl (g/L)
Ara (g/L)
HAc (g/L)
HQ (g/L)
HF (g/L)
GlyOH (g/L)
EtOH (g/L)
DW (g/L)
pH
XOH (g/L)
ArOH (g/L)
Time (h)

Fig. 2.

was sustained for a longer period, so that final biomass concentrations were higher than in the reference fermentation. This may be a consequence of the broth pH, that does not decrease so sharply, being maintained in values greater than 4.5 for the entire experiment.

After glucose depletion, acetic acid was consumed simultaneously with xylose. Formic acid consumption began by the end of acetic acid consumption, and its consumption rate increased after acetic acid depletion. Hydroquinone was metabolized simultaneously with formic acid, but at a lower rate, being the last inhibitor to be depleted.

These kinetic profiles show similar trends as described for this yeast when grown in brewery's spent grains hemicellulosic hydrolysates *(19,33)*.

The overall results for the modified central composite design as a function of the real inhibitor concentrations used in each assay are summarized in Table 2, in which the calculated monosaccharide and inhibitor consumption rates, product productivities and yields are presented, together with the specific growth rate for exponential phase and the broth pH at the maximum xylitol concentration.

Regardless the concentration, the inhibitors always induced a marked effect on the initial fermentation period, reducing the glucose metabolism and the rates of glucose consumption (Q_{glc}), product formation (Q_{glyOH}, Q_{etOH}), and the carbon assimilation into biomass (μ, Q_x, Y_x), clearly indicating a change in the carbon flux to maintenance processes. Regarding pentose metabolism, the inhibitor effects on xylose consumption are more complex. Although some mixtures did not seem to affect, (some even favor), xylose consumption, the majority tends to reduce it. Both biomass formation and yield from xylose were also decreased. Conversely, a different trend was observed for xylitol production and yield, that are stimulated in many of the inhibitor mixtures assayed, clearly indicating that a carbon flux shift from biomass to xylitol production occured. Similar effects, although not so marked, can also be found for arabinose and arabitol metabolism. Because arabitol is also produced during xylose metabolism, the reported values for arabitol yield from arabinose may have been slightly overestimated. The inhibitor consumption rates were much lower than monosaccharide consumption rates. Amongst the inhibitors, acetic acid always displayed the highest consumption rate and hydroquinone the lowest.

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As stated earlier no significant differences were found among the replicates, both in the reference fermentations and in the central point.

Fig. 2. *(Opposite page)* Time-course of pH, biomass, monosaccharides, and metabolic products during the growth of *D. hansenii* CCMI 941 in a medium containing the central concentrations of all inhibitors (Exp. II.10).

Table 2
Experimental Matrix and Kinetic and Stoichiometric Parameters for *D. hansenii* Growth in Synthetic Media According to the Modified Central Composite Design[a]

