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Effects of Aliphatic Acids, Furfural, and Phenolic Compounds on *Debaryomyces hansenii* CCMI 941

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Abstract

Debaryomyces hansenii is a polyol overproducing yeast that can have a potential use for upgrading lignocellulosic hydrolysates. Therefore, the establishment of its tolerance to metabolic inhibitors found in hydrolysates is of major interest. We studied the effects of selected aliphatic acids, phenolic compounds, and furfural. Acetic acid favored biomass production for concentrations <6.0 g/L. Formic acid was more toxic than acetic acid and induced xylitol accumulation (maximum yield of 0.21 g/g of xylose). All tested phenolics strongly decreased the specific growth rate. Increased toxicity was found for hydroquinone, syringaldehyde, and 4-methylcatechol and was correlated to the compound's hydrophobicity. Increasing the amount of furfural led to longer lag phases and had a detrimental effect on specific growth rate and biomass productivity.

Index Entries: Lignocellulosic byproducts; acetic acid; formic acid; hydroquinone; syringaldehyde; 4-methylcatechol; furfural; inhibition.

Introduction

The chemical hydrolysis of lignocellulosic materials to obtain monosaccharide-rich fermentable hydrolysates always coproduces many different byproducts. Their concentration in the hydrolysates depends on the raw material, but mostly on the extent of the hydrolysis and possibly on the detoxification methods applied. Careful optimization of both the hydrolysis and detoxification conditions may reduce the amount of byproducts but does not eliminate them all. Therefore, their impact on any biologic process must always be assessed, because they may have a significant effect on bioprocess performance. These effects are well studied for bioethanol production using both bacteria *(1–4)* and yeast (e.g., see refs. *5–9)*, but less is known about their effects for other

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biological systems, such as on the growth and metabolism of xylitol-producing yeasts.

The impact of these compounds on microbial metabolism depends on several factors, including the overall chemical composition of the hydrolysate and cultivation conditions, such as initial biomass concentration, pH, temperature, and oxygen availability, but one of the most important is the nature of the microorganism and its intrinsic characteristics *(10)*. Consequently, many of the data concerning the effect of hydrolysis byproducts on bioethanol production do not fully apply to xylitol production. For example, some evidence has been accumulating that lower xylitol productivities and/or yields can be found for some treatments that exhibit a higher removal of inhibitors *(11–13)*. Furthermore, some compounds generally recognized as inhibitors may have a positive effect on xylitol bioproduction. This is the case for formic acid, which was reported to enhance xylitol production by *Candida tropicalis* grown in continuous culture on a chemically defined medium *(14)*.

In the present work, we studied the effects induced by selected hydrolysis byproducts on the aerobic growth and metabolism of *Debaryomyces hansenii*. This yeast naturally assimilates pentoses and overproduces xylitol *(15–17)*. The compounds were chosen to be representative of the major categories of potential microbial inhibitor compounds usually present in hydrolysates, namely aliphatic acids (acetic and formic acid), furan derivatives (furfural), and phenolic compounds (hydroquinone, syringaldehyde, and 4-methylcatechol).

Materials and Methods

Microorganism

D. hansenii CCMI 941 *(18)* was used in all experiments. The yeast was maintained on YM agar slants containing 20 g/L of glucose, 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 20 g/L of agar.

Media

Chemically defined media were used in all experiments. The monosaccharides content in the culture media was chosen to simulate the composition of pentose-rich hemicellulosic acid hydrolysates. The reference medium contained: 5 g/L of D-glucose, 15 g/L of D-xylose, 5 g/L of L-arabinose, 12 g/L of $(NH_4)H_2PO_4$, 4 g/L of $(NH_4)_2HPO_4$, 2 g/L of KH_2PO_4, 0.05 g/L of $CaCl_2 \cdot 2H_2O$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.188 g/L of Na_2-EDTA, 35 mg/L of $FeSO_4 \cdot 7H_2O$, 7 mg/L of $MnSO_4 \cdot H_2O$, 11 mg/L of $ZnSO_4 \cdot 7H_2O$, 1 mg/L of $CuSO_4 \cdot 5H_2O$, 2 mg/L of $CoCl_2 \cdot 6H_2O$, 1.3 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 2 mg/L of H_3BO_3, 0.3 mg/L of NaI, 0.1 g/L of myo-inositol, 20 mg/L of calcium pantothenate, 5 mg/L of thiamine hydrochloride, 5 mg/L of pyridoxal hydrochloride, 5 mg/L of nicotinic

