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Identification of Inhibitory Components Toxic Toward *Zymomonas mobilis* CP4(pZB5) Xylose Fermentation

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ABSTRACT

Zymomonas mobilis CP4(pZB5) is a recombinant bacterium that can produce ethanol from both xylose and glucose. The ethanol-producing efficiency of this organism is substantially impeded by toxic substances present in pretreated hydrolyzates or solid biomass substrates. Acetic acid and furfural (a pentose degradation product) are highly toxic to this organism at levels envisioned for a pretreated-hardwood liquid hydrolyzate. In addition, lignin degradation products and 5-hydroxymethylfurfural (a hexose degradation product) have a moderately toxic effect on the organism. Of the compounds studied, organic acids and aldehydes were found to be more inhibitory than lignin acids or the one alkaloid studied. Acetone:water and methanol extracts of solid biomass samples from red oak, white oak, and yellow poplar are toxic to *Zymomonas* cell growth and ethanol production, with the extracts from white oak being the most toxic.

Index Entries: *Zymomonas*; recombinant; ethanol; oak; toxicity; yellow-poplar; xylose.

INTRODUCTION

Zymomonas mobilis is an ethanologenic bacterium, generally considered an economical high-performance biocatalyst for ethanol production from

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glucose originating from various substrates *(1–4)*. The CP4 (pZB5) mutant of this organism has been genetically modified also to produce ethanol from the pentose sugar, xylose *(5)*.

Lignocellulosic materials from woody biomass have been targeted as a potential substrate for industrial ethanol production. Ethanologenic microorganisms such as *Z. mobilis* and *Saccharomyces cerevisiae* have been favorably used to convert biomass to ethanol *(6–9)*. However, when considering the economics of ethanol production, these microorganisms must be able to completely convert all potential carbohydrates in lignocellulosics. Xylose is a major hydrolysis product of hardwood hemicelluloses, and constitutes 10–26% of the dry weight of temperate-zone hardwoods *(10)*. This pentose is a difficult sugar for most microorganisms to convert to ethanol *(11,12)*. The combined xylose- and glucose-fermenting capabilities of the recombinant *Z. mobilis* CP4 (pZB5) make this organism a potentially ideal biocatalyst for economical ethanol production.

The conversion of woody biomass to ethanol typically involves a two-stage process. An initial dilute sulfuric acid treatment of wood affords deacetylation and depolymerization of the hemicelluloses. The resulting sugar solution is fermented to ethanol, and the remaining solid material is submitted to a simultaneous saccharification and fermentation process to convert the available glucose.

The ethanol-fermentation efficiency of *Z. mobilis* CP4 (pZB5) with the hemicellulose hydrolyzate is substantially hindered by the toxic substances present in this stream. In addition to acetic acid, many other inhibitory compounds such as organic acids, phenolics, and carbohydrate degradation products are suspected to be present in the hydrolyzates at levels that will affect the overall xylose-to-ethanol conversion process *(13)*. Xylose fermentation inhibition seriously jeopardizes the economic success of wood-to-ethanol producing facilities.

Efforts are now underway to improve process yields by increasing the efficiency of carbohydrate conversion to ethanol. There are several reasons for inefficient conversion, and the quantitative aspects of these limitations are not completely understood. The objective of this study was to obtain a firmer, quantitative understanding of the role inhibitors play in the conversion of xylose-to-ethanol by the recombinant *Zymomonas CP4* (pZB5), and the results of this investigation are presented below.

EXPERIMENTAL METHODS

General

The recombinant *Z. mobilis* CP4 (pZB5) used in this study was obtained from M. Zhang at the National Renewable Energy Laboratory

(NREL). The bacterium was stored at –70°C in cryovials containing RM media (1X conc., yeast extract 10 g/L, KH_2PO_4 2g/L) and glycerol (20% W/V). Inoculum preparation was as follows. One thawed vial of *Z. mobilis* was mixed with a medium that contained RM medium (10X conc., 20 mL), D-glucose (500 g/L, 10 mL), D-xylose (500 g/L, 10 mL), and tetracycline stock solution (12 mg/mL, 0.25 mL). The total liquid volume was adjusted to 200 mL with deionized (DI) water. The cells were cultivated in a shaker at 30°C (18 h), spun down for 10 min at 4700*g*, and the supernatant was discarded. The cells were resuspended in sterile RM media (10X conc., 5 mL) plus DI water (45 mL), and subsequently diluted with DI water to to a final optical density ($OD_{600\,nm}$) value of 25–30.

