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Influence of Lignocellulose-Derived Aromatic Compounds on Oxygen-Limited Growth and Ethanolic Fermentation by *Saccharomyces cerevisiae*

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Abstract

Phenolic compounds released and generated during hydrolysis inhibit fermentation of lignocellulose hydrolysates to ethanol by *Saccharomyces cerevisiae*. A wide variety of aromatic compounds form from lignin, which is partially degraded during acid hydrolysis of the lignocellulosic raw material. Aromatic compounds may also form as a result of sugar degradation and are present in wood as extractives. The influence of hydroxy-methoxy-benzaldehydes, diphenols/quinones, and phenylpropane derivatives on *S. cerevisiae* cell growth and ethanol formation was assayed using a defined medium and oxygen-limited conditions. The inhibition effected by the hydroxy-methoxy-benzaldehydes was highly dependent on the positions of the substituents. A major difference in inhibition by the oxidized and reduced form of a diphenol/quinone was observed, the oxidized form being the more inhibitory. The phenylpropane derivatives were examined with respect to difference in toxicity depending on the oxidation-reduction state of the γ-carbon, the presence and position of unsaturated bonds in the aliphatic side chain, and the number and identity of hydroxyl and methoxyl substituents. Transformations of aromatic compounds occurring during the fermentation included aldehyde reduction, quinone reduction, and double bond saturation. Aromatic alcohols were detected as products of reductions of the

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corresponding aldehydes, namely hydroxy-methoxy-benzaldehydes and coniferyl aldehyde. High molecular mass compounds and the corresponding diphenol were detected as products of quinone reduction. Together with coniferyl alcohol, dihydroconiferyl alcohol was identified as a major transformation product of coniferyl aldehyde.

Index Entries: Aromatic compounds; inhibitors; *S. cerevisiae*; ethanolic fermentation; growth.

Introduction

Fuels produced from renewable resources do not contribute to a net increase of carbon dioxide in the atmosphere, in contrast to fossil fuels. Lignocellulose is considered an attractive raw material for the production of fuel ethanol owing to its abundance, e.g., as residues from forestry. The polysaccharides of lignocellulose, cellulose and hemicellulose, can be hydrolyzed with acids or enzymes to sugars. During acid hydrolysis, byproducts such as aliphatic acids (predominantly acetic, levulinic, and formic) and furan derivatives (furfural and 5-hydroxymethylfurfural), are generated along with the fermentable sugars following the degradation of hemicellulose and cellulose. These compounds have a minor inhibitory effect on the fermentability of spruce hydrolysates by *Saccharomyces cerevisiae*, mainly in terms of maximum ethanol productivity *(1,2)*. The concentrations of the inhibitors depend on the type and severity of the hydrolysis *(1)* as well as on the type of raw material.

Lignin, a complex macromolecule composed of phenylpropane units, comprises 20–32% of wood *(3)*. It originates from the random oxidative polymerization of hydroxylated cinnamyl alcohols. During the pretreatment of the lignocellulosic raw material prior to microbial fermentation, a minor part of lignin is degraded to a wide range of aromatic compounds *(4–6)*. Low molecular mass aromatic compounds are formed not only as the result of partial degradation of lignin. It has previously been shown that catechols form as products from pentoses and hexuronic acids owing to acid-catalyzed reactions under heating *(7)*. Another source of phenolic compounds is wood extractives *(3,8)*.

Previous studies have shown that the removal of phenolic compounds from the hydrolysate prior to fermentation with *S. cerevisiae* results in a substantial improvement of the fermentability *(2,9)*. A wide variety of phenolic compounds can be identified in lignocellulose hydrolysates with modern analytical techniques. Elucidating the connection between the structure and toxicity of lignocellulose-derived phenolic compounds is important (I) for the identification of the fraction among the phenolics that cause the inhibition of the fermentation, and (II) for the development of techniques for specific removal of the inhibitors.

Aromatic monomers, presumed to inhibit alcoholic fermentation, have been identified and quantified in poplar *(6)*, red oak *(5)*, birch *(10)*, willow *(9)*, and spruce *(2)* hydrolysates. The mechanism of inhibition by aromatic

compounds has not yet been elucidated. However, low molecular mass phenolic compounds have been found inhibitory to the fermenting microorganisms *(4–6,11–13)*. Clark and Mackie *(4)* tested the effect of two benzylic compounds, i.e., aromatic compounds with a one-carbon side chain, on *S. cerevisiae* and concluded that low molecular mass phenolics were more inhibitory than carbohydrate degradation products. Ando et al. *(6)* examined the effect of nine aromatic compounds, primarily benzylic compounds, on *S. cerevisiae*. Mikulásová et al. *(12)* studied the toxicity of 20 aromatic compounds, primarily benzylic, on *Candida utilis* and *Candida albicans*. Tran and Chambers *(11)* assayed the effect of nine phenolics, most of which were benzylic compounds, on *Klebsiella pneumoniae* and concluded that the lignin model compounds were more toxic than the nonphenolic extractive model compounds, which were also tested. Nishikawa et al. *(13)* studied the effect of seven benzylic compounds on *K. pneumoniae*. As a result of these studies, it was proposed that aromatic alcohols are less toxic than corresponding acids, which, in turn, are less toxic than corresponding aldehydes *(5,12)*. The effect of various aromatics was also examined by Ando et al. *(6)*, who arranged the functional groups in order of inhibitory effect. However, this study was based on direct comparison of nine compounds of which two were phenylpropane derivatives and seven were benzaldehydes or benzoic acids. Therefore, more than one structural element at a time was varied in some of the comparisons.

