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Identification of Microbial Inhibitory Functional Groups in Corn Stover Hydrolysate by Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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Abstract

Dilute-acid biomass hydrolysates contain biomass degradation products that are inhibitory to cell growth and fermentation. Overliming with $Ca(OH)_2$ has been found to be one of the most effective methods for detoxifying the dilute-acid hydrolysate for ethanol production. However, the mechanism of overliming is not well understood. Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy was used to elucidate the functional groups involved in the overliming reaction. The ^{13}C-NMR spectra showed that the major functional groups removed during the overliming process were aliphatic and aromatic acids or esters, and other aromatic and aliphatic compounds. Ketone and aldehyde functionalities were not detected in the spectra. This is the first time that ^{13}C-NMR has been used to elucidate the overliming reaction.

Index Entries: Overliming; inhibition; corn stover hydrolysate; carbon-13 nuclear magnetic resonance; acetone.

Introduction

The conversion of biomass feedstocks into fuels and chemicals is a major research area, which has been identified by the US Department of

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Energy (DOE) and the National Research Council in their reports on biobased products development road maps *(1,2)*. The National Research Council projected that by 2010 about 25% of carbon-based chemicals would derive from biomass resources *(2)*.

Corn stover is one of the major feedstocks identified by DOE for short-term production of fuels and chemicals *(3)*. However, corn stover, like other lignocellulosic biomass feedstocks, requires pretreatment to improve access and hydrolysis of the biopolymers. Most pretreatment processes, including dilute-acid hydrolysis, generate extractives and degradation products of lignin and carbohydrates, which have toxic microbial properties *(4–10)*. These products can inhibit cell growth, fermentation, and product formation. The composition of the toxic components is a function of the feedstock and the pretreatment process. The toxicity of the compounds is also a function of the microorganism. Because of the complexity of the problem, several detoxification schemes have been investigated, including overliming *(9–12)*, solvent extraction *(13)*, steam treatment *(9,13)*, and fungal treatment *(14)*. Overliming has been found to be one of the most effective methods for detoxifying various biomass hydrolysates. However, the mechanism of overliming is not well understood. Several methods have been used to identify some of the toxic components of biomass hydrolysates, including gas chromatography/mass spectroscopy *(9,15,16)*, high-performance liquid chromatography *(13)*, and ultraviolet spectroscopy *(17)*.

Carbon-13 nuclear magnetic resonance (^{13}C-NMR) has not been used in the past to examine toxicants in biomass hydrolysates because of the low concentration of the toxic components. In this article, we report the use of ^{13}C-NMR to identify functional groups removed or complexed with calcium ions during overliming.

Materials and Methods

Dilute-acid corn stover hydrolysates were prepared at National Renewable Energy Laboratory's (NREL's) pilot plant facility in Golden, CO. The first batch consisted of a dilute hydrolysate of the composition shown in Table 1. The second batch was more concentrated, but its analysis was not provided.

The hydrolysates were treated according to the scheme in Table 2. The batch 1 sample (composition in Table 1) was concentrated on a rotary vacuum evaporator (40°C, 84 kPa) from 300 to 25 mL. The concentrated sample was divided into two samples. One half was overlimed with $Ca(OH)_2$ according to a modified NREL protocol. Thus, $Ca(OH)_2$ solution was added dropwise to the hydrolysate until the pH was between 9.3 and 10.0. The sample was then placed in a shaker bath at 50°C and 100 rpm for 30 min. The sample was not filtered and the pH was not adjusted before NMR analysis. The other half of the concentrated sample was neither overlimed nor filtered and was analyzed "as is" (CSHB1).

Table 1
Composition of Corn Stover Hydrolysate
(Analysis by NREL)

Compound	Average composition (g/L)
Cellobiose	0.40 ± 0.03
Glucose	6.01 ± 0.28
Xylose	36.83 ± 1.17
Arabinose	6.56 ± 0.18
Galactose	3.09 ± 0.18
Mannose	2.89 ± 0.11
Lactic acid	0.00
Acetic acid	4.24 ± 0.25
Hydroxymethylfurfural	0.46 ± 0.66
Furfural	1.58 ± 0.09