Toxicity testing was carried out in sterile 12 mL screw-capped test tubes containing 10 mL of positive control medium (yeast extract, 10g/L; KH_2PO_4, 2g/L; xylose, 44.7g/L; glucose, 2.4g/L; tetracycline, 1.2 mg/mL). The initial sugars in the positive control represent the amounts in the liquid hydrolyzate (preparation of which is described in the next section). The inhibitory compounds were added to the culture tubes dissolved either in ethanol (0.1 mL) or in ethanol:water (1:1 mixture). Each tube was inoculated with *Zymomonas* to achieve an initial net OD_{600nm} of 0.2. Finally, the tubes were incubated at 30°C in a shaker with agitation for up to 72 h. The cell growth was monitored by measuring the nonlinear OD of the culture tubes at 600 nm using a Milton-Roy Spectronic 601 spectrophotometer (slit width ~5 mm). The nonlinear OD measurements for each test condition were corrected with an appropriate blank control (medium without *Zymomonas* cells). The net OD values reported are obtained by subtracting the OD of test sample from the OD of medium blank. Toxic effects were also monitored by determining ethanol concentrations with a Varian gas chromatograph equipped with a flame ionization detector, and a Porapak Q 80/100 glass column (2 m × 4 mm id) packed in-house. Isopropanol was used as the internal standard.

Preparation of the Liquid Hydrolyzate

The mixed sawdust feedstock (1:1:1 red oak:white oak:yellow poplar) was milled and prescreened to pass through an 8-mm screen. The pretreatment run was conducted at a solids concentration of 20% (based on dry weight) using 0.82% (w/w) sulfuric acid at 160°C for 10 min. A high-solids paddle reactor, which is a steam-jacketed, horizontal cylindrical vessel of approximately 100 L total volume fitted with an internal paddle mixer, was utilized in this run. Biomass and deionized water are charged to the vessel through a flanged access port. After the vessel is sealed, steam is supplied to the shell to heat the vessel contents. When the target temperature is approached, a calculated amount of sulfuric acid solution is pumped

into the reactor and the reactor is held at the desired temperature for the designated time. Then, the reactor is cooled by water supplied through the shell and its contents are discharged and separated by filtration. The liquid hydrolyzate and the pretreated solids were stored refrigerated at 4°C.

Preparation of Various Hydrolyzate Samples for Toxicity Testing

The overlimed hydrolyzate was prepared by adjusting the hydrolyzate pretreatment liquor to pH 10.0–10.5 with solid $Ca(OH)_2$. The hydrolyzate was then kept at 50°C for 30 min. Finally, the pH was adjusted to 7.0 using 96% (v/v) H_2SO_4. The mixture was sterile filtered through a 0.2-μm sterile filter and stored at 4°C for a minimum of 5 d. The direct neutralized hydrolyzate was prepared by neutralizing with $Ca(OH)_2$ to achieve a final pH of 6.0. On the day of the experiment the hydrolyzates, overlimed or direct neutralized (30 mL), were combined with yeast extract (1g), KH_2PO_4 (200 mg), tetracycline (0.1 mL stock), xylose (3.1 g), glucose (168 mg), and water to achieve a sugar concentration the same as the liquid hydrolyzate from the reactor.

An organic phase was also prepared from the liquid hydrolyzate by extracting with methyl *tert*-butyl ether (MTBE) *(14)*. Essentially the crude hydrolyzate (15 mL) was extracted with MTBE (50 mL) three times, and the combined organic phase was evaporated under reduced pressure and subsequently under high vacuum.

Processing of Untreated and Treated Solid Biomass Substrates

The freeze-dried, untreated (red oak, white oak, and yellow poplar) and treated (mixed hardwood) solid biomass samples were Wiley-milled to a fine powder (1-mm screen), with precautions to minimize the sample heating. The resulting powders were subjected to three 48-h, room temperature acetone:water (7:3) extractions. The combined extracts (obtained by filtration) were evaporated under reduced pressure at room temperature to remove acetone, with the water subsequently removed by freeze-drying. After the extractions, the fibers were air-dried, weighed, and subjected to 48-h soxhlet extractions with methanol. The methanol extracts were evaporated under reduced pressure followed by freeze-drying. The treated mixed hardwood was processed in the same fashion except that prior to processing, the material was washed with distilled water until the wash water and distilled water pHs were the same.

Chemical Analyses

Glucose, xylose, acetic acid, ethanol, HMF, and furfural concentrations present in the hydrolyzate were determined using a Waters HPLC system equipped with a refractive index (RI) detector, and an HPX87-H

analytical column (Bio-Rad). The column was kept at 65°C, and 0.01N H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL/min. All compounds were quantitated using an external standard method.