acid, 1 mg/L of 4-aminobenzoic acid, and 0.1 mg/L of D-biotin. When required, acetic acid (Merck, Darmstadt, Germany), sodium formate (Riedel-de Haën, Seelze, Germany), 4-methylcatechol (Sigma, St. Louis, MO), syringaldehyde (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), hydroquinone (Sigma), or furfural (Sigma-Aldrich Chemie, GmbH) was added to the reference medium at appropriate concentrations. The pH of the media was set to 5.5 before filter sterilization using 0.22-µm Gelman membrane filters (Pall, Ann Arbor, MI).

Growth Conditions

A 24-h-grown YM slant was used to seed 100 mL of reference medium in a 1000-mL baffled Erlenmeyer flask capped with a cotton wool stopper. After 17 h, 2.5 mL of this culture was used to seed a similar flask containing the different test media. Initial cell dry weight concentration was about 0.4 g/L. All cultures were grown aerobically in an Infors HT® Unitron (Bottmingen, Switzerland) orbital incubator set at 30°C and 150 rpm. All assays were done at least in duplicate.

Analytical Methods

D-xylose, D-glucose, L-arabinose, formic and acetic acids, ethanol, furfural, furfuryl alcohol, and furoic acid were analyzed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column from Bio-Rad (Hercules, CA). The HPLC system was a Waters LC1 module l plus (Millford, MA) equipped with both a refractive index and an ultraviolet detector set at 280 nm. The mobile phase was 5 m*M* H_2SO_4, the column temperature was 50°C, and the flow rate was 0.4 mL/min. The system was equipped with a Micro-Guard Cation-H Refill Cartridge from Bio-Rad prior to the HPX-87H column. Owing to the partial overlap of arabinose, xylitol, and arabitol, samples were also analyzed by HPLC using a Waters Sugar Pak 1 column. The column was used with a Merck Hitachi HPLC system (Tokyo, Japan) equipped with a refractive index detector (L-7490). The mobile phase was 50 mg/L of calcium EDTA, the column temperature was 90°C, and the flow rate was 0.5 mL/min. Because both methods do not distinguish between D- and L-arabitol, the latter was used as arabitol standard. All samples were filtered through 0.45-µm Gelman membrane filters prior to analysis.

Phenolic compounds were quantified spectrophotometrically utilizing a modification of the Prussian blue method as described in ref. *19* using an LKB Biochrom Ultrospec II spectrophotometer (Cambridge, UK). The specific phenolic compounds assayed (hydroquinone, syringaldehyde, and 4-methylcatechol) were used as calibration standards.

Cellular dry weight was quantified gravimetrically by filtering of 5 mL of culture broth through 0.45-µm Gelman membrane filters, washing with an equal volume of water, and drying overnight at 100°C to constant weight.

Results

Hexose and pentose mixtures induce quite different microbial performances as compared to single-substrate cultures *(20–22)*. Therefore, to better investigate the effect of inhibitor compounds produced during chemical hydrolysis of lignocellulosic materials on yeast metabolism, a culture medium that simulates the hydrolysate's composition, especially addressing monosaccharide composition, should preferably be used.

Figure 1 depicts the time course of biomass and monosaccharide concentration during the growth of *D. hansenii* CCMI 941 in the reference medium. Glucose was the preferred monosaccharide, being rapidly consumed from the culture medium in about 8 h. Xylose assimilation began before glucose depletion, albeit at a slower rate. Additionally, before the total consumption of xylose, arabinose assimilation slowly started, but at the end of the fermentation most of the arabinose was still present (65%).

