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Comparison of aromatic monomers in lignocellulosic biomass prehydrolysates

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Differences in the relative toxicity of xylose-rich prehydrolysates derived from woody and herbaceous feedstocks are likely due to the relative abundance of a variety of inhibitory compounds. Acetate, as well as several aromatic monomers, has been shown to be an inhibitor of the xylose-fermenting yeast, *Pichia stipitis*. Comparative information on the concentration of known and likely inhibitors, other than acetate, is lacking. The present study provides data on the aromatic monomer composition of representative herbaceous and woody prehydrolysates. Dilute-acid prehydrolysates were prepared from three feedstocks; two herbaceous, corn stover and switchgrass (*Panicum virgatum* L.), and one woody (poplar). The prehydrolysates were neutralized with $Ca(OH)_2$, extracted with ethyl acetate, trimethylsilylated, and analyzed by GC-MS. Fourteen aromatic monomers were tentatively identified by comparison with published mass spectra. The concentrations of the aromatic monomers totalled 112, 141 and 247 mg L^{-1} for corn stover, switchgrass and poplar prehydrolysates, respectively. This is also the order of increasing inhibition of growth and ethanol productivity observed for *Pichia* fermentations. The woody prehydrolysate contained approximately four-fold more syringyl-based monomers than did the herbaceous prehydrolysates, while guaiacyl-containing compounds were more evenly distributed.

Keywords: lignocellulosic biomass; prehydrolysate; *Pichia stipitis*; aromatic; inhibitors

Introduction

The production of fuel ethanol from lignocellulosic biomass feedstocks has several benefits including domestic availability, pollution reduction and ease of introduction into existing gasoline and diesel distribution networks [12,25]. One widely studied process for converting lignocellulosic biomass to ethanol involves a pretreatment of the feedstock with dilute acid (approximately 1.0% (w/w) sulfuric acid) at temperatures over 140°C, conditions which catalyze the hydrolysis of biomass hemicellulose, but leave the cellulose fraction largely intact [17]. This dilute-acid pretreatment yields a solids stream, consisting of mostly lignin and cellulose, and an aqueous stream, called the prehydrolysate, which contains hemicellulose-derived xylose, lesser amounts of other carbohydrates, sugar degradation products, lignin degradation products, acetic acid, and other compounds [15]. Sugars (mostly xylose) in the prehydrolysate typically represent 15–30% of the original dry weight of the biomass [7]. Rapid and efficient fermentation of these sugars is essential for making biomass-to-ethanol conversion processes economically viable [25].

Dilute-acid prehydrolysates contain many compounds other than sugars, and some of these inhibit fermentation by and growth of microorganisms. These inhibitors can be divided into groups based on their origin [15]: (1) compounds released during pretreatment, eg acetic acid; (2) sugar degradation products, eg furfural; (3) lignin degradation products, eg syringaldehyde; (4) fermentation products, eg ethanol; and (5) contaminants released by processing equipment, eg chromium. for a review of prehydrolysate fermentation and a discussion of the role of inhibitors, see Olsson and Hahn-Hägerdahl [15].

Several papers describing ethanol yields from *Pichia stipitis* fermentations of dilute-acid prepared lignocellulosic prehydrolysates have been published, including studies of prehydrolysate derived from aspen wood [26], red oak [22], sugar cane bagasse [20], corn cob [10], mixed wood chips [18], eucalyptus [9], switchgrass [8], corn stover [8,16], poplar [8,16], rice straw [13], *Pinus radiata* [19] and wheat straw [4]. *Pichia*, although an efficient fermenter of xylose is susceptible to inhibition by toxic compounds in lignocellulosic prehydrolysates, leading to lower ethanol productivities (g ethanol L^{-1} h^{-1}) and yields (g ethanol g sugar consumed^{-1}) compared to those achieved in fermentations of control media [15]. Among the compounds identified in prehydrolysates which are known to inhibit *Pichia* fermentations are vanillin, syringealdehyde, acetic acid and furfural [5,22,23]. The lignin-derived compounds, ie syringealdehyde and vanillin, have been shown to be particularly potent inhibitors of ethanol production and cell growth [5,22], especially when compared on a concentration basis to acetic acid or sugar degradation compounds. In particular, vanillin, a guaiacyl-containing compound, was shown to be more inhibitory than representative syringyl- and hydroxybenzyl-containing compounds [5].