The present investigation is largely devoted to phenylpropane derivatives, which deserve particular attention among lignocellulose-derived aromatics since they represent the basic units of lignin *(3)*. Among the 20 compounds (Fig. 1) assayed for inhibition of growth and ethanol formation by *S. cerevisiae*, 14 were phenylpropane-derivatives (Fig. 1, compounds 7–20). Coniferyl aldehyde (Fig. 1, compound 12) was selected after identification in a spruce hydrolysate *(2)*. Isoeugenol (Fig. 1, compound 14) was identified in a willow hemicellulose hydrolysate *(9)*. Other selected phenylpropane-derivatives (such as compounds 8, 11, 13, 15, and 20) also represent naturally occurring *p*-hydroxyphenyl, guaiacyl, and syringyl elements in lignin, and some (compounds 7, 9, 10, and 16–19) were included in order to make the comparison systematic from a structural point of view. In addition to phenylpropane-derivatives, diphenols/quinones (Fig. 1, compounds 4–6) and vanillins (Fig. 1, compounds 1–3) were included. Catechol was identified in a willow hydrolysate *(9)*, hydroquinone in a spruce hydrolysate *(2)*, and an oxidized derivative of hydroquinone, benzoquinone, was included for comparison. Among the hydroxy-methoxybenzaldehydes or vanillins, vanillin (compound 1) was found in both a spruce *(2)* and a willow hydrolysate *(9)*. The isomeric forms isovanillin (compound 2) and *o*-vanillin (compound 3) were also included. The concentrations tested for inhibition of *S. cerevisiae* cell growth and ethanol formation (0.02–1 g/L) are below the total concentration of phenolic compounds determined in a spruce hydrolysate (1.86 g/L) *(2)*.