As expected for oxygen-sufficient conditions, no other product besides biomass and carbon dioxide was found. After a short lag phase (approx 1.3 h), the culture reached the exponential phase, exhibiting a specific growth rate of 0.39 h^{-1}, that decreased after glucose depletion. Xylose was not able to sustain such a high specific growth rate, and also the culture pH sharply decreased after 8 h, reaching a value of 2.9 after 15 h of incubation (Fig. 1). The overall biomass productivity was high (0.38 g/[L·h]), as well as the yield (0.43 g/g).

Effects of Aliphatic Acids

Table 1 shows the effects of acetic and formic acids on monosaccharide assimilation and on the kinetic and stoichiometric parameters of *D. hansenii* growth. Aliphatic acid toxicity is related to the concentration of the undissociated form, and initial acid concentrations are presented for this form, to give a better comparison between the two acids. Those concentrations were estimated using the Hendersson-Hasselbach equation, based on initial pH and pK_a values of the acids; 4.8 and 3.8, respectively, for acetic and formic acids (*2*).

Acetic acid is the major aliphatic acid present in hemicellulosic hydrolysates. Its concentration depends mainly on the composition of feedstock material and on the hydrolysis treatments applied. In nonconcentrated hydrolysates, it can vary in a wide range, between 0.6 *(23)* and 11 g/L *(24,25)*, although values between 1.5 and 9 g/L are most common, and was the range studied in the present work.

At 25 m*M* undissociated acetic acid (9 g/L) no growth occurred, and the data are omitted from Table 1. For the acid concentrations enabling growth, the duration of lag phase was not significantly increased in relation to the reference culture. The specific growth rate decreased for all concentrations tested and for the highest acid concentration reached 75%

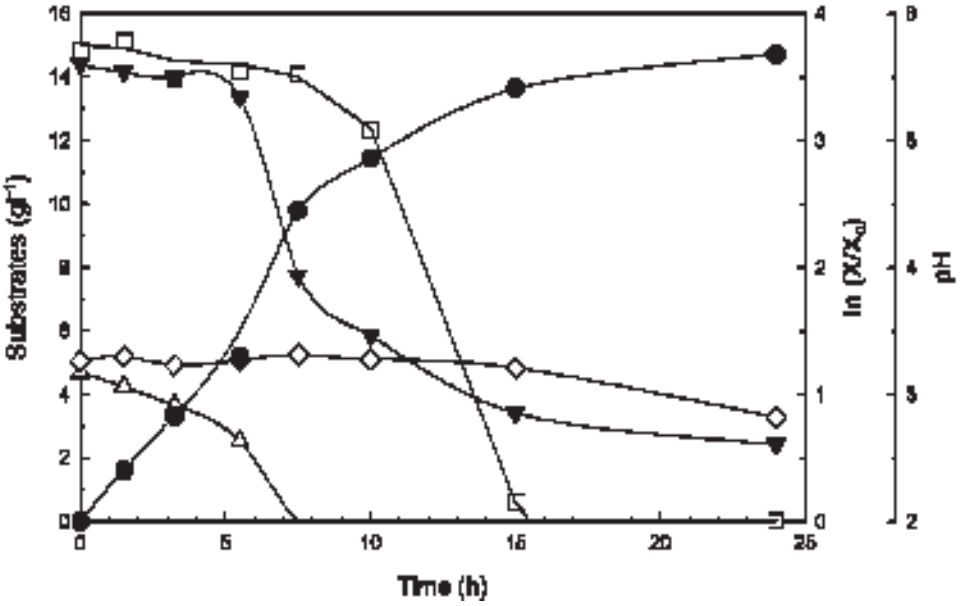

Fig. 1. Time course of pH, and biomass and monosaccharide concentrations during growth of *D. hansenii* CCMI 941 in reference culture: (Δ) glucose; (❑) xylose; (◊) arabinose; (●) biomass; (▼) pH.

of the value obtained in the reference cultivation. Acetic acid was always totally consumed. Arabinose assimilation increased considerably compared to growth in the absence of inhibitors (2.9-fold higher at 8.32 m*M*), and biomass yield and productivity were also enhanced by 35 and 50%, respectively. Another noteworthy difference induced by acetic acid is the evolution of the broth pH. Although dependent on acid concentration, pH always remained close to 5.5 for longer periods than in the reference cultivation (data not shown). Final pH increased with acid concentrations, reaching 6.2 for a concentration of 16.63 m*M* undissociated acid.