The gradient HPLC analyses were carried out using a Gilson HPLC system, equipped with a dual wavelength UV-detector (Model 119) and a C_{18} column (Whatman RAC-II, 5-mm particle size). The gradient used is based on a previously reported method *(15)* that utilizes methanol (solvent A) and 0.2% trifluoroacetic acid in water (solvent B). The linear gradient consisted of an initial concentration of 100% solvent B. The linear gradient was begun at 1 min so that at 40 min the concentration of solvent B had decreased to 5%. This concentration was held for 5 min (40–45 min) and the gradient was returned to the original concentration in 5 min. After a 10-min re-equilibration, the system was ready for another injection. The flow rate was maintained at 0.75 mL/min, and the dual wavelength UV detector set at 280/320 nm, with the 280-nm data channel used for data collection.

The GC/MS analysis of derivatized extracts was carried out using a Fisions gas chromatograph/mass spectrometer equipped with a DB-5 capillary column (J & W Scientific). The extracts were silylated using a commercially available BSTFA/TMCS (99:1) reagent (Supleco) as described previously *(14)*.

The carbohydrate analyses of fiber samples were carried out by a method adopted from Kaar and co-workers *(16)*. The Klason and acid-soluble lignins as well as ash determinations were performed according to standard protocols *(17,18)*. All the samples analyzed for carbohydrates were quantitated using a Gilson HPLC system equipped with an Aminex HPX-87P column (Bio-Rad, maintained at 85°C) and an RI detector. HPLC grade water was used as the eluent at a flow rate of 0.6 mL/min, and the samples were injected to the column through a 20-µL loop. Sugars were quantitated using an external standard analysis.

RESULTS AND DISCUSSION

The major compounds present in the pretreated liquid hydrolyzate prepared from the mixture of red oak, white oak, and yellow-poplar were glucose (2.40 ± 0.48 mg/mL), xylose (44.75 ± 2.02 mg/mL), acetic acid (9.22 ± 0.43 mg/mL), and furfural (0.91 ± 0.051 mg/mL). The amount of 5-hydroxymethylfurfural (HMF) in the hydrolyzate was negligible compared to the other three components. Acetic acid, furfural, and HMF are formed during the pretreatment process. The xylan component of wood is deacetylated and depolymerized, and under well-controlled conditions provides xylose, acetic acid, and 4-*O*-methyl-D-glucuronic acid. However, such conditions are not achieved during industrial processing and some of the xylose is degraded to furfural *(19)*. Furthermore, glucuronoxylans of

Table 1
Extractive Yields (Mass Percent on a Dry Basis)

Sample	Acetone:water	Hot methanol	Total extracts	% Extractives[a]
Yellow poplar	1.51	1.39	2.90	2.4
Red oak	5.36	2.30	7.66	4.4–9.6
White oak	2.42	4.33	6.75	5.3–6.6
Treated solids	8.67	1.95	10.12	—

[a] Values adapted from ref. *10*.

hardwoods contain an average of one uronic acid group per seven xylose residues *(20)*. The 4-*O*-methyl-α-D-glucopyranosiduronic acid linkage to xylose is the most acid-stable of all glycosidic linkages in wood *(21)*. Thus, approx 1/7 of all xylose in temperate zone hardwoods can potentially remain unavailable for fermentation. HMF results from the dehydration of a portion of the hexoses formed during the pretreatment process.

Purification and Analysis of Solid Biomass Samples

The predominant extractives in the oaks are hydrolyzable tannins, and the compounds in yellow-poplar range from alkaloids (glaucine) to sesquiterpenes and lignans *(22)*. As these compounds provide passive protection for pathogen invasion in trees *(23)*, it can be expected that some of the extractives may also be toxic to *Zymomonas* and may be found in the hemicellulose hydrolyzate stream. Biomass samples were first extracted with acetone:water (7:3) to remove most of the hydrolyzable tannins *(24)*, and subsequently by a hot methanol-soxhlet extraction. The yields for the acetone:water and methanol extractives are given in Table 1. Interestingly, the treated solid sample afforded the highest percentage of acetone:water extracts, which is probably because of lignin degradation products that are insoluble in acidic water but soluble in acetone:water.