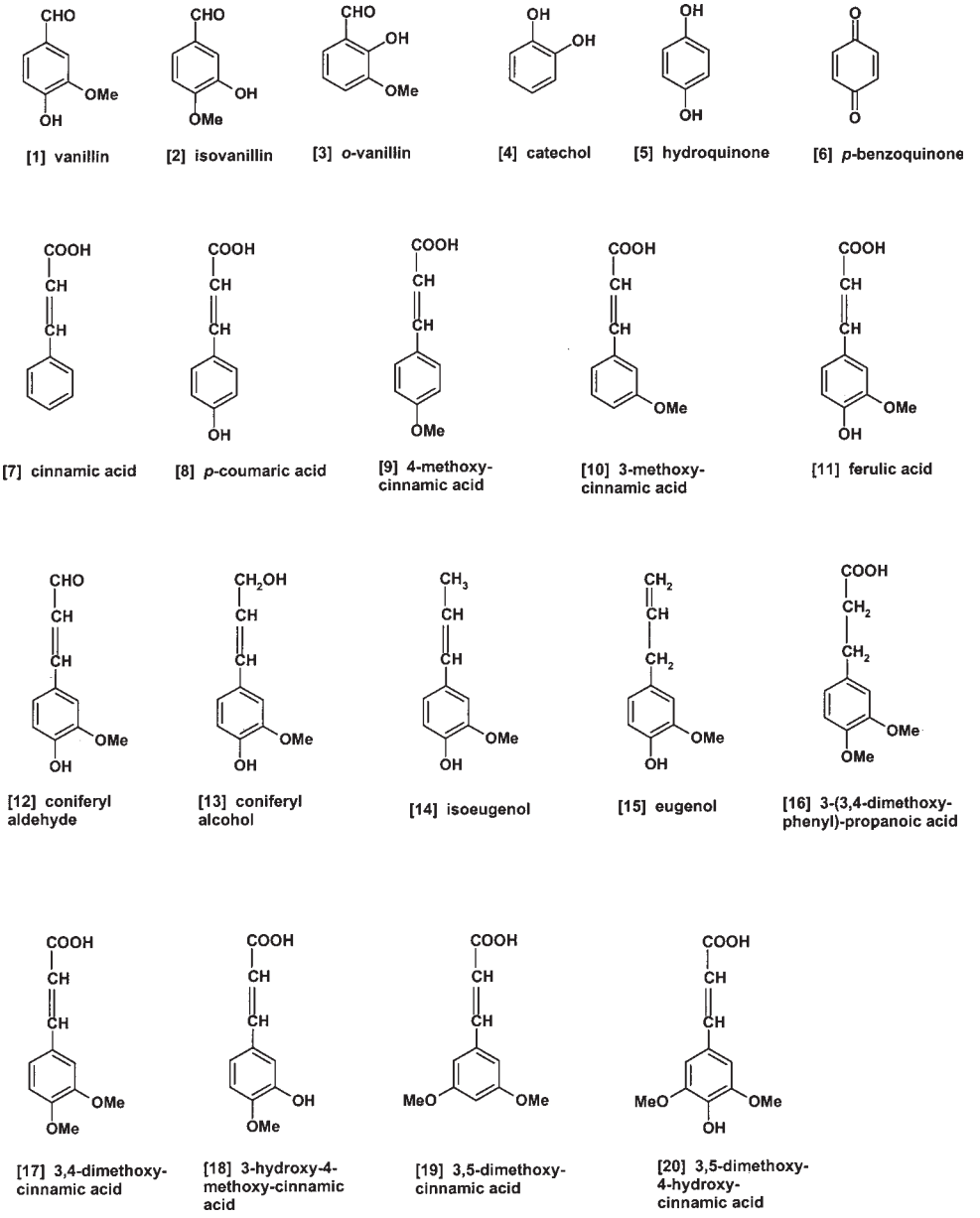

Fig. 1. Examined compounds.

Materials and Methods

Microorganism and Growth Conditions

Baker's yeast, *S. cerevisiae*, obtained from Jästbolaget AB (Rotebro, Sweden), was maintained on agar plates (20 g/L of agar, 15 g/L of malt extract broth). Inocula were grown aerobically in defined medium *(14)* supplemented with 20 g/L of glucose. Cells were harvested in exponential

growth phase by centrifugation at 1200*g* at 4°C for 10 min and washed with 9 g/L of NaCl.

Fermentations

Defined medium *(14)* supplemented with 20 g/L of glucose was used in all fermentations. The fermentations were performed under oxygen-limited conditions, in 150-mL fermentors sealed with rubber stoppers with a working volume of 140 mL. Fermentors were equipped with cannulas for carbon dioxide removal and sampling. Fermentors were inoculated to a final optical density (OD) (measured at 620 nm) of 0.50, corresponding to 0.16 g/L of yeast cells (dry wt). The cells were incubated at 30°C with stirring, and fermentations were run for 24 h under aseptic conditions. Cell growth was determined during fermentations by measuring culture OD every hour for 12 h and at the end of the fermentations. Samples for high-performance liquid chromatography (HPLC) analysis were withdrawn after 0, 2, 4, 6, 8, and 12 h and at the end of the fermentation. Vanillin, isovanillin, *o*-vanillin, catechol, hydroquinone, benzoquinone, cinnamic acid, *p*-coumaric acid, ferulic acid, coniferyl alcohol, 3-(3,4-dimethoxy-phenyl)-propanoic acid, 3,4-dimethoxy-cinnamic acid, and 3,5-dimethoxy-4-hydroxy-cinnamic acid were added to achieve initial concentrations of 0.02, 0.2, and 1 g/L. Because of lower solubility, 4-methoxy-cinnamic acid, 3-methoxy-cinnamic acid, coniferyl aldehyde, isoeugenol, eugenol, 3-hydroxy-4-methoxy-cinnamic acid, and 3,5-dimethoxy-cinnamic acid were added to achieve initial concentrations of 0.02 and 0.2 g/L.

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fluka (Buchs, Switzerland) and were of analytical grade, unless stated otherwise. The cinnamic acids were supplied as *trans*, or predominantly *trans*, preparations. The isoeugenol was supplied as a *cis-trans* mixture. Chemicals were dissolved in double-deionized water (Millipore, Bedford, MA), sterile-filtered through AcroCap™ filters with a 0.2-μm pore size (Gelman Sciences, Ann Arbor, MI) and added to the fermentors prior to inoculation with yeast. Reference fermentations of 20 g/L of glucose in defined medium were carried out in each set of fermentations. All fermentations were repeated at least twice. The results presented in Table 1 are average values from repeated fermentations.

Analyses

Cell concentrations were determined gravimetrically (as dry wt) at the beginning and the end of the fermentations. For dry wt determinations, 5 mL of culture broth was filtered through predried nitrocellulose filters with a 0.45-μm pore size (Gelman Sciences) The filtered pellets were then washed with 15 mL of double-deionized water (Millipore) and dried in a microwave oven (Whirlpool, Benton Harbor, MI) at a power scale of 3.5 for 15 min.

OD at 620 nm was determined with a U-2000 spectrophotometer (Hitachi, Tokyo, Japan) using plastic cuvets with a 1-cm path length. The samples

Table 1
Influence of Vanillins, Diphenols/Quinones and Phenylpropane Derivatives on Cell Growth and Ethanol Formation by *S. cerevisiae*