Formic acid is a product of furan compounds degradation. In general, its concentration is lower than for acetic acid and does not exceed 1.7 g/L *(26,27)*. Nevertheless, hydrolysates obtained in harsher conditions can present a quite different pattern: formic acid concentrations can reach 10 g/L, exceeding acetic acid content *(5)*.

In the present work, the effects of formic acid were studied on the broader range, the same used for acetic acid, in order to compare easily the two aliphatic acids; the results are presented in Table 1. Growth was possible in the entire range studied. Higher concentrations of formic acid induced longer lag phases, reaching 4 h at the highest concentration assayed. Specific growth rate decreased significantly with increased concentrations. Biomass productivity was slightly enhanced for the lower concentration assayed, but for concentrations above 1.91 m*M* a steady

Table 1
Effects of Acetic and Formic Acids, Furfural, and Selected Phenolic Compounds on Physiology of *D. hansenii*[a].

Category	Compound	Concentration (g/L)	Inhibitor consumption (%)[b]	Assimilation			λ/λ_r	μ/μ_r	Q_x/Q_{xr}	Y_x/Y_{xr}
				Glc/Glc_r	Xyl/Xyl_r	Ara/Ara_r				
Aliphatic acids	Acetic acid	1.5 (4.16)[c]	100	1.0	1.0	2.6	0.8	0.83	1.32	1.05
		3.0 (8.32)[c]	100	1.0	1.0	2.9	1.2	0.93	1.51	1.24
		6.0 (16.63)[c]	100	1.0	1.0	2.0	1.6	0.75	1.49	1.35
	Formic acid	1.5 (0.64)[c]	100	1.0	1.0	2.8	—	0.80	1.20	1.00
		3.0 (1.28)[c]	90	1.0	1.0	2.7	0.9	0.78	1.04	0.88
		4.5 (1.91)[c]	73	1.0	1.0	1.6	0.9	0.73	0.88	0.80
		6.0 (2.55)[c]	55	1.0	0.9	1.6	1.3	0.74	0.80	0.77
		7.5 (3.19)[c]	53	1.0	0.8	0.6	2.6	0.68	0.66	0.73
		9.0 (3.83)[c]	48	1.0	0.6	0.4	3.0	0.47	0.51	0.73
Phenolic compounds	Hydroquinone	0.25	98	1.0	1.0	0.8	0.9	0.87	0.90	0.92
		0.50	99	1.0	0.9	0.6	1.0	0.78	0.88	0.98
		1.00	99	1.0	0.8	0.4	1.4	0.79	0.86	0.98
		1.50	100	1.0	0.6	0.2	1.7	0.80	0.77	1.07
		2.00	76	1.0	0.6	0.0	2.2	0.79	0.67	1.05
		3.00	24	1.0	0.4	0.0	2.6	0.75	0.54	1.07
	Syringaldehyde	0.25	n.m.	1.0	1.0	0.8	1.6	0.75	0.97	0.89
		0.50	n.m.	1.0	1.0	0.8	1.6	0.68	0.95	0.89
		0.75	n.m.	1.0	1.0	0.5	1.5	0.58	0.9	0.84
		1.00	n.m.	1.0	0.9	0.5	1.4	0.43	0.87	0.90
		1.50	n.m.	1.0	0.8	0.3	1.2	0.20	0.63	0.73
	4-Methylcatechol	0.25	60	1.0	0.9	0.6	0.6	0.68	0.99	1.05
		0.50	n.a.	1.0	0.7	0.1	1.4	0.54	0.74	0.97
		0.75	28	1.0	0.2	0.0	0.4	0.35	0.34	0.88
		1.00	n.a.	0.5	0.2	0.0	2.4	0.26	0.10	0.38
Furan derivatives	Furfural	0.50	96	1.0	0.6	1.0	1.8	0.78	0.45	0.61
		1.00	93	1.0	0.5	0.8	3.9	0.74	0.41	0.58
		1.50	99	1.0	0.5	0.8	5.3	0.67	0.43	0.62
		2.00	100	1.0	0.2	0.1	9.7	0.70	0.41	1.05

[a]λ, duration of lag phase; μ, specific growth rate; Q_x, biomass productivity (calculated after 24-h cultivation time); Y_x biomass yield (calculated at 24-h cultivation time, based on total consumed sugars). Subscript $_r$ denotes values obtained in the reference cultivation.