Separation and Analysis of the Pretreatment Liquid Hydrolyzate

Pretreatment hydrolyzate liquors contain variety of inhibitory components that are toxic to various microorganisms *(11)*: metals/minerals, carbohydrate decomposition products, lignin degradation products, and/or compounds derived from wood extractives are all potential inhibitors *(25–27)*. In order to separate the nonpolar organics from the polar, fermentable fraction, the hydrolyzate was fractionated with MTBE *(14)*.

The amount of material recovered from the extraction was reproducible (3.14 ± 0.08 mg/mL), and HPLC analysis of the aqueous phase and the MTBE extract confirmed that MTBE separated most of the phenolics and furans from the polar carbohydrates (which remain in the aqueous fraction).

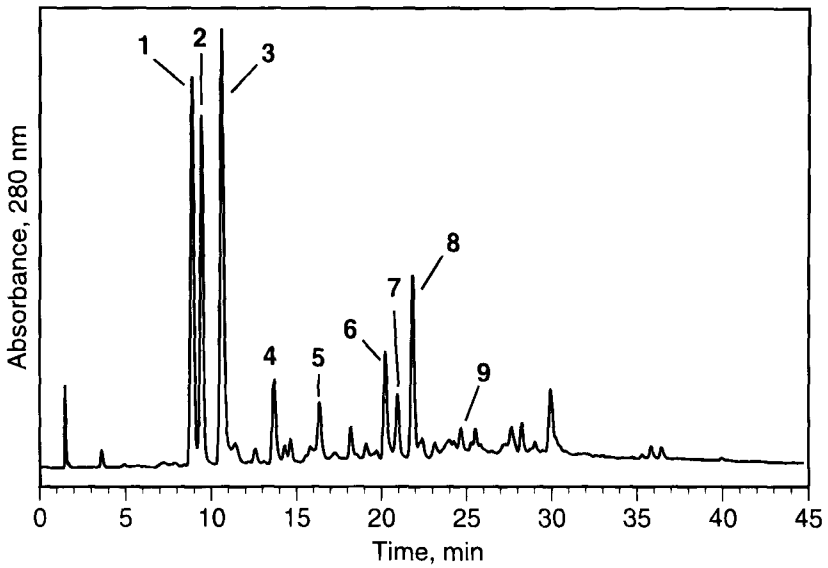

Fig. 1. HPLC separation of the MTBE extract. Conditions are as described in the text. Compound key: **1**, gallic acid; **2**, HMF; **3**, furfural; **4**, protocatechuic acid; **5**, unknown; **6**, vanillin; **7**, coniferyl alcohol; **8**, syringaldehyde; **9**, sinapic acid.

The GC/MS analysis of the trimethylsilylated MTBE-extracts revealed gallic acid, HMF, vanillin, protocatechuic acid, and syringaldehyde. These compounds were identified by comparing the mass spectral-fragmentation patterns and retention times with those of commercially available compounds. Reverse-phase HPLC analysis of the MTBE-extracted material confirmed the GC/MS study, and it was further determined that gallic acid, HMF, and furfural were the major compounds present (Fig. 1). These peaks were identified by comparing the retention times with authentic compounds, and by spiking the MTBE fraction. Other compounds present in significantly lower concentrations were protocatechuic acid, vanillin, coniferyl alcohol, syringaldehyde, and sinapic acid. Thus, the major phenolic compounds in the ether extract are the result of the acid degradation of tannin and lignin.

The degradation of lignin during acidolysis is mainly owing to the cleavage of the ether bonds; during dilute acid treatment the β-O-4 end units of lignin are preferentially cleaved. After cleavage of this bond, the released phenolic can undergo rearrangement reactions to generate various low-mol-wt compounds. For example, some phenolic compounds identified in wood hydrolyzates and lignin acidolysis mixtures include vanillin, coniferylaldehyde, syringaldehyde, vanillic acid, and syringic acid *(25,26)*. The hydrolyzable tannins of oak will decompose forming gallic acid, ellagic acid, and glucose. Several of the compounds found in

Table 2
Summative Analysis of the Biomass Samples

Component(%)	Red oak	White oak	Yellow-poplar	Treated solids
Hexosan[a]	35.20	36.51	38.99	53.28
Pentosan[b]	16.70	17.70	14.53	0.02
Acid soluble lignin	3.14	3.49	3.48	1.14
Klason lignin	24.50	24.50	23.0	28.0
Ash	0.8	2.0	1.2	0.0
Moisture(105°C)	7.99	7.46	7.59	8.99
Total[c]	94.5	98.1	95.0	97.3

[a] Corrected for mass change according to the eqn.: Hexosan = (glucose)(0.9) + (HMF + 162/110).