Group	Compound[a]	Concentration (g/L)	Q_{8h} (g/[L · h])	Y_{EtOH} (g/g)	μ_{max} (h^{-1})	Y_x (g/g)
Reference			0.67 ± 0.09	0.405 ± 0.008	0.110 ± 0.004	0.076 ± 0.003
Hydroxy-methoxy-benzaldehydes	Vanillin [1]	0.02	0.73	0.403	0.110	0.079
		0.20	0.73	0.401	0.100	0.075
		1.00	0.58	0.409	0.070	0.054
	Isovanillin [2]	0.02	0.76	0.415	0.110	0.080
		0.20	0.76	0.409	0.110	0.076
		1.00	0.63	0.410	0.090	0.059
	o-Vanillin [3]	0.02	0.31	0.405	0.090	0.070
		0.20	0.00	0.000	0.000	0.000
		1.00	0.00	0.000	0.000	0.000
Diphenols/quinones	Catechol [4]	0.02	0.76	0.411	0.110	0.070
		0.20	0.75	0.405	0.110	0.070
		1.00	0.72	0.417	0.080	0.061
	Hydroquinone [5]	0.02	0.73	0.402	0.110	0.071
		0.20	0.73	0.409	0.110	0.067
		1.00	0.71	0.416	0.080	0.060
	p-Benzoquinone [6]	0.02	0.00	0.000	0.000	0.000
		0.20	0.00	0.000	0.000	0.000
		1.00	0.00	0.000	0.000	0.000
Phenylpropane derivatives	Cinnamic acid [7]	0.02	0.50	0.400	0.090	0.066
		0.20	0.28	0.434	0.045	0.018
		1.00	0.00	0.171	0.001	0.004
	p-Coumaric acid [8]	0.02	0.70	0.403	0.110	0.078
		0.20	0.68	0.405	0.100	0.078
		1.00	0.42	0.410	0.070	0.073
	4-Methoxy-cinnamic acid [9]	0.02	0.73	0.398	0.110	0.076
		0.20	0.54	0.428	0.050	0.019
		1.00	—	—	—	—

3-Methoxy-cinnamic acid [10]	0.02	0.75	0.407	0.110	0.072
	0.20	0.54	0.425	0.050	0.025
	1.00	—	—	—	—
Ferulic acid [11]	0.02	0.68	0.405	0.110	0.072
	0.20	0.31	0.412	0.070	0.063
	1.00	0.14	0.407	0.030	0.008
Coniferyl aldehyde [12]	0.02	0.60	0.401	0.110	0.072
	0.20	0.24	0.363	0.070	0.067
	1.00	—	—	—	—
Coniferyl alcohol [13]	0.02	0.75	0.415	0.110	0.076
	0.20	0.74	0.414	0.110	0.073
	1.00	0.69	0.413	0.100	0.073
Isoeugenol [14]	0.02	0.59	0.400	0.110	0.077
	0.20	0.24	0.430	0.060	0.035
	1.00	—	—	—	—
Eugenol [15]	0.02	0.59	0.401	0.100	0.076
	0.20	0.24	0.414	0.060	0.048
	1.00	—	—	—	—
3-(3,4-Dimethoxy-phenyl)-propanoic acid [16]	0.02	0.75	0.415	0.110	0.070
	0.20	0.75	0.413	0.110	0.070
	1.00	0.68	0.423	0.070	0.058
3,4-Dimethoxy-cinnamic acid [17]	0.02	0.48	0.417	0.110	0.070
	0.20	0.28	0.426	0.090	0.051
	1.00	0.00	0.000	0.000	0.003
3-Hydroxy-4-methoxy-cinnamic acid [18]	0.02	0.50	0.403	0.100	0.068
	0.20	0.38	0.397	0.060	0.050
	1.00	—	—	—	—
3,5-Dimethoxy-cinnamic acid [19]	0.02	0.68	0.397	0.100	0.067
	0.20	0.55	0.400	0.080	0.047
	1.00	—	—	—	—
3,5-Dimethoxy-4-hydroxy-cinnamic acid [20]	0.02	0.76	0.424	0.110	0.073
	0.20	0.76	0.417	0.110	0.074
	1.00	0.73	0.433	0.110	0.072

[a]Numbers in brackets refer to compounds in Fig. 1.

were, if required, diluted with double-deionized water into the linear range. Water was also used as the blank.

The concentrations of glucose, ethanol, glycerol, acetic acid, and lactic acid were determined by using a high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) using a refractive index detector (RID-10A; Shimadzu) operating at 45°C, with 5 m*M* H_2SO_4 as the mobile phase, at a flow rate of 0.5 mL/min. Five standards containing the analyzed compounds were injected separately from samples. Each standard and sample was injected twice. The calculation was made by computer integration (Gilson UniPoint 1.52; Gilson, Middleton, WI).

The concentrations of the tested model compounds and their degradation products were determined by using reversed-phase HPLC. The samples were separated on a Gynkotek system 480 (Gynkotek, Germering, Germany) equipped with a reversed-phase Nucleosil 100-5 C18 column (Merck, Darmstadt, Germany) and monitored by a UVD 340 S diode array detector (Gynkotek).

The model compounds and products were eluted at a flow rate of 0.8 mL/min with a gradient of methanol and water, both containing trifluoroacetic acid at a concentration of 0.025%. The methanol concentration was increased from 5 to 100% in 35 min. Syringic acid, dissolved in methanol, was used as an internal standard. In the event of interference with peaks in the sample, syringic acid was used as an external standard.

After separation by HPLC, the products obtained from the fermentation with coniferyl aldehyde were identified by gas chromatography-mass spectroscopy. The quantification of dihydro-coniferyl alcohol was based on the relative response factors obtained from the total ion current.

Prior to analysis all samples were diluted and filtered through 0.2-μm membrane filters (Advantech MFS, Pleasanton, CA).