		Factors			Responses																		
		HAc	HQ	HF	μ	pH	Q_{glc}	Q_{xyl}	Q_{ara}	Q_x	Q_{glyOH}	Q_{etOH}	Q_{xOH}	Q_{x_xOH}	Q_{arOH}	Q_{HAc}	Q_{HQ}	Q_{HF}	Y_x	Y_{glyOH}	Y_{etOH}	Y_{xOH}	Y_{x_xOH}	Y_{arOH}
Block	Exp.	(g/L)			(h^{-1})		(g/[Lh])												(g/g)					
	1	0.00	0.00	0.00	0.14	3.23	1.94	0.65	0.13	0.53	0.20	0.48	0.34	0.19	0.07[b]	0.00	0.00	0.00	0.27	0.10	0.25	0.52	0.19	0.54
	2	0.00	0.00	0.00	0.14	3.16	2.00	0.66	0.03[b]	0.64	0.21	0.51	0.34	0.20	0.05	0.00	0.00	0.00	0.32	0.11	0.26	0.53	0.21	1.79[b]
	3	1.11	0.64	0.97	0.12	4.71	1.72	0.68	0.16	0.38	0.16	0.45	0.41	0.19	0.10	0.01	0.00	0.01	0.22	0.09	0.26	0.60	0.20	0.62
	4	5.02	0.64	0.96	0.07	5.47	1.53	0.67	0.16	0.17	0.07	0.30	0.32	0.13	0.10	0.05	0.00	0.01	0.11	0.05	0.19	0.48	0.13	0.63
	5	1.29	0.64	3.50	0.11	5.33	1.72	0.59	0.16	0.28	0.12	0.41	0.35	0.15	0.10	0.01	0.00	0.02	0.16	0.07	0.24	0.60	0.17	0.61
I	6	5.10	0.65	3.55	0.06	5.67	0.92	0.56	0.15	0.11	0.09	0.25	0.30	0.13	0.08	0.05	0.00	0.02	0.12	0.09	0.28	0.54	0.14	0.54
	7	1.26	2.40	0.97	0.09	4.99	1.63	0.72	0.15	0.20	0.14	0.51	0.45	0.17	0.03	0.02	0.01	0.01	0.12	0.09	0.31	0.62	0.14	0.21
	8	5.67	1.65	1.09	0.08	5.58	0.91	0.73	0.19	0.14	0.05	0.28	0.38	0.16	0.09	0.07	0.01	0.01	0.15	0.05	0.31	0.52	0.15	0.47
	9	1.31	2.37	3.40	0.08	5.19	1.16	0.62	0.13	0.14	0.10	0.34	0.34	0.11	0.03	0.01	0.00	0.02	0.12	0.09	0.29	0.54	0.12	0.22
	10	5.08	2.45	3.53	0.06	5.60	1.35[b]	0.55	0.13	0.13	0.10	0.40	0.25	0.09	0.04	0.04	0.01	0.02	0.10	0.08	0.30	0.49	0.10	0.29
	11	3.20	1.50	2.25	0.09	5.34	1.21	0.70	0.16	0.13[b]	0.09	0.32	0.40	0.14	0.07	0.04	0.01	0.02	0.11[b]	0.08	0.26	0.57	0.13	0.42
	1	0.00	0.00	0.00	0.15	3.15	1.81	0.71	0.13	0.51	0.24	0.57	0.31	0.22	0.05	0.00	0.00	0.00	0.28	0.13	0.32	0.43	0.22	0.41
	2	0.00	0.00	0.00	0.15	3.03	1.79	0.73	0.13	0.56	0.23	0.54	0.31	0.23	0.04	0.00	0.00	0.00	0.31	0.13	0.30	0.43	0.22	0.32
	3	0.00	1.69	2.85	0.10	5.07	1.53	0.79	0.15	0.34	0.18	0.45	0.39	0.16	0.05	0.00	0.01	0.02	0.22	0.11	0.29	0.49	0.12	0.30
	4	6.50	1.43	2.38	0.06	5.79	0.85	0.63	0.16	0.18	0.06	0.32	0.33	0.12	0.08	0.06	0.01	0.01	0.18	0.08	0.38	0.53	0.13	0.52
	5	3.76	1.68	0.00	0.07	5.56	1.46	0.91	0.27	0.24	0.16	0.38	0.46	0.19	0.07	0.06	0.01	0.00	0.16	0.11	0.26	0.50	0.13	0.32
II	6	3.14	1.38	4.57	0.08	5.71	0.89	0.59	0.13	0.17	0.10	0.29	0.34	0.14	0.06	0.03	0.01	0.03	0.17	0.11	0.33	0.57	0.16	0.35
	7	3.79	0.00	2.81	0.09	5.79	1.35	0.60	0.17	0.29	0.13	0.37	0.22	0.20	0.08	0.04	0.00	0.02	0.21	0.10	0.27	0.37	0.22	0.52
	8	3.03	2.74	2.21	0.05[b]	5.51	0.82	0.62	0.13	0.15	0.07	0.30	0.27	0.11	0.03	0.03	0.01	0.01	0.15	0.08	0.37	0.44	0.12	0.30
	9	3.17	1.44	2.31	0.09	5.49	1.17	0.71	0.15	0.20	0.13	0.37	0.38	0.15	0.05	0.04	0.01	0.02	0.17	0.11	0.32	0.54	0.15	0.33
	10	3.12	1.35	2.30	0.08	5.53	1.12	0.68	0.15	0.22	0.11	0.35	0.37	0.14	0.05	0.04	0.01	0.02	0.19	0.10	0.31	0.55	0.14	0.35
	11	3.17	1.42	2.35	0.08	5.51	1.12	0.68	0.15	0.21	0.12	0.36	0.35	0.15	0.07	0.04	0.01	0.02	0.19	0.11	0.32	0.51	0.16	0.47

[a]Values were calculated for the duration of maximum relevant monosaccharide consumption rate.

[b]Value was omitted from analysis.