Table 2
Treatment of Corn Stover Hydrolysate Samples Before ^{13}C-NMR Analyses

Sample	Treatment	Label
Batch 1 concentrated corn stover hydrolysate	None	CSHB1
Batch 1 concentrated corn stover hydrolysate	Overlimed to pH 10.0	CSHB1V10
Batch 2 corn stover hydrolysate	None	CSHB2
Batch 2 corn stover hydrolysate	Overlimed to pH 10.0	CSHB2V10
Batch 2 corn stover hydrolysate	Overlimed to pH 7.0	CSHB2V7
Sugar mixture with no acetic acid	None	SM1
Sugar mixture with acetic acid	None	SMAc

The batch 2 hydrolysate was more concentrated than the batch 1 and thus was not concentrated by vacuum evaporation. This hydrolysate was overlimed "as received" with $Ca(OH)_2$ to pH 10.0 and then heated in a shaker bath at 50°C and 100 rpm for 30 min. The pH of this sample (CSHB2V10) was not adjusted but was filtered before analysis. Another batch 2 sample was overlimed to pH 10.0, the pH was adjusted to 7.0 using sulfuric acid, and the sample was heated in the water bath as just described. This sample (CSHB2V7) was filtered and analyzed. A third sample of batch 2 hydrolysate was not overlimed but was analyzed "as received" (CSHB2).

In addition to the hydrolysate samples, two model sugar mixtures of similar composition to the batch 1 hydrolysate (Table 2) were prepared. The first sugar mixture had the same composition as that in Table 2 (SM1), and the second mixture had a sugar composition similar to that in Table 1, except 4.24 g/L of acetic acid was added to the mixture (SMAc).

All samples were dissolved in deuterium oxide (D_2O) and analyzed on a Varian Unity 400-MHz NMR, utilizing a Broad Banded-Direct Detection

probe operating at 100 MHz for ^{13}C detection. A WALTZ-16 modulated BB proton decoupling was applied to the data. Data were acquired for 12 h.

Results and Discussion

Although some of the inhibitors could be evaporated during the course of the concentration and thus reduce their effects on the toxicity of the hydrolysates, previous experiments in our laboratory justified the concentration method. Buhner *(18)* investigated the effect of concentration on the detoxification and fermentability of dilute-acid corn fiber hydrolysate. It was demonstrated that the raw hydrolysate could support *Candida tropicalis* cell growth and fermentation. However, when the sample was concentrated to two and three times the original concentration, the microorganism could neither grow nor ferment any of the concentrated substrates. Clearly, the toxicity of the sample increased even when some components were lost during the vacuum evaporation process.

The ^{13}C-NMR spectra of the six samples are shown in Figs. 1–5. The model sugar mixture spectrum showed well-defined sugar peaks between 60 and 100 ppm (Fig. 1). The acetic acid in the sugar mixture resulted in additional peaks in the spectrum at 20 and 176 ppm, which were assigned to CH_3 and carboxylic carbon of the acetic acid, respectively.

The concentrated hydrolysate (CSHB1) spectrum (Fig. 2A) clearly showed strong signals for the sugar peaks (xylose, glucose, mannose, galactose, and arabinose) in the 60- to 100-ppm region. The assignment of these peaks to sugar carbons agrees with peaks from the model sugar mixture spectrum (Fig. 1).

In addition to the sugar peaks (Fig. 3A), there were relatively weak signals in both the aliphatic and aromatic regions of the spectrum, which suggests the presence of lignin degradation products and other aromatic compounds. The aliphatic and aromatic regions of the spectrum were enhanced to provide more details (Fig. 2B,C). Assignment of the nonsugar peaks in the spectrum (Table 3) was based on our model compound studies and published grass lignin data *(19–23)*. The peaks between 10 and 21 ppm (Fig. 2B) were assigned to saturated hydrocarbons, and, in particular, the relatively strong peak at 20.6 ppm was assigned to the methyl carbon of acetic acid (C1) in accordance with the model sugar spectrum (Fig. 1). The peaks between 22 and 25 ppm were assigned to methyl groups in aliphatic ethers and those between 25 and 35 ppm were assigned to C-α and C-β in methylenes. The peak at 55.4 was due to methoxyl groups in sinapyl or guaiacyl. The peaks between 60 and 110 ppm were assigned to carbons in the sugar mixture. The peaks at 110 to 111 ppm were assigned to C2 in coniferyl with α and β unsaturated.