A Sephadex G-25 column (Amersham-Pharmacia, Uppsala, Sweden) was used for size exclusion chromatography of benzoquinone incubated in double-deionized water and in fermentation medium. A 50 m*M* potassium phosphate buffer, pH 5.5, was used to elute the fractions. Eighteen fractions from each experiment were eluted, and the absorbance at 246 nm was measured with a U-2000 spectrophotometer. Blue dextran (Amersham-Pharmacia) (measured at 670 nm) and sodium hydroxide (measured as an increase in pH) were used as size markers.

Calculations

Ethanol yield (Y_{EtOH}) was based on grams of ethanol formed per grams of glucose consumed. Biomass yield (Y_X) was based on the increase in dry wt per grams of glucose consumed. Mean maximum volumetric productivity (Q), hereafter referred to as productivity, was calculated as grams of ethanol produced per liter of culture medium per hour during the first 8 h of the fermentation, since this period corresponded to the period of the mean maximum volumetric productivity in the reference fermentations.

The maximum growth rate (μ_{max}), hereafter referred to as the growth rate, was calculated as the increase in logarithmical OD per hour during the exponential growth phase.

Results

Twenty different aromatic compounds were tested (*see* Fig. 1). All investigated compounds inhibited yeast cell growth at the highest concentration applied, with the exception of coniferyl alcohol (Fig. 1, compound 13) and 3,5-dimethoxy-4-hydroxy-cinnamic acid (Fig. 1, compound 20) (Table 1).

Ethanol yield in the reference fermentations was 0.405 ± 0.008 g/g, the productivity was 0.67 ± 0.09 g/(L·h), the growth rate was 0.110 ± 0.004 g/(L·h), and the biomass yield was 0.076 ± 0.003 g/g.

Vanillin and isovanillin (Fig. 1, compounds 1 and 2, respectively) slightly inhibited cell growth at the concentration of 1 g/L, vanillin being marginally more inhibitory. The difference in ethanol productivity, however, was within the experimental deviation. *o*-Vanillin (Fig. 1, compound 3) was inhibitory at the lowest concentration (0.02 g/L), slightly decreasing the growth rate. Ethanol productivity was decreased to 46% of the reference. This concentration, however, had no influence on the ethanol yield. Neither growth nor ethanol production was observed at 0.2 and 1 g/L.

Catechol and hydroquinone (Fig. 1, compounds 4 and 5, respectively) slightly inhibited growth at 1 g/L. No difference in inhibitory potential between these two compounds was observed, whereas *p*-benzoquinone (Fig. 1, compound 6) completely inhibited cell growth and ethanol production at a concentration of 0.02 g/L.

Cinnamic acid (Fig. 1, compound 7) inhibited cell growth rate and ethanol productivity to approx 41% of the reference fermentation at a concentration of 0.2 g/L. Ethanol yield was slightly increased. At a concentration of 1 g/L, growth inhibition was almost complete, achieving <6% of the biomass yield and 1% of the growth rate of the reference fermentations. Ethanol yield was about 42% of the reference, but productivity after 8 h was zero.

p-Coumaric acid (Fig. 1, compound 8) at 1 g/L decreased growth rate and ethanol productivity to approx 63% of the reference fermentation. Biomass and ethanol yield, however, were comparable to the reference. No difference between 4-methoxy-cinnamic acid and 3-methoxy-cinnamic acid (Fig. 1, compounds 9 and 10, respectively) was observed, the slight variations being within the experimental deviations. At a concentration of 0.2 g/L, both ethanol productivity and cell growth were inhibited; however, growth was inhibited more. Ethanol yield, on the other hand, was slightly increased. Ferulic acid (Fig. 1, compound 11) at 0.2 g/L decreased biomass yield to 83%, growth rate to 64%, and ethanol productivity to 46% of the reference. At 1 g/L productivity was decreased to approx 20% of the reference and biomass yield was decreased to 10% of the reference fermentation. Ethanol yield was unaffected. Coniferyl aldehyde (Fig. 1, compound 12) at a concentration of 0.02 g/L slightly inhibited ethanol

productivity (to about 90% of the reference), but not the other parameters. With an increase in concentration to 0.2 g/L, productivity decreased to 36%, whereas growth was less influenced. Ethanol yield was slightly decreased, to 90% of the reference. On the other hand, no inhibition was observed after addition of coniferyl alcohol (Fig. 1, compound 13).

Isoeugenol and eugenol (Fig. 1, compounds 14 and 15, respectively) showed quite similar inhibitory properties. A slight inhibition of ethanol productivity was observed at a concentration of 0.02 g/L; however, this value also can be attributed to the standard deviation. With an increase in concentration to 0.2 g/L, inhibition became more noticeable. Ethanol productivity was decreased to approx 35% and growth rate to 55% of the reference for both compounds. Biomass yields decreased to 46 and 63% of the reference when isoeugenol and eugenol, respectively, were added to the fermentations at this concentration. Ethanol yield was slightly increased after addition of isoeugenol, whereas after addition of eugenol it was comparable to the reference.

3-(3,4-Dimethoxy-phenyl)-propanoic acid (Fig. 1, compound 16) slightly inhibited cell growth at 1 g/L by decreasing the growth rate to 64% and biomass yield to 76% of the reference fermentation. Ethanol yield, on the other hand, was slightly increased.