[b]n.a., not available; n.m., not measurable.

[c]Corresponding concentration (m*M*) of undissociated acid.

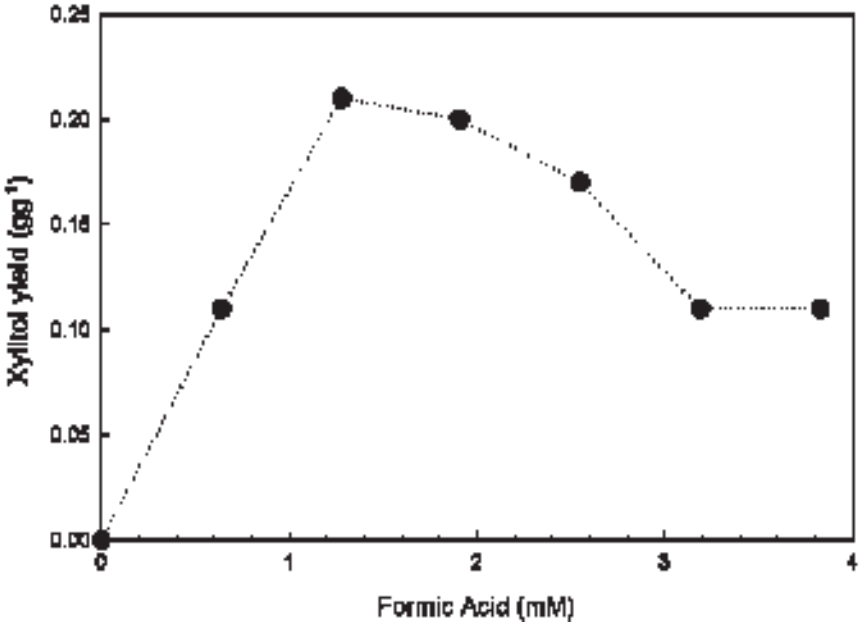

Fig. 2. Xylitol yield as function of undissociated formic acid concentration. The values were calculated when xylitol attained its maximal concentration.

decrease was found. Converse to what was found for acetic acid, for formic acid concentrations >0.64 m*M*, significant amounts of acid were still present in the culture after 24 h. For concentrations <1.91 m*M*, an increase in arabinose assimilation occurred, but for concentrations > 3.19 m*M*, pentoses assimilation was limited. Although the cultures were carried out in aerobic conditions, *D. hansenii* produced xylitol, reaching a concentration of 2.96 g/L in the presence 1.28 m*M* of the undissociated form, corresponding to a xylitol yield of 0.21 g/g (Fig. 2). As occurred for acetic acid, broth pH remained close to 5.5 for longer incubation periods (about 15 h). Final fermentation pH also increased for higher initial acid concentrations, ranging from 4.3 (0.64 m*M*) to 6.8 at 3.19 and 3.83 m*M* (data not shown).

Effects of Phenolic Compounds

During the acid hydrolysis of lignocellulosic materials, a partial degradation of lignin usually occurs, which together with the degradation of carbohydrates generates a wide range of phenolic compounds each one usually present at low concentrations *(5,7,29)*. In our study, hydroquinone, syringaldehyde, and 4-methylcatechol were selected as representatives of such phenolic compounds and studied separately up to 3 g/L, which is close to the higher concentration range of total phenolic compounds reported for hydrolysates *(3)*. The effects of these compounds on yeast growth and metabolism are presented in Table 1.

Hydroquinone enabled growth for all concentrations assayed, but that was not the case for syringaldehyde and 4-methylcatechol, for which the higher concentrations enabling growth were 1.5 and 1.0 g/L, respectively.