In the carbonyl region (Fig. 2C), there were relatively strong signals due to aromatic and aliphatic carboxylic carbons (165–180 ppm). The peak at 176 ppm was assigned to acetic acid, as discussed earlier. In addition to the aliphatic carboxylic carbon, there were aromatic carboxylic carbons

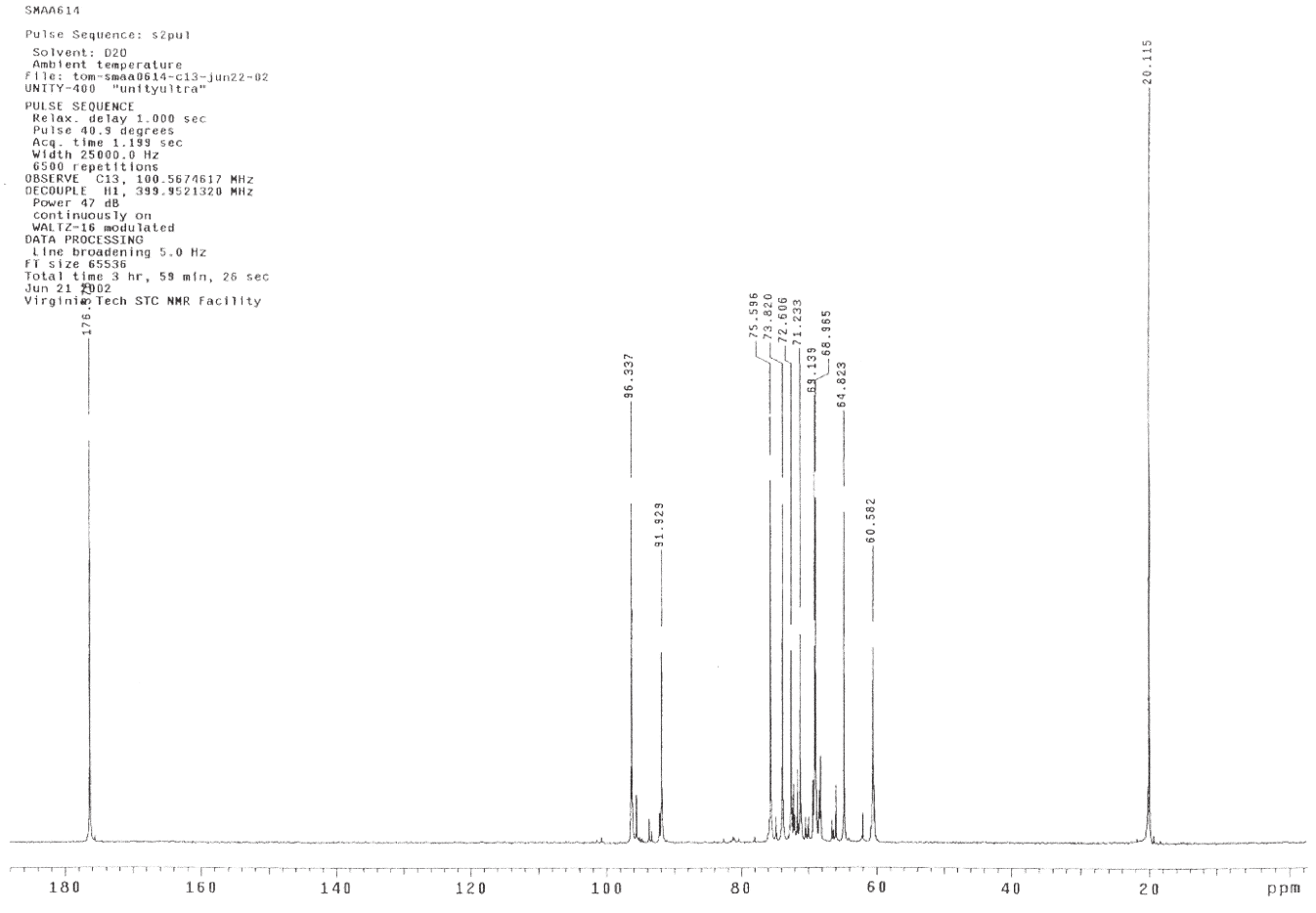

Fig. 1. ^{13}C-NMR spectrum of model sugar mixture spiked with acetic acid.