At 0.02 g/L, 3-hydroxy-4-methoxy-cinnamic acid (Fig. 1, compound 18) decreased growth rate and biomass yield to about 90% and ethanol productivity to 75% of the reference fermentation. With an increase in the concentration to 0.2 g/L, the inhibition of growth rate and ethanol productivity decreased to approx 55% of the reference.

Both 3,4-dimethoxy-cinnamic acid and 3,5-dimethoxy-cinnamic acid (Fig. 1, compounds 17 and 19, respectively) decreased biomass yield slightly at a concentration of 0.02 g/L. 3,4-Dimethoxy-cinnamic acid inhibited ethanol productivity more than 3,5-dimethoxy-cinnamic acid, while increasing the final yield of ethanol, whereas at this concentration 3,5-dimethoxy-cinnamic acid did not influence ethanol yield. The decrease in all parameters was more pronounced at a concentration of 0.2 g/L, except for ethanol yield. 3,5-Dimethoxy-cinnamic acid did not dissolve to the concentration necessary for performing the fermentation at 1 g/L. The inhibition by 3,4-dimethoxy-cinnamic acid was almost complete at 1 g/L, except for biomass yield, which, however, was only 4% of the reference.

The 3,5-dimethoxy-4-hydroxycinnamic acid (Fig. 1, compound 20) did not inhibit the cell growth or the ethanol formation even at the highest concentration applied, 1 g/L. Ethanol yield was slightly higher than the yield in the reference fermentations.

The fermentations with hydroxy-methoxy-benzaldehydes, diphenols/quinones, and coniferyl aldehyde were analyzed with respect to transformation of the added aromatic compound by using HPLC. Most of the compounds were transformed during the fermentations, but not the diphenols. Hydroquinone was detected as a product in fermentations with benzoquinone. The transformation of benzoquinone took place both with and

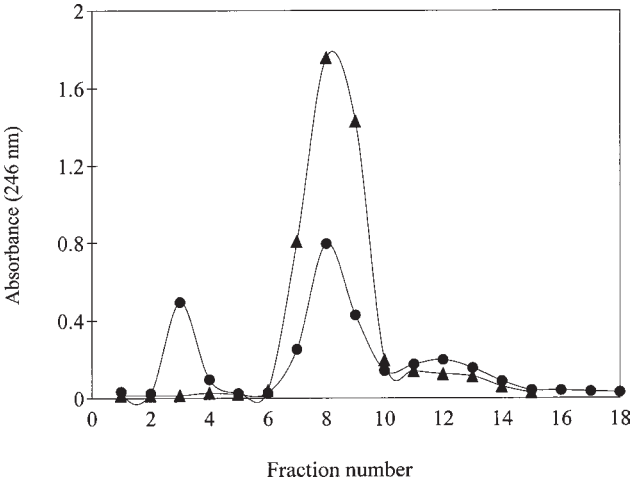

Fig. 2. Size exclusion chromatography of benzoquinone and reaction products. Benzoquinone incubated in double-deionized water (▲) is compared with benzoquinone incubated in growth medium without yeast cells added (●).

without yeast cells added to the medium, but not when benzoquinone was incubated only in double-deionized water. Therefore, it could be concluded that the medium components are involved in the transformation of benzoquinone. A brown fraction, containing products from this reaction was separated by size exclusion chromatography and had a high relative molecular mass, since it was eluted in the same fraction as Blue dextran (~2000 kDa) (Fig. 2).

The hydroxy-methoxy-benzaldehydes were converted to the corresponding alcohols, with the exception of *o*-vanillin at a concentration of 1 g/L, at which neither growth nor ethanol production was observed. *o*-Vanillin at 0.02 g/L inhibited cell growth rate and ethanol production (Table 1); however, conversion to *o*-vanillyl alcohol still occurred with a rate comparable to that of vanillin and isovanillin. At 0.2 g/L of *o*-vanillin, there was no growth, but still the conversion occurred to some extent (Fig. 3). When the concentration of *o*-vanillin was increased to 1 g/L, neither growth nor conversion was observed. Coniferyl aldehyde was converted to coniferyl alcohol and 3-(4-hydroxy-3-methoxy-phenyl)-propanol (dihydroconiferyl alcohol) (Fig. 4). In addition, the occurrence of small amounts of polymerization products was detected.

Discussion

At the concentrations tested, inhibition of growth was observed for all compounds examined except 3,5-dimethoxy-4-hydroxy-cinnamic acid and coniferyl alcohol. Cell growth, determined as biomass yield and growth rate, was generally influenced more than ethanol yield whenever the test compound was inhibitory.