[b] Corrected for mass change according to the eqn.: Pentosan = (xylose)(0.88) + (furfural + 132/96).

[c] Total = %Hexosan + %Pentosan + %Klason lignin + %Acid-soluble lignin + %Ash + %Moisture. This total does not account for acetate, uronic acids, or other hemicellulosic/pectic sugars.

this study were also reported by Tran and Chambers to be present in an extractive-free oak-prehydrolyzate stream, and also found to be inhibitory to *Pichia stipitis (13)*.

Analysis of the Extractive-Free Biomass Samples

The extractive-free biomass of red oak, white oak, yellow poplar, and treated solids were subjected to summative biomass analyses *(16)*, and the values are given in Table 2. Glucose and xylose were the only carbohydrates quantified. In this protocol, a correction is made based on the assumption that all carbohydrates destroyed during the acidolysis process are converted to either HMF or furfural. Thus, any decomposition of sugars should be quantified by the HMF and furfural values, and these values can be added to glucose and xylose values, respectively. The hexosan and pentosan values are corrected for mass changes associated with hydrolysis. The method employed to estimate the percent biomass does not account for acetate, uronic acids, and the other hemicellulosic and pectic sugars. Therefore, the values owing less than 100%. The ash contents reported are probably higher than the true values owing to the high degree of error associated with the gravimetric determinations (ash contents were determined from 100 mg of starting material). The ash contents for oak and yellow poplar are on the order of 0.1 to 1.3% and 0.3% (dry-mass basis), respectively *(10)*. The ash content of the treated solids was negligible, because the acid pretreatment solubilized the inorganics.

Table 3
Net OD_{600nm} Values and Ethanol Fermentation Yields for the Positive Control and the Overlimed and Direct Neutralized Hydrolyzates

Sample[a]	Net OD_{600nm}	Ethanol (mg/mL)	% Performance[b]
Positive control	1.051	18.70	100
Overlimed hydrolyzate (30% loading)	0.587	2.37	13
Direct neutralized hydrolyzate (30% loading)	0.999	0.60	3

[a] The initial amounts of xylose and glucose in samples are 44.7 mg/mL and 2.4 mg/mL, respectively. Fermentation time was 72 h.

[b] Calculated by the equation: [(Final EtOH conc. sample)/(Final EtOH conc. positive control)]100.

Toxicity Testing of the Hydrolyzate Against Zymomonas Mobilis

The goal of this study was to investigate the relative toxicity of hydrolyzate fractions at various dilutions toward *Zymomonas* cell growth and ethanol production. All the fractions and controls prepared were supplemented with glucose and xylose to provide concentrations matching that of the original hydrolyzate, and these toxic effects were compared to a positive control, which functions as a pure-sugar control. Initial studies were concerned with characterization of the positive control relative to overlimed and direct neutralized hydrolyzate fractions at various loading levels. A 100% loading level represents sugar concentrations in bioassay tubes that match that of the original hydrolyzate, and lower-percentage loading values represent the corresponding lower-hydrolyzate concentrations. For example, a 30% level represents a bioassay (direct neutralized or overlimed) that contains 3 parts hydrolyzate and 7 parts DI water. The overlimed hydrolyzate is currently considered the "best-case scenario" for industrial processing as previous studies have indicated that treatment of hydrolyzate liquors with strong bases helps reduce inhibition *(11)*.

All hydrolyzate bioassays were performed at 30% loading levels because precipitation in the bioassay tube increased with increased hydrolyzate levels. The relative toxicity studies were carried out by measuring the OD_{600nm} of the cultures to determine cell growth, and by ethanol yield to ascertain fermentation performances. The net OD_{600nm} values, and ethanol yields for positive and overlimed controls as well as for the neutralized 30% hydrolyzate are shown in Table 3. All values are averages of duplicate samples.

These data reveal inhibition of cell growth and ethanol production at the 30% level. The increased OD_{600nm} value for the 30% (v/v) hydrolyzate level relative to the negative control is attributed to the turbidity caused by

precipitation that tended to cling to the sides of the tubes. In support of this statement are the data obtained from the HPLC analysis of the supernatant, which indicate a lower ethanol yield for the 30% hydrolyzate (v/v) relative to the overlimed negative control (0.6 mg/mL vs 2.37 mg/mL, respectively).