Phenolic compounds in prehydrolysates prepared from woody substrates have been described [1,3,22,26]. In contrast, relatively little is known about the lignin-derived compounds in prehydrolysate obtained from herbaceous

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lignocellulosics such as switchgrass (*Panicum virgatum* L.) and corn stover. Switchgrass and corn stover are considered to be excellent candidate feedstocks for conversion to ethanol [6,21], and thus are of particular interest.

The aim of the present study was to identify and quantify phenolics and lignin-derived compounds found in the prehydrolysate derived from two herbaceous feedstocks (switchgrass and corn stover) and one woody feedstock (poplar). These feedstocks were chosen based on their potential for use in biomass-to-ethanol processes and because the relative fermentability of their prehydrolysates has been established [8]. The results provide analytical information that should be useful in determining the chemical basis for differences in rates and extents of prehydrolysate fermentations as well as for designing detoxification processes [15].

Materials and methods

Sample preparation

Milled and dried feedstocks (poplar, corn stover and switchgrass) were treated in a 0.6-L stainless steel Parr reactor at 10% solids (180°C, 1% (w/w) H_2SO_4, 1 min) as described [7]. The resulting material was filtered through VWR 413 grade paper, and neutralized to pH 6.0 with $Ca(OH)_2$ as described [8]. The pretreatment conditions were chosen to optimize the yield of xylose in the liquid prehydrolysate. One milliliter of 0.13 g L^{-1} *o*-vanillin (Sigma, St Louis, MO, USA) internal standard solution was added to 10 ml of prehydrolysate. Ten milliliters of ethyl acetate were added and mixed by inversion. After separation of the two phases, the upper layer was transferred to a test tube and dried under N_2 at 40°C for 15 min. The residue was redissolved in 0.5 ml of ethyl acetate and transferred to a vial to which was added 50 μl pyridine and 300 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma. The mixture was allowed to react at room temperature overnight.

GC-MS

Gas chromatography/mass spectrometry was performed on a 10-m SE-54 capillary column using a Finnigan GC/MS. Mass spectra were obtained at 70 eV. For quantification of tentatively identified phenolic compounds, a 1 : 1 response ratio with *o*-vanillin was assumed.

Results/discussion

Partial total ion count (TIC) chromatograms obtained from TMS-derivatised ethyl acetate extracts from corn stover, switchgrass and poplar prehydrolysates are shown in Figure 1. In all cases, chromatograms reflect neutralized prehydrolysates, ie suitable for fermentation. This approach was taken to facilitate comparison of the GC-MS data with fermentation results. Ethyl acetate was used for extraction because it has been shown to remove phenolic compounds such as parahydroxybenzoic acid and vanillin from aspen prehydrolysates. Ethyl acetate was also shown to reduce inhibition of microbial growth and fermentation of prehydrolysates [26]. Thus it can be expected that microbial inhibitors, along with non-inhibitory compounds, are to be found in an ethyl acetate extract.