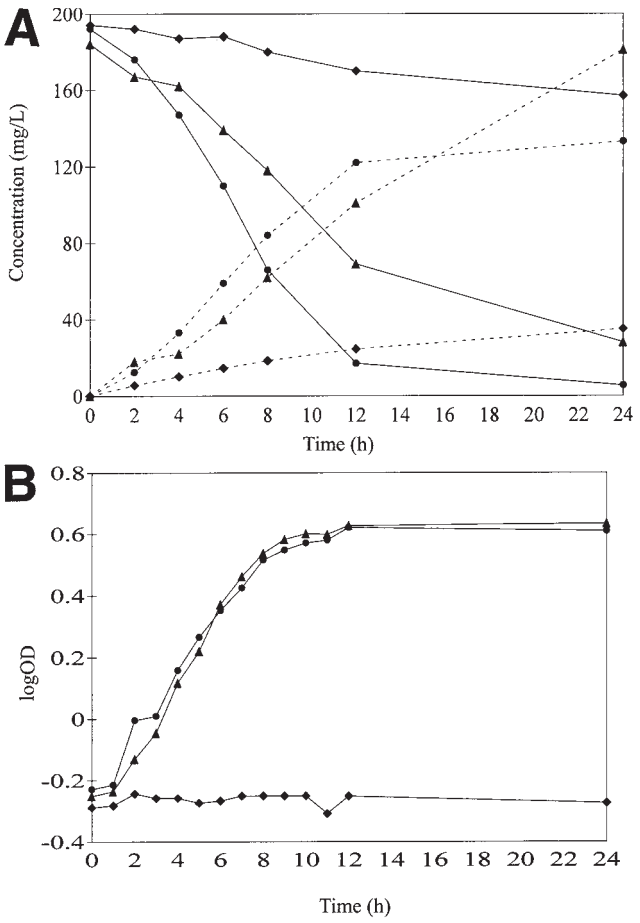

Fig. 3. **(A)** Transformation of vanillin (▲), isovanillin (●), and *o*-vanillin (◆) during fermentation with *S. cerevisiae* (solid lines) to corresponding alcohols (same symbols, dashed lines). **(B)** The same symbols show the effect of the vanillins on cell growth, indicated as logOD (OD at 620 nm).

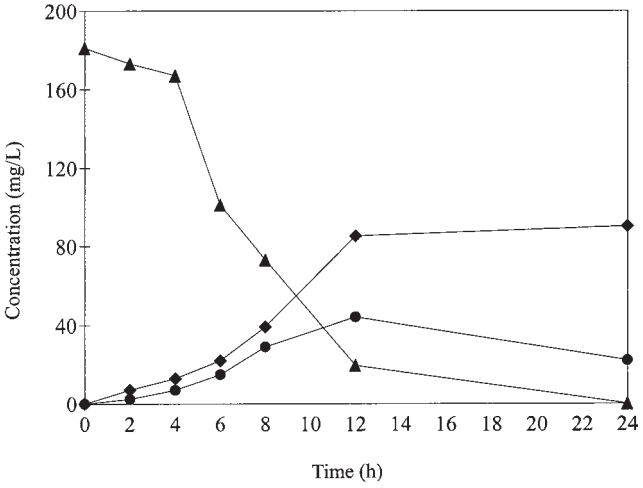

Fig. 4. Conversion of coniferyl aldehyde (▲) during fermentation and formation of coniferyl alcohol (●) and dihydroconiferyl alcohol (◆).

The results from the fermentations with vanillins suggest that the position of the substituents has a major effect (cf. vanillin and *o*-vanillin), whereas the identity of the substituent (hydroxyl or methoxyl) is of less importance (cf. vanillin and isovanillin). Specifically, if the hydroxyl substituent is in the *o*-position, as in *o*-vanillin, rather than in the *p*-position, as in vanillin, the inhibitory effect becomes considerable, which also agrees with previous results obtained with *Candida* sp. *(12)*. Whether the methoxyl and hydroxyl substituents are in the *m*- and *p*-positions, respectively (vanillin), or, alternatively, in the *p*- and *m*-positions (isovanillin) does not have any major effect on toxicity. Based on the comparison between *p*- and *m*-hydroxybenzoic acids, it has previously been concluded that *p*-OH is more toxic than *m*-OH *(6)*. Our results do not support this trend, since there was no significant difference between the parameters of growth and ethanol formation for vanillin (*p*-OH) and isovanillin (*m*-OH).

The degree of inhibition by the diphenols was not affected by whether the hydroxyl substituents were in the *o*- (catechol) or *p*-position (hydroquinone). Both catechol and hydroquinone were slightly inhibitory, influencing cell growth at 1 g/L but not ethanol production. By contrast, benzoquinone, the oxidized form of hydroquinone, was very inhibitory.

Previous results from fermentations with benzylic compounds have indicated that aromatic aldehydes are more toxic than the corresponding carboxylic acid, which, in turn, is more toxic than the corresponding alcohol *(5,6,12)*. In our comparison of phenylpropane derivatives, coniferyl alcohol showed less inhibition than the corresponding oxidized (coniferyl aldehyde) and reduced (isoeugenol) forms. Coniferyl aldehyde inhibited cell growth about as much as the corresponding carboxylic acid, ferulic acid, in contrast to previously reported results. However, ethanol yield was more inhibited by coniferyl aldehyde. When the γ-carbon was fully reduced (isoeugenol), the influence on ethanol formation was similar to that of ferulic acid. Cell growth, on the other hand, was more inhibited by isoeugenol than by ferulic acid and coniferyl aldehyde. In conclusion, among compounds 11–14 (Fig. 1) coniferyl alcohol was much less inhibitory for both growth and ethanol formation than the others.