The duration of the lag phase was not significantly increased by any of the compounds, reaching, at most, approx 3 h for hydroquinone and 4-methylcatechol at 3 and 1 g/L, respectively. The specific growth rate decreased linearly with the increasing concentrations of syringaldehyde and 4-methylcatechol. Hydroquinone at concentrations of 0.25 and 0.50 g/L induced a decrease in specific growth rate, but further increases in concentration had no effect.

A negative correlation for biomass productivity and concentration was found for all phenolics tested. All the compounds induced a minor pentose assimilation compared to reference conditions, whereas glucose assimilation was only affected at 1.0 g/L of 4-methylcatechol. Biomass yield was not significantly influenced by hydroquinone, whereas higher concentrations of the other phenolic compounds decreased it.

Effects of Furfural

Furfural, the dehydration product of pentoses, is commonly found in hemicellulosic hydrolysates, and depending on the raw material, it can be the major furan derivative present. In nonconcentrated hydrolysates it can reach about 4 g/L *(25)*, although values up to 2 g/L are more common *(28)*. In our work concentrations in the range of 0.5–5 g/L were assayed.

The effects of furfural on yeast growth and metabolism are given in Table 1. For cases of 3.5 g/L and above no growth was observed until 24 h, and no further discussion is presented. For the cultures in which growth occurred, a positive correlation between higher concentrations and longer lag phases was observed, reaching approx 13 h in the presence of 2 g/L of furfural. Specific growth rate at the exponential phase exhibited a significant decrease in the presence of 0.5 g/L of furfural, but further increases in furfural concentration had only a minor effect, a pattern similar to that found for overall biomass productivity.

For the conditions that enabled growth, furfural was always metabolized into furfuryl alcohol, and at least part of this generated furoic acid (data not shown). No other extracellular metabolite was detected. Pentoses assimilation decreased considerably compared to growth in the absence of inhibitors, as well as the rate of glucose assimilation (data not shown). Biomass yield decreased to approx 60% of the yield obtained in the reference culture, except for 2 g/L, which exhibited a similar value.

Discussion

High biomass concentration is a key factor to ensure a high efficiency in xylitol bioproduction. This bioprocess should, therefore, be preferably

carried out as a two-step process. The first step, run with excess oxygen and low monosaccharides concentration, can rapidly produce biomass. The second step, oxygen limited, produces xylitol using concentrated media (*30–33*). Thus, it is important to understand not only the direct impact that the toxic byproducts generated during hydrolysis may have on the second step, but also the impact on the biomass production step, giving its importance to the overall process economics.

Effects of Aliphatic Acids

Although *D. hansenii* tolerated higher total formic acid concentrations than acetic acid, considering the effective undissociated form, acetic acid proved to be less inhibitory than formic acid, in a pattern similar to that already described for *Saccharomyces cerevisiae (5)*. A possible explanation for the higher sensitivity toward formic acid may be related to the differential permeability of the cell membrane for the two acids, because formic acid has a higher mobility across the cell membrane *(2,5)*. Despite a slight decrease in the specific growth rate, acetic acid induced a positive impact on growth, at both the kinetic (overall biomass productivity) and stoichiometric level at considerably higher concentrations of the undissociated acid. This is probably owing to the conjugation of two different effects. First, there is a buffering effect that allows the yeast to be maintained at more favorable pH for longer times, enabling a more efficient assimilation of monosaccharide. Second, the acetic acid acts as an energy and/or carbon source. Roberto et al. (*23*) found similar results for *Candida guilliermondii* and stated that low acetic acid concentrations might favor growth. Is should be noted that both our results and those of Roberto et al. *(23)* were obtained under uncontrolled pH culture systems, and, therefore, the beneficial effect of acetic acid on growth may be minimized for controlled pH cultures, only prevailing its function as an additional carbon and energy source.

A similar discussion can be made for formic acid. For the lowest concentrations assayed, this acid generates a buffering effect, allowing a higher assimilation of monosaccharides. In contrast to acetic acid, formic acid was not used for cell growth but induced xylitol production. Indeed, a metabolic flux shift from biomass to xylitol production (0.21 g/g xylose) was observed, even though the cultures were carried out under aerobic conditions, in which xylitol accumulation is not favored *(34,35)*. Granström and Leisola (*14*) reported a similar result for another pentose-assimilating yeast, *Candida tropicalis (14)*, but under oxygen-limiting conditions. Nevertheless, as in their study, the results that we obtained may be explained by an increased redox imbalance owing to the formation of NADPH at the formate dehydrogenase level.