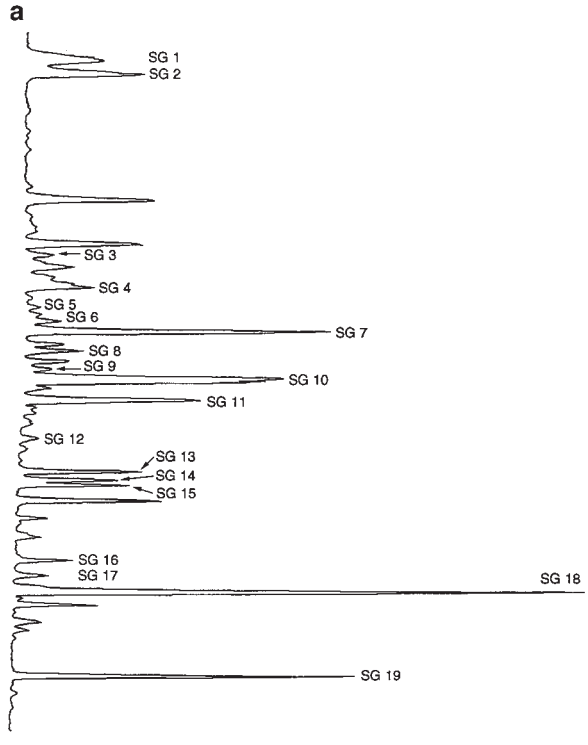
By matching mass spectra with those in the literature [11,14,27], 14 compounds with structures indicative of lignin monomers or esterified phenolics were tentatively identified. Table 1 summarizes the identification and quantification of the chromatogram peaks from the three feed stocks. The concentrations of the identified lignin-derived components total 247, 141 and 112 mg L^{-1} for poplar, switchgrass and corn stover prehydrolysates, respectively. This is also the order of decreasing microbial inhibition, as measured by ethanol yield, productivity, and cell mass increase in *Pichia* fermentations, as shown in Table 2. In addition, this is also the order of decreasing Klason lignin content for the feedstocks [7]. Similarly, the Klason lignin content of native woody feedstocks was found to be negatively associated with ethanol yields using *Saccharomyces* in simultaneous saccharification and fermentation experiments [24]. The acetate concentrations averaged 1.3, 2.0 and 2.9 g L^{-1} for corn stover, switchgrass and poplar prehydrolysates, respectively. Furfural and hydroxymethylfurfural concentrations were less than 0.01 g L^{-1} for all neutralized prehydrolysates.

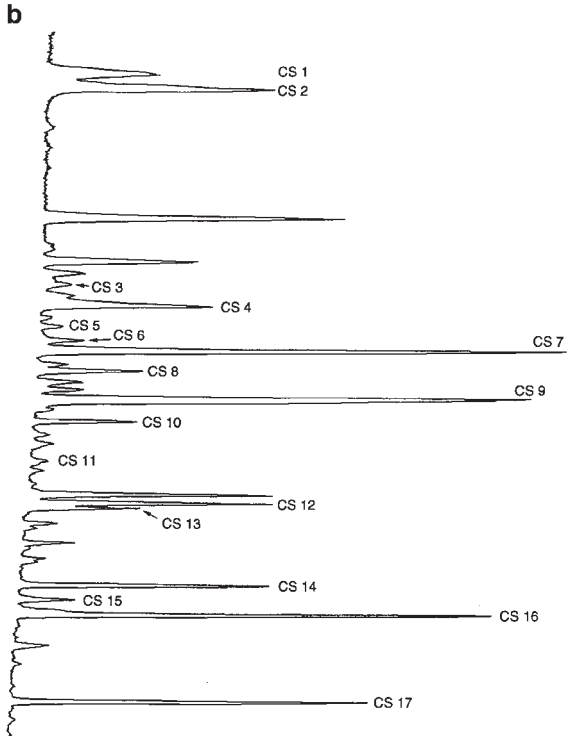
The most striking difference in both the type and amount of aromatic compounds was found among the syringyl compounds. In popular prehydrolysate, the concentration of syringyl-containing compounds was approximately fourfold higher than in switchgrass or corn stover prehydrolysates. The guaiacyl compounds were more evenly distributed among the three prehydrolysates, with corn stover having the lowest concentration of identified guaiacyl derivatives, and switchgrass the highest. Although other work indicates that guaiacyl-containing monomers are highly toxic to *Pichia* [5], the concentrations used in that study were far higher than anything seen in the prehydrolysates examined here. The work currently described was conducted on native, unextracted biomass, so it is difficult to determine whether differences in the ratios of the three main phenyl derivatives are due to differences in the core lignin structures of the three feedstocks, or if they represent differences in more-easily solubilized non-core lignin materials.