The presence (3,4-dimethoxy-cinnamic acid) or absence (3-(3,4-dimethoxyphenyl)-propanoic acid) of an unsaturated bond in the side chain of a phenylpropane derivative was of major importance for the degree of inhibition (Table 1). This finding is in agreement with what has been reported previously regarding the effect of coniferyl alcohol and dihydroconiferyl alcohol on *K. pneumoniae (11)*. By contrast, the comparison of isoeugenol and eugenol (Table 1) indicates that the position of the unsaturated bond does not influence toxicity.

Previous investigations concerning benzylic compounds *(6,13)* have revealed that compounds carrying fewer hydroxyl and methoxyl substituents are more inhibitory. Our data for phenylpropane derivatives are partially in agreement, considering that cinnamic acid (Fig. 1, compound 7) inhibited growth more (Table 1) than corresponding compounds with one,

two, or three hydroxyl and methoxyl substituents (Fig. 1, compounds 8–11 and 17–20) and that 3,5-dimethoxy-4-hydroxy-cinnamic acid (Fig. 1, compound 20) did not inhibit growth rate whereas the corresponding compounds with fewer substituents did (Fig. 1, compounds 7–11 and 17–19). However, the degree of inhibition caused by the corresponding compounds with one (compounds 8–10) and two (compounds 11 and 17–19) hydroxyl and methoxyl substituents overlaps.

In contrast to the results by Ando et al. *(6)*, we did not find that the compounds with hydroxyl substituents instead of methoxyl substituents should be more inhibitory to ethanol formation (cf. compounds 8 and 9, and compounds 11, 17, and 18). With respect to inhibition of growth, there is no clear trend when compounds with either hydroxyl or methoxyl substituents in corresponding positions (compounds 8 and 9, and 11, 17, and 18) are compared (Table 1).

Regarding the identity of the substituents, it can be concluded that the replacement of a *p*-hydroxyl substituent (compounds 8 and 11) with a methoxyl substituent (compounds 9 and 17) leads to increased inhibition.

When compounds with methoxyl substituents in the *p*-position (compounds 9 and 17) were compared with corresponding compounds with the methoxyl substituent in the *m*-position (compounds 10 and 19), no major difference in toxicity was observed. Also, in accordance with the result from the comparison between vanillin and isovanillin (compounds 1 and 2, respectively), no major effect was obtained when the positions of the hydroxyl and methoxyl substituents of a guaiacyl-type phenylpropane derivative (compound 11) were switched (compound 18), the slight differences being within the experimental errors.

No obvious correlation between toxicity and solubility was observed. For instance, isoeugenol and eugenol, which were poorly soluble, rather strongly inhibited cell growth and ethanol formation. On the other hand, ferulic acid and cinnamic acid had a similar inhibitory effect, the solubility being much higher. 3,5-Dimethoxy-cinnamic acid and 3,4-dimethoxy-cinnamic acid did not have the same solubility in water, but the inhibitory effect on cell growth was quite similar.

All three vanillins examined were converted to the corresponding alcohols. It is noteworthy that this reaction occurred with similar rate regardless of whether the aromatic aldehyde showed a low (vanillin, isovanillin) or high (*o*-vanillin) toxicity. However, if the concentration of the inhibitor (*o*-vanillin) was increased so that cell growth ceased, the conversion of the inhibitor decreased considerably or stopped. The transformation of vanillin to vanillyl alcohol has been observed previously *(13,15)*. De Wulf et al. *(15)* suggested that the conversion to vanillyl alcohol is not catalyzed by alcohol dehydrogenase (ADH) but by a vanillin-induced oxidoreductase. The data supporting this was, however, not shown. The conversion of other studied aldehydes (4-hydroxybenzaldehyde, syringaldehyde, and benzaldehyde) was also reported *(15)*.

Two steps in the transformation of coniferyl alcohol were identified: the reduction to coniferyl alcohol and the reduction resulting in the formation of dihydroconiferyl alcohol. In addition, the occurrence of small amounts of polymerization products was detected, indicating the presence of radical intermediates.

The transformation of benzoquinone occurred in the presence of the growth medium, but in the presence or absence of yeast cells, suggesting that medium components are at least in part responsible. The eluted high molecular mass compounds result from polymerization, indicating the formation of a semiquinone radical intermediate. The formation of the semiquinone was most probably catalyzed by metal ions present in the growth medium. This semiquinone radical may be toxic to the cell, which could explain why no cellular activities were observed in the presence of benzoquinone, even at the lowest concentration used in this study (0.02 g/L).

This investigation focused on the relationship between the structure of selected mononuclear aromatics and the inhibition of ethanolic fermentation by *S. cerevisiae*. The experimental conditions used were similar to previous investigations, in which the actual wood hydrolysates were fermented *(1,2)*. Most compounds examined were related to the basic phenylpropane structural element of lignins. Some of the results show similarity to what has previously been found for fermentation inhibition by benzylic compounds, whereas other results differ or are of novel character. Several of the aromatic compounds examined occur as *cis-trans* isomers. Whether different stereoisomers have different inhibitory effects on the growth and ethanol production characteristics of *S. cerevisiae* remains to be elucidated. It is probable that the lignocellulose-derived aromatic compounds exert an additive or even synergistic inhibitory effect, which also needs to be studied. Further characterization of the transformation and action of the aromatic inhibitors will be required to unravel the mechanisms behind the relationships found between structure and inhibition of fermentation.

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