Two important facts were revealed from this experiment: First, the precipitation caused by the hydrolyzate makes the OD values for ascertaining cell growth somewhat unreliable. Although these nonlinear OD measurements have been corrected with an appropriate blank, when precipitation takes place the method should be used only for obtaining a rough estimate of the cell growth of organisms in a specific medium. Second, based on the ethanol yields, the hydrolyzate clearly inhibits the ability of the recombinant *Zymomonas* to ferment xylose to ethanol, and can only be marginally improved by overliming protocols.

Inhibitory Effects of Suspected Toxins

In order to determine the most toxic components in the liquid hydrolyzate, the data obtained from the HPLC analysis of the liquid hydrolyzate were used to estimate the loading levels for bioassays performed with individual suspected inhibitors (*see* Table 4). In addition to compounds detected in the hydrolyzate, toxic effects of several other potential inhibitors *(13)*, including the alkaloid glaucine (a major compound present in yellow poplar), were analyzed. Three loading levels were chosen for each compound tested. No precipitation was observed during the addition of pure compounds to the test media, and thus cell-growth efficiency was evaluated from OD values at all three loading levels. Ethanol yields were measured at the 100%, and the results are given in Table 4.

In general, the cell-growth values (OD) followed that of the ethanol yields. Inconsistent results from the cell-growth inhibition studies preclude their complete presentation herein. Although complete explanation of the observed inconsistencies is not possible, part of the problem may reside with the organism itself and its response to the compounds added. For example, visualization of the furfural and HMF-bioassay samples under the microscope revealed that the organisms responded differently to the two compounds. In the presence of HMF, the cells remain viable but tend to agglomerate, modifying the OD response. These results clearly suggest that care must be taken in relying on growth measurements (OD_{600nm}) in assessing the toxicity of hydrolyzate components. The only completely reliable method of ascertaining fermentation efficiency is through determination of ethanol yields.

The ethanol yields indicate that acetic acid is by far the most toxic compound, followed by caproic acid and furfural (Table 4). Except for vanillin and syringaldehyde, the other phenolic compounds tested have

Table 4
Ethanol Fermentation Yields Obtained after a 48–h Fermentation with Individual Suspected *Zymomonas* Inhibitors (100% Level)

Sample	Inhibitor concentration[a] (mg/mL)	Ethanol[b] (mg/mL)	% Performance
Positive control	—	13.43–14.83	100
Overlimed hydrolyzate	—	1.37–1.43	9
Acetic acid	9.03	<1	0
Furfural	0.95	8.52	58
HMF	0.09	11.86	80
Gallic acid	0.173	11.39	77
Syringaldehyde	0.130	9.48	64
Coniferyl alcohol	0.050	12.10	82
Vanillin	0.043	8.71	65
Vanillic acid	0.084	13.63	101
Sinapic acid	0.060	11.93	89
Syringic acid	0.093	12.80	95
Protocatechuic acid	0.050	9.68	72
Glaucine	0.052	10.13	75
Caproic acid	0.064	7.64	57

[a] Inhibitor concentrations used in this study were estimated from the HPLC analyses of compounds detected in the hydrolyzate. Inhibitor concentrations for other compounds were based on the values from ref. *13*. The glaucine level was estimated based upon extractive content of yellow poplar, assuming that glaucine was 10% of the extract.

[b] Samples were added to media dissolved in 0.1 mL of ethanol. Thus, the ethanol yields reported are subtracted from the initial ethanol in the media.

only a moderate effect on ethanol production. Most of the compounds are therefore only slightly inhibitory to cell growth and ethanol production, with organic acids being the strongest inhibitors. There is also a trend in which organic acids and aldehydes are more inhibitory than lignin acids, alcohols, or the one alkaloid tested. These results suggest that efforts to minimize toxicity should initially be directed at removing the acetic acid formed during the acid hydrolysis.

Toxicity Testing of Extracts from Treated and Untreated Solid Biomass Samples

Lignin and extractives in wood can be solubilized during the acid pretreatment of woody biomass, without formation of compounds such as acetic acid. These extracts may or may not be toxic to the organisms that convert sugars to ethanol. However, as a major role of extractives in a growing tree is to prevent pathogen invasion, the compounds are inherently toxic. Oaks contain a high concentration of hydrolyzable tannins, whereas yellow poplar is noted for its alkaloid content *(22,24)*. Therefore, the toxicity of these fractions

Table 5
Ethanol Fermentation Yields after 48 h for the Controls and Wood Extracts