μ, specific growth rate; pH, broth pH at the maximum xylitol concentration; Q_{glc}, volumetric glucose consumption rate; Q_{xyl}, volumetric xylose consumption rate; Q_{ara}, volumetric arabinose consumption rate; Q_x, volumetric biomass production rate; Q_{glyOH}, volumetric glycerol production rate; Q_{etOH}, volumetric ethanol production rate; Q_{xOH}, volumetric xylitol production rate; Q_{x_xOH}, volumetric biomass production rate at maximum xylitol productivity; Q_{arOH}, volumetric arabitol production rate; Q_{HAc}, volumetric acetic acid consumption rate; Q_{HQ}, volumetric hydroquinone consumption rate; Q_{HF}, volumetric formic acid consumption rate; Y_x, biomass yield on glucose; Y_{glyOH}, glycerol yield on glucose; Y_{xOH}, xylitol yield on xylose; Y_{x_xOH}, biomass yield on consumed monosaccharides at maximum xylitol productivity; Y_{arOH}, arabitol yield on arabinose.

Parameter values differ typically less than 10% independently of the block. Therefore, the statistical treatment was simplified by assuming that the intercept (β_0) is equal in all the blocks. There were minor differences between the values established by the design and the real inhibitor concentrations, but when using the correct natural variables *(15,18)* instead of the codified ones, the possible impacts that this may impose on the regression is negligible.

Statistical validation of the polynomial equations was made by analysis of variance. Model analysis by the coefficient of multiple determination (R^2) was also performed. Table 3 shows the regression results for the physiological parameters for which a significant regression could be found. ($p \leq 0.05$ and $R^2 > 0.8$). For simplicity, only statistically significant regression coefficients estimates ($p \leq 0.05$) are presented. The pure quadratic terms (β_{HAcHAc}, β_{HQHQ}, and β_{HFHF}), even if statistically significant, were omitted both from the table and the discussion. It is important to state that the forward stepwise procedure used for the regression is an iterative algorithm, that does not give unique definite solutions for the estimates. Thus, it is preferable to interpret the factors inclusion/exclusion from the model and their signal, but not their magnitude. For the 20 kinetic and stoichiometric parameters studied, only for arabitol productivity and product yields, it was not possible to identify significant effects of the assayed compounds, because it was not possible to find a significant regression model for these parameters. For those parameters for which it was possible to establish a model, a good agreement between experimental data for the reference fermentations (*see* Table 2) and the β_0 estimates was found, together with high correlation coefficients.

The inhibitory effects observed on the initial fermentation period can be partially explained by the presence of acetic acid. Essentially, this aliphatic acid negatively affected glucose consumption rate, specific growth rate, and product formation, both for products deriving from glucose metabolism and xylitol. Arabinose metabolism was not significantly affected, which may be explained by the fact that acetic acid is usually depleted when arabinose metabolism sets on. The negative impact of acetic acid on xylitol bioproduction in semisynthetic medium had already been described, although acetic acid could also improve both the xylitol yield and productivity for concentrations up to 1 g/L *(24)*, a value below the lower concentration assayed in this work. Recently, the acetic acid direct impact on intracellular xylose metabolism was evaluated *(25)* and it was found that it does not have a direct influence on the xylose metabolism specific enzymes. These, together with the results presented here, that suggest an unspecific cellular target for acetic acid, support the assumption that acetic acid inhibition occurs by means of a combined effect of a intracellular pH decrease and anions accumulation arising by the undissociated acid form diffusion across cell membrane *(34)*.

Table 3
Statistically Significant Regression Coefficients Estimates (± Standard Error) for the Polynomial Model for the Specific Growth Rate, Broth pH at Maximum Xylitol Productivity and Kinetic Parameters for *D. hansenii* Growth in Synthetic Media Containing Inhibitors According to the Modified Central Composite Design ($p \leq 0.05$)[a]

	β_0	β_{HAc}	β_{HQ}	β_{HF}	$\beta_{HAc\ HQ}$	$\beta_{HAc\ HF}$	$\beta_{HQ\ HF}$	$\beta_{HAc\ HQ\ HF}$	R^2
μ	0.149 ± 0.003	–0.018 ± 0.002	–0.042 ± 0.008		0.006 ± 0.001				0.98
pH	3.150 ± 0.118	0.454 ± 0.090	0.960 ± 0.236	0.511 ± 0.120				–0.042 ± 0.014	0.98
Q_{glc}	1.824 ± 0.054	–0.092 ± 0.024					–0.050 ± 0.022		0.89
Q_{xyl}	0.702 ± 0.026		0.197 ± 0.052	–0.078 ± 0.026				–0.006 ± 0.002	0.84
Q_{ara}	0.131 ± 0.008				0.015 ± 0.004			–0.007 ± 0.002	0.89
Q_x	0.538 ± 0.022	–0.082 ± 0.015	–0.109 ± 0.019	–0.018 ± 0.007					0.95
Q_{glyOH}	0.221 ± 0.013	–0.027 ± 0.004					–0.013 ± 0.004		0.86
Q_{etOH}	0.550 ± 0.015	–0.075 ± 0.011					–0.037 ± 0.009	0.007 ± 0.002	0.96
Q_{xOH}	0.312 ± 0.010	–0.020 ± 0.003	0.204 ± 0.020				–0.024 ± 0.004		0.95
Q_{x_xOH}	0.218 ± 0.010	–0.012 ± 0.003					–0.013 ± 0.003		0.81
Y_{x_xOH}	0.226 ± 0.009		–0.092 ± 0.019		0.009 ± 0.003				0.86
Q_{HAc}		0.013 ± 0.001			0.003 ± 0.001			–0.001 ± 0.000	0.99
Q_{HQ}			0.011 ± 0.002						0.87
Q_{HF}				0.009 ± 0.001					0.99