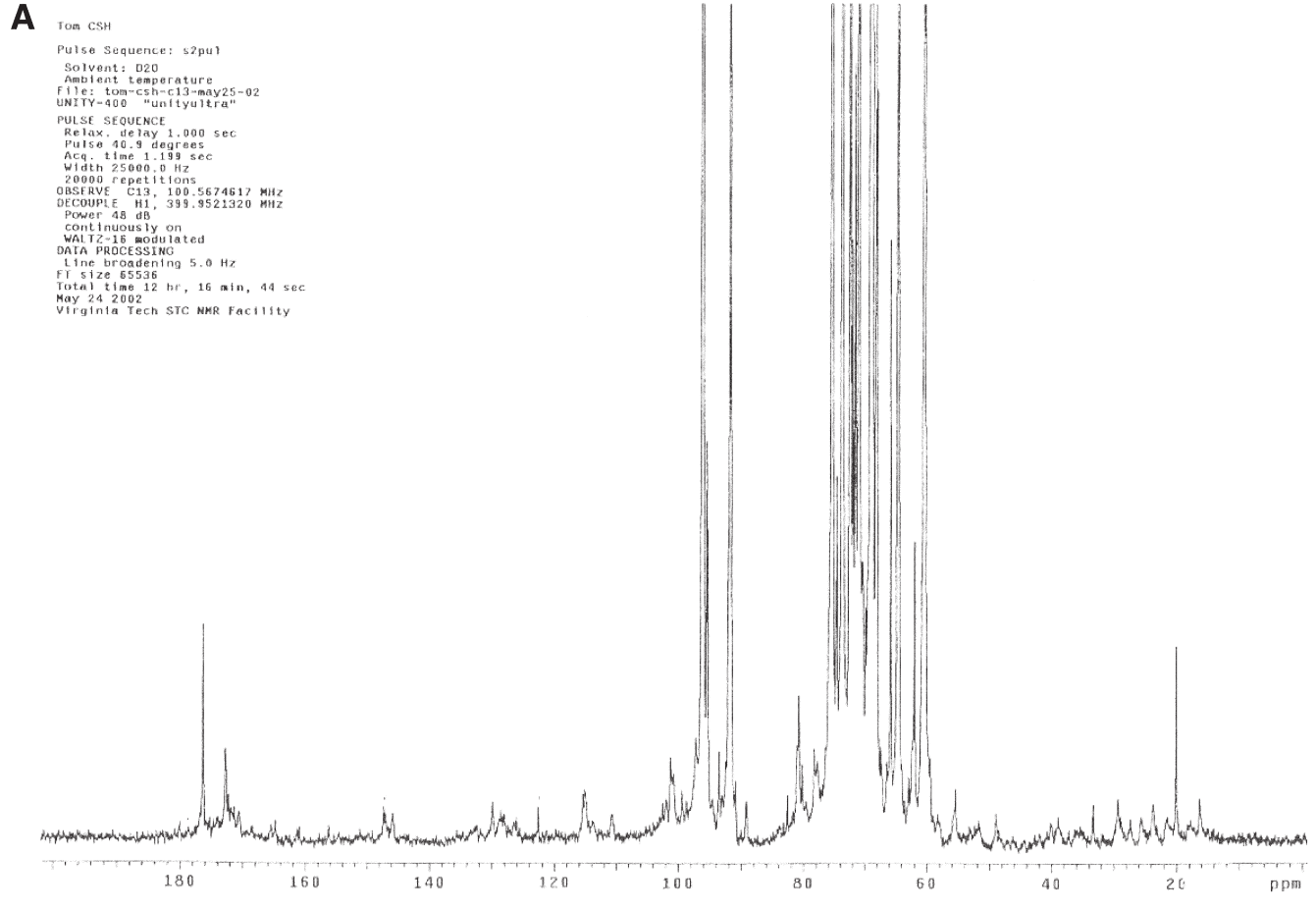

Fig. 2. **(A)** ^{13}C-NMR spectrum of original (nonoverlimed) concentrated corn stover hydrolysate (CSHB1).