Sample (% Loading)[a]	Ethanol (mg/mL)[b] Acetone:Water Extract (% Performance)	Ethanol (mg/mL) Hot Methanol Extract (% Performance)
Positive control	16.79 (100)	16.79 (100)
Overlimed hydrolyzate	1.12 (7)	1.12 (7)
Red oak (30%)	1.26 (8)	7.23 (43)
Red oak (15%)	1.40 (9)	12.47 (74)
Red oak (5%)	6.81 (41)	11.87 (71)
White oak (30%)	<1 (0)	<1 (0)
White oak (15%)	1.32 (8)	<1 (0)
White oak (5%)	4.71 (28)	3.51 (21)
Yellow poplar (30%)	11.26 (67)	11.33 (67)
Yellow poplar (15%)	13.72 (82)	12.87 (77)
Yellow poplar (5%)	11.86 (70)	12.43 (74)
Treated solids (30%)	2.82 (17)	13.76 (82)
Treated solids (15%)	10.03 (60)	14.60 (87)
Treated solids (5%)	13.59 (81)	12.03 (72)

[a] The levels of extractives added to the media were estimated based on the extractive yields obtained from the samples, and the amount of xylose found in the wood hydrolyzate using the relationship: (% yield of extract)/(% xylose in wood) = (extract conc. in hydrolyzate)/(xylose conc. in hydrolyzate). This represents the relationship between extractive and xylose contents, if one assumes that all of the extractives and xylose were solubilized by the pretreatment process.

[b] All the extracts were freeze-dried to ensure complete removal of solvents, and the extractives were added to the media dissolved in ethanol:water (1:1, 0.2 mL). Ethanol values reported are values obtained after subtracting initial ethanol in media (at T_0).

should be ascertained, as species with highly toxic extracts may not be desirable as substrates for industrial bioconversion.

Owing to the poor solubility of the extractives either in ethanol or ethanol:water, and the severe precipitation that occurred when these extractives were added to the media, testing was performed at 30%, 15%, and 5% loading levels (*see* Table 5 for details on estimating loading levels). Even at these levels, adding extractives to media tended to cause precipitation, thereby limiting the usefulness of OD measurements.

The results indicate that the red oak and white oak acetone:water extracts are more toxic than the methanol extracts toward *Zymomonas* ethanol fermentation. In both instances, white oak extracts were the most toxic, and yellow poplar extractives were the least toxic. These results correlate with what is known about wood and its resistance to microbial decay. Of the three species, white oak is the most durable, followed by red

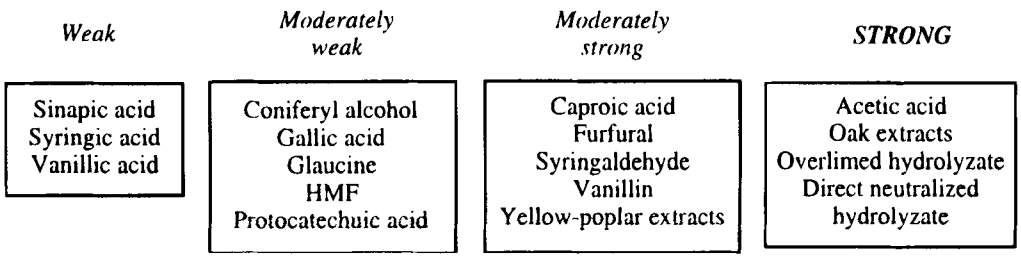

Fig. 2. Relative compound/extract inhibition scale for *Zymomonas mobilis* CP4(pZB5) xylose fermentation.

oak and yellow poplar. This resistance to decay is mostly owing to the tannins present in the white and red oaks. The mechanism of biological resistance is not completely clear, but tannins are well known for their ability to precipitate proteins *(29)*. Extracellular degradatory enzymes secreted by a wood-invading organism would, when in intimate contact with a tannin molecule, undergo precipitation and subsequent inactivation *(24)*. Thus, it is also understandable that significant precipitation occurred in the growth inhibition bioassay.

The fact that oak extractives are much more inhibitory than that of yellow poplar suggests that yellow poplar or fast-growing wood species such as cottonwood, aspen, or poplar (low tannin content woods) would be much more desirable substrates for bioconversion. Our future studies will concentrate on a hydrolyzate liquor prepared from yellow poplar sawdust.