The concentrations of syringealdehyde (S-CHO, Table 1) and vanillin (G-CHO, Table 1) in the poplar prehydrolysate are similar to those reported by Buchert *et al* [3] for birch wood prehydrolysate prepared by steam treatment and extracted with dimethylchloride without neutralization, although the concentrations of 1-guaiacyl acetol (G-CH_2-CO-CH_2OH, Table 1) and 1-syringyl acetol (S-CH_2-CO-CH_2OH, Table 1) in poplar prehydrolysate were found to be 20–30 fold higher. In comparison to red oak prehydrolysate pretreated under similar conditions [22], poplar prehydrolysate contained 5-fold less vanillin and 10-fold less syringealdehyde, although the extraction and preparation regimens were different.

Compounds which were unique to specific prehydrolysates included vanillyl propanol (G-CH_2-CH_2-CH_2OH, Table 1) in switchgrass, syringyl methyl ketone (S-CO-CH_3, Table 1) in corn stover and para-hydroxybenzoic acid (H-COOH, Table 1) in poplar. Para-hydroxybenzoic acid is a major component in poplar-derived hydrolysates [1,2].

In previous work [8] it was shown that prehydrolysates





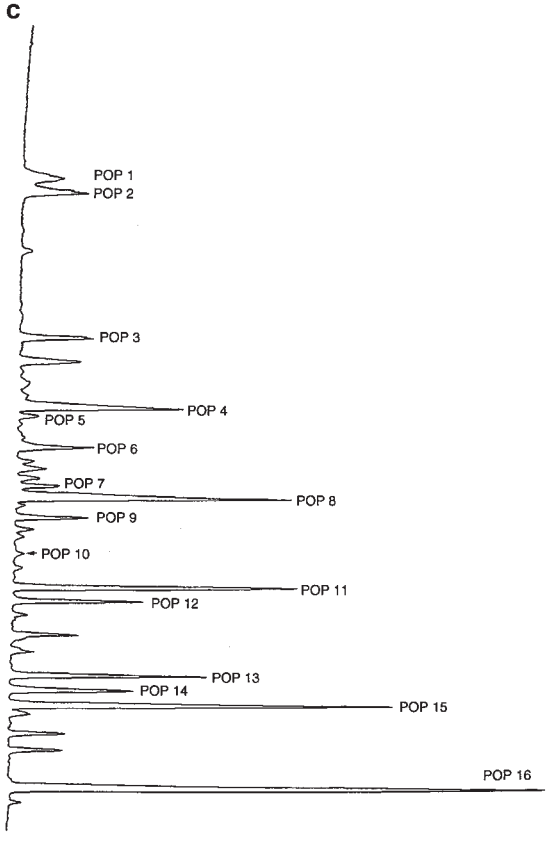


Figure 1 TIC chromatograms obtained from TMS-derivatized ethyl acetate extracts of switchgrass (a), corn stover (b) and poplar (c) prehydrolysates. Tentative identifications based on mass spectra are given below. See Table 1 for explanation of H, G, and S.

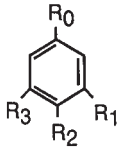
Peak label			Tentative identification
SG 1	CS 1	POP 1	ortho-vanillin (internal standard)
SG 2	CS 2	POP 2	G—CHO
		POP 3	H—COOH
SG 3			arabinose
	CS 3		arabinofuranose
	CS 4	POP 4	S—CHO
SG 4	CS 5		arabinose
SG 5			arabinose
SG 6			levo-glucosan
SG 7		POP 5	xylose
SG 8			arabinose
	CS 6	POP 6	xylopyranose
	CS 7		xylose
SG 9		POP 7	G—CH_2—CO
	CS 8		S—CO—CH_3
SG 10	CS 9	POP 8	xylose + aromatic moieties
	CS 10	POP 9	G—C═C—COH
SG 11			G—CH_2—CH_2—CH_2OH
SG 12	CS 11	POP 10	G—CO—CHOH—CH_3 (ketonic)
SG 13		POP 11	S—CHOH—CO—CH_3
SG 14	CS 12		H—C═CH—COOH
SG 15	CS 13	POP 12	G—CH_2—CO—CH_2OH (ketonic)
SG 16	CS 14	POP 13	S—CO—CO—CH_3
SG 17	CS 15	POP 14	S—CH_2—CO—CH_2OH (ketonic)
SG 18	CS 16	POP 15	G—CH_2—CO—CH_2OH (transenolic)
SG 19	CS 17	POP 16	S—CH_2—CO—CH_2OH (transenolic)