Comparison of the different levels of inhibition induced by both acids for *D. hansenii* and other microorganisms, not considering the impact of

the culture conditions, reveals that *D. hansenii* showed a fairly high tolerance to both acetic acid and formic acid (undissociated form) compared to ethanologenic recombinant bacteria (e.g., *Escherichia coli [2]*, *Zymomonas mobilis [1]*) and yeasts (e.g., *Pichia stipitis [36]*, *C. guilliermondii [23]*, *Kluyveromyces marxianus [6]*). Compared with other yeasts, *D. hansenii* also has the ability to grow at high pH values (at least up to 8.3) *(37)*, a feature that can be used as a tool to increase further both the tolerance to aliphatic acids and xylitol productivity *(38,39)*.

Effects of Phenolic Compounds

Compared to other metabolic inhibitors, the toxicity of phenolic compounds has not been extensively studied in the literature. A possible reason is that phenolic compounds vary greatly in type and amount, among the lignocellulosic materials *(29)*. Furthermore, the phenolic hydrolysate's content may even change within different hydrolysis conditions for the same material *(43)*. In addition, accurate qualitative and quantitative analyses of phenolics are not easily done, thus limiting their study.

The phenolics selected for this work are usually present in hydrolysates, and their hydrophobicities are markedly different *(4)*. Increased toxicity was found for hydroquinone, syringaldehyde, and 4-methylcatechol and could be correlated to the compound's hydrophobicity. This supports the mechanism proposed for the toxicity of phenolics, i.e., a partition of the phenolic compound into the biologic membranes causing loss of integrity, thereby preventing their function as selective barrier and enzyme matrices *(4,44,45)*. Furthermore, it was also found that these compounds could limit both pentose consumption and assimilation into biomass, because, as discussed for furfural, they also promoted a metabolic shift from biomass production to maintenance processes.

Comparison of the different levels of inhibition induced by all phenolic compounds in *D. hansenii* and other microorganisms, not considering the impact of the culture conditions, demonstrates that *D. hansenii* always showed a higher tolerance to these compounds than *E. coli (4)* and *K. marxianus (6)*.

Effects of Furfural

The most significant effect of furfural in *D. hansenii* growth was the increase in the lag phase with an increase in furfural concentration. In *S. cerevisiae (40)*, this was suggested to occur owing to a reversible inactivation of cell replication by furfural, as also described for *P. stipitis (41)*. In contrast to *S. cerevisiae (40)*, no correlation between furfural concentration and specific growth rate was found.

Converse to what occurred for glucose metabolism, in which no significant effect on biomass yield from glucose was observed, furfural

specifically limited both pentose consumption and its assimilation into biomass, promoting a metabolic shift to maintenance processes since no metabolic products were detected. Similar data were previously described for *Candida blankii (42)*.

Comparison of the different levels of inhibition induced by furfural in *D. hansenii* and other microorganisms, not considering the impact of the culture conditions, indicates that *D. hansenii* showed a similar or even higher tolerance to furfural compared to ethanologenic recombinant bacteria, such as *E. coli (3)* and *Z. mobilis (1)*, and yeast such as *K. marxianus (6)*, *S. cerevisiae (40)*, and *C. blankii (42)*, the latter also grown in fully aerobic conditions.

Conclusion

Our study highlights the effects induced by selected compounds from the three major groups of potential inhibitors present in hydrolysate based media on the physiology and metabolism of *D. hansenii* when grown in a completely defined medium containing pentoses and hexoses. Specifically, the results indicate that furfural and phenolic compounds, even at low concentrations, have a negative effect on growth and should be minimized when working with hydrolysate-based media, at both hydrolysis and detoxification steps. Conversely, aliphatic acids, if maintained at controlled concentrations, may have a positive effect on the biomass production step, and it is foreseen that a similar effect can occur for xylitol production.

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