Removing compounds from wood hydrolyzates that are inhibitory to microorganisms should improve the overall efficiency of the biomass conversion process. Previous studies indicate that treating wood hydrolyzate with sodium hydroxide, calcium hydroxide, or a strong anionic-exchange resin helps remove these compounds *(11,27)*. The results obtained here suggest that with respect to xylose utilization, the increase in fermentation efficiency is low when overliming is used. A qualitative ranking for the compounds and extracts tested is shown in Fig. 2. Acetic acid and the oak extracts were the most inhibitory compounds in the liquid hydrolyzate, furfural exhibited a moderate effect. Solvent extraction methods such as this may be useful in separating a fairly toxic organic fraction from the liquid hydrolyzate, and this should be explored in more detail. For example, a wood-to-ethanol facility may be able to use ethyl acetate as the extraction solvent with the ester made from the ethanol and acetic acid produced at the facility. This and other amelioration strategies are currently under investigation.

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REFERENCES

1. Lawford, H. G. (1988), *Appl. Biochem. Biotechnol.* **17,** 203–219.
2. Baratti, J. and Bu'Lock, J. D. (1986), *Biotechnol. Adv.* **4,** 95–115.
3. Rogers, P. L., Lee, K. J., Skotnicki, M. L., and Tribe, D. E. (1982), *Adv. Biochem. Eng.* **23,** 27–84.
4. Lee, K. J., Tribe, D. E., and Rogers, P. L. (1979), *Biotechnol. Lett.* **1,** 421–426.
5. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. (1995), *Science* **267,** 240–243.
6. Keim, C. R. (1983), *Enzyme Microbiol. Technol.* **5,** 103–114.
7. Rodriguez, E., and Callieri, D. A. S. (1986), *Biotechnol. Lett.* **8,** 745–748.
8. Beavan, M., Zawadzki, B., Droniuk, R., Lawford, H., and Fein, J., (1989), *Appl. Biochem. Biotechnol.* **20/21,** 319–326.
9. Parekh, S. R., Parekh, R. S., and Wayman, M. (1989), *Process Biochem.* **24,** 88–91.
10. Pettersen, R. C. (1984), *ACS Symp. Ser.* **207,** 57–126.
11. McMillan, J. D. (1994), *ACS Symp. Ser.* **556,** 411–437.
12. Ingram, L. O., Alterthum, F., Ohta, K., and Beall, D. S. (1990), *Dev. Ind. Microbiol.* **31,** 21–30.
13. Tran, A. V., and Chambers, R. P. (1986), *Enzyme Microb. Technol.* **8,** 439–444.
14. Örsa, F., and Holmbom, B. (1994), *J. Pulp and Paper Sci.* **20,** 361–366.
15. Kermasha, S., Goetghebeur, M., and Dumont, J. (1995), *J. Agric. Food Chem.* **43,** 708–716.
16. Kaar, W. E., Cool, L. G., Merriman, M. M., and Brink, D. L. (1991), *J. Wood Chem. Technol.* **11,** 447–463.
17. Morris, W. E. and Johnson, D. B. (1967), in *Procedures for the Chemical Analysis of Wood Products*, USDA Forest Products Lab, Madison, WI.
18. *Technical Association of the Pulp and Paper Industry (TAPPI) Standard Methods*, T13, T222, T250, and T211.
19. Antal, M. J., Leesomboon, T., Mok, W. S., and Richards, G. N. (1991), *Carbohydr. Res.* **217,** 71–85.
20. Timell, T. E. (1964), *Adv. Carbohydr. Chem.* **19,** 247–302.
21. Fengel, D. and Wegener, G. (1989), *Wood: Chemistry, Ultrastructure, Reactions*, 2nd ed., Walter de Gruyter, Berlin.
22. Rowe, J. W. and Conner, A. H., (1979), in *Extractives in Eastern Hardwoods, A Review*, Gen. Tech. Rep., FPL-18, USDA Forest Products Laboratory, p. 67.
23. Hillis, W.E. (1987), in *Heartwood and Tree Exudates*, Springer-Verlag, Berlin, p. 268.
24. Scalbert, A. (1992), in *Plant Polyphenols*, Hemingway, R. W. and Laks, P. E., eds., Plenum, New York, pp. 259–280.
25. Frazer, F. R. and McCaskey, T. A. (1989), *Biomass* **18,** 31–42.
26. Lapierre, C., Rolando, C., and Monties, B. (1983),*Holzforchung* **37,** 189–198.
27. Stanek, D. A. (1958), *TAPPI* **41,** 601–609.
28. McCaskey, T. A., Rice, M. D., and Smith, R. C. (1986), in *Biomass Energy Development*, Smith, W. H., ed., Plenum, New York, pp. 573–585.
29. Luck, G., Liao, H., Murray, N. J., Grimmer, H. R., Warminski, E. E., Williamson, M. P., Lilley, T. H., and Haslam, E. (1994), *Phytochemistry* **37,** 357–371.