Fig. 2. *(continued)* **(B)** Enhanced ^{13}C-NMR spectrum of original (nonoverlimed) concentrated corn stover hydrolysate showing aliphatic region.

Fig. 2. (*continued*) **(C)** Enhanced ^{13}C-NMR spectrum of original (nonoverlimed) concentrated corn stover hydrolysate showing aromatic region (CSHB1V10).

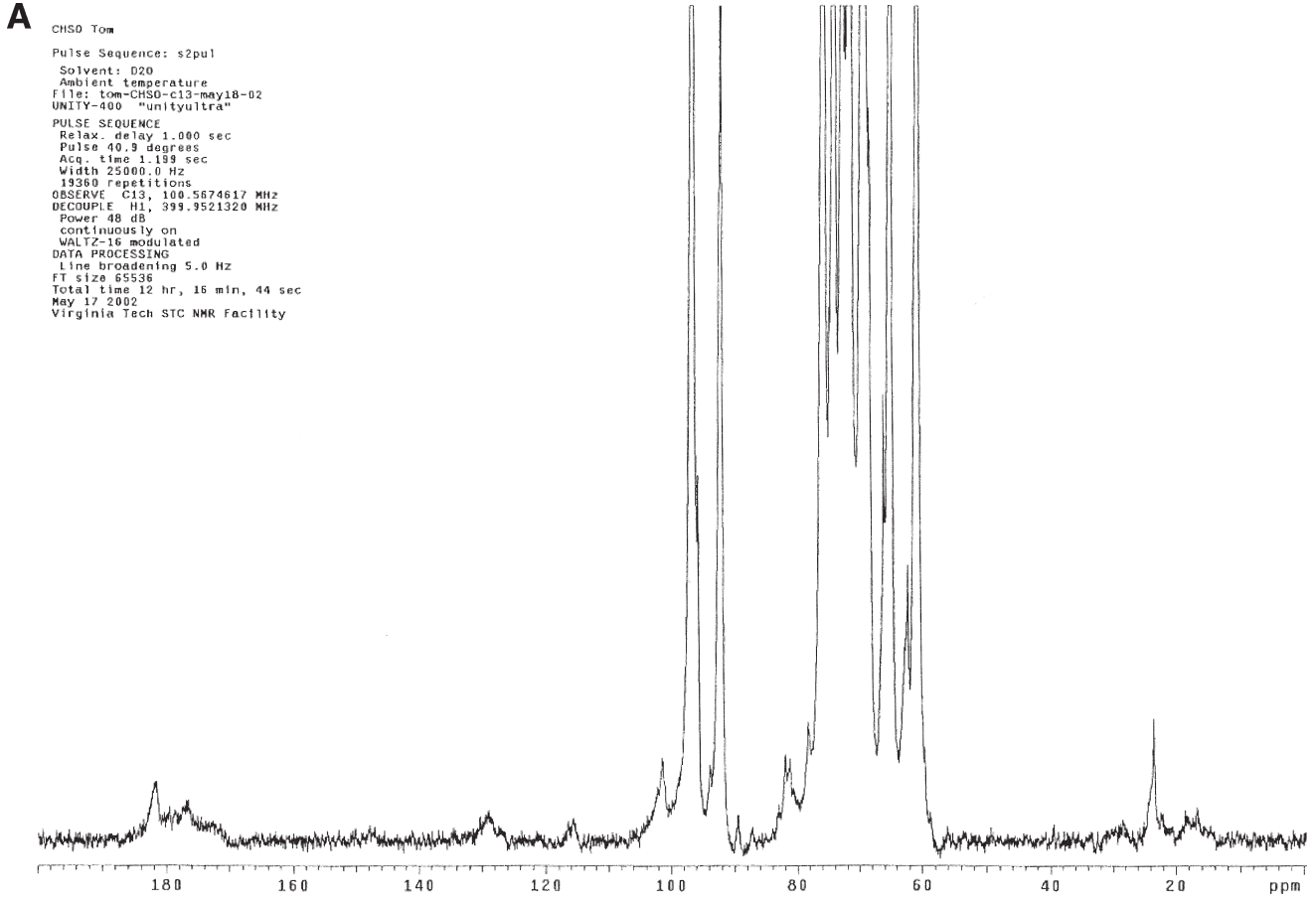

Fig. 3. **(A)** ^{13}C-NMR spectrum of pH 10.0 overlimed concentrated corn stover hydrolysate.