from herbaceous feedstocks were more easily fermentable than those from woody feedstocks, and that the discrepancies could be only partially attributed to acetate concentration differences. It is demonstrated here that prehydrolysates derived from herbaceous feedstocks (switchgrass and corn stover) contain much less phenolic monomers than poplar-derived prehydrolysate. When considered alongside the lower acetate concentrations of the herbaceous prehy-

Table 1 Lignin-derived compounds tentatively identified in dilute-acid prehydrolysates prepared from poplar, switchgrass and corn stover

Phenolic moietie[c]	R_0	Chromatogram peak labels	Poplar (mg L^{-1})	Switchgrass (mg L^{-1})	Corn stover (mg L^{-1})
H	—COOH	POP3	11.1 (0.8)[a]	nd[b]	nd
H	—C═CH—COOH	SG14,CS13	nd	7.7 (0.2)	10.9 (0.2)
S	—CHO	CS4, POP4	29.3 (2.0)	nd	10.0 (0.3)
S	—CHOH—CO—CH_3	SG13, POP11	35.3 (2.2)	9.1 (0.3)	10.0 (0.6)
S	—CH_2—CO—CH_2OH	SG17,19; CS15,17; POP14, 16	74.4 (4.0)	26.3 (1.5)	17.2 (0.8)
S	—CO—CH_3	CS8	nd	nd	5.2 (0.1)
S	—CO—CO—CH_3	SG16, CS14, POP13	17.0 (0.8)	2.6 (0.1)	9.5 (0.3)
G	—CO—CHOH—CH_3	SG12, CS11, POP10	1.0 (0.1)	2.0 (0.1)	nd
G	—CHO	CS2, SG2, POP2	15.1 (0.4)	16.5 (0.6)	19.6 (0.2)
G	—CH_2—CO	SG9, POP7	4.4 (0.4)	1.5 (0.1)	nd
G	—CH_2—CH_2—CH_2OH	SG11	nd	18.4 (0.8)	nd
G	—C═CH—COH	CS10, POP9	8.5 (0.5)	trace	4.0 (0.6)
G	—CH_2—CO—CH_2OH	SG15, 18; CS13, 16; POP12, 15	51.2 (2.5)	56.7 (3.0)	26.0 (1.1)
Total			247.3	140.8	112.4

[a]Values in parentheses are standard errors of the means.
[b]nd = not detected.
[c]



Moietie	R_1	R_2	R_3
Hydroxyphenyl (H)	H	OH	H
Guaiacyl (G)	OCH_3	OH	H
Syringyl (S)	OCH_3	OH	OCH_3

Table 2 Inhibitor concentrations and fermentation results obtained from poplar, switchgrass and corn stover predydrolysates

Prehydrolysate source	*p*-Hydroxy-phenyl monomers (mg L^{-1})	Guaiacyl monomers (mg L^{-1})	Syringyl monomers (mg L^{-1})	Acetate[a] (g L^{-1})	Ethanol yield[a] (g ethanol g $sugar^{-1}$)	Ethanol productivity[a] (g ethanol L^{-1} h^{-1})	Final dry cell mass[a] (relative to control)
Poplar	11.1	80.2	156.0	2.9	0.34	0.25	–15%
Switchgrass	7.7	95.1	38.0	2.0	0.38	0.54	+5%
Corn stover	10.9	49.6	51.9	1.3	0.41	0.79	+7%

[a]Calculated from Reference [8].

drolysates, the evidence presented here provides a good rationale for the lower toxicity of herbaceous prehydrolysates. Whether this trend can be generalized to other feedstocks can only be determined by direct comparison. These data provide a basis for comparison in assessing the feasibility of feedstocks for biomass-to-ethanol processes.

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