[a] β_{HAcHAc}, β_{HQHQ} and β_{HFHF}, even if statistically significant, were omitted both from the table and the discussion. Missing values correspond to statistically nonsignificant coefficients.

Formic acid had a quite diverse inhibitory pattern, negatively affecting xylose consumption and the initial biomass production. Conversely to previous reports *(26,35)*, in which formic acid was described to enhance xylitol production in chemically defined media and at similar initial pH, in this work, formic acid per se had no statistically significant effect on xylitol productivity. This may be owing to the observed dissociation between xylose and formic acid consumption, suggesting that formic acid effect on xylose consumption may be owing to an indirect effect of acid inhibition on biomass production. Furthermore, an unspecific cell uptake and its related effects in a similar way as described for acetic acid, does not seem to occur, because formic acid concentrations remained almost unchanged for long periods and only decreased after acetic acid depletion. This is probably owing to the lower concentrations of the undissociated formic acid form at the fermentation pH.

Both aliphatic acids had a positive impact on broth pH, which is probably a direct consequence of buffering capacities, as consequence of their weak acid nature. However, the coefficient for the acids interaction was not statistically significant to explain any response, given that this factor was never incorporated in the explanatory model for any parameter (Table 3). Hydroquinone inhibited both the specific growth rate and the initial biomass productivity and statistically it did not significantly influence glucose or arabinose metabolism. Conversely, for values around 1.5 g/L hydroquinone specifically increased both xylose consumption, and xylitol productivity. Hydroquinone and acetic acid interaction had a significant positive impact on growth, arabinose consumption, and biomass yield. The interactions between hydroquinone and formic acid had a negative impact on glucose metabolism and all productivities. Hydroquinone seems to partially lessen the acetic acid and amplify formic acid effects. The effects of phenolic compounds on xylitol production is still poorly known, being reported that a phenolic content reduction in hydrolysates could increase both xylitol yield and productivity *(20)*. On the contrary, their effect on yeast ethanol production from xylose has been studied and these compounds have been described to inhibit ethanol production, mainly owing to growth inhibition *(10)*. Nevertheless, some evidences have been reported that lower xylitol productivities and/or yields can be found for some detoxification treatments that exhibited a higher removal of such compounds *(19,36)*. The inhibitory mechanism of the phenolic compounds is considered to be related to a partition and loss of integrity of biological membranes, thereby affecting their ability to serve as selective barriers and enzyme matrices. However, these effects can be reverted if an energy source is available *(37)*. This may explain why xylose consumption increased, but not its assimilation (hydroquinone negatively affects biomass yield). The observed increase in xylitol production would then be a consequence of overflow metabolism occurring at limited oxygen conditions. Furthermore, this membrane dysfunction might be helpful to

diminish the stress induced by aliphatic acids. Formic acid and hydroquinone consumption are mainly dependent on their concentration. Conversely, acetic acid consumption rate is increased by hydroquinone, highlighting the possible mechanism involved in the positive interaction that hydroquinone exerts on lessen acetic acid inhibition.

Conclusions

Although the main effects induced by aliphatic acids and hydroquinone are important, the results obtained in this study highlight that the interactions among these compounds are essential to explain the overall performance, specifically the interactions involving hydroquinone. Furthermore, it was observed that the impacts induced by these compounds are not only in the induction of inhibitory effects. Remarkably, hydroquinone per se induced some positive effects, namely, on xylose consumption and xylitol production. Furthermore, hydroquinone and acetic acid combinations seem to limit the inhibitory impact of acetic acid. Finally, this work confirmed the existence of interaction effects between phenolic compounds and aliphatic acids, which overlay the need of further studies on the nature of the inhibitors interaction and on the microbial response mechanisms involved.

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