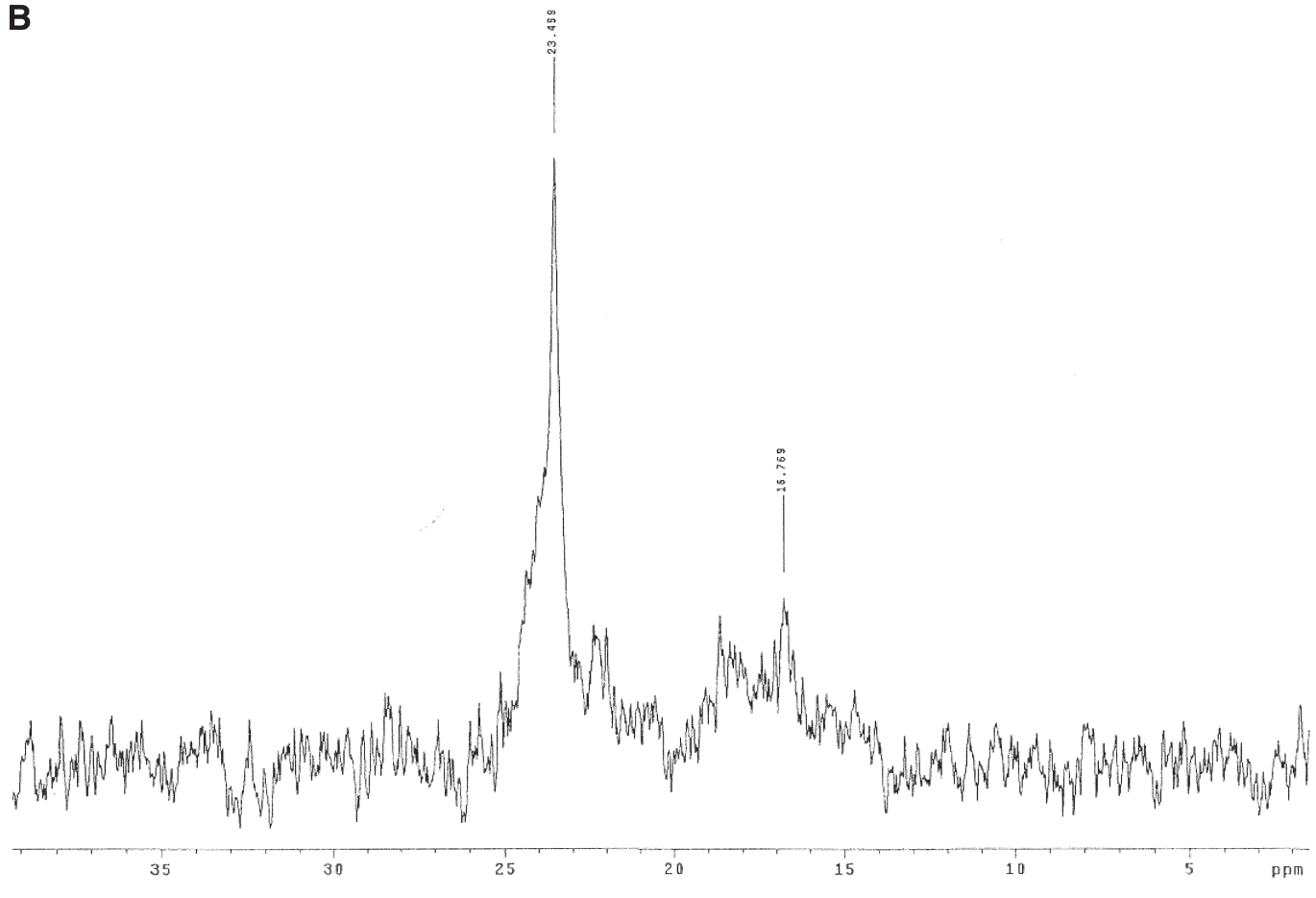

Fig. 3. *(continued)* **(B)** Enhanced ^{13}C-NMR spectrum of pH 10.0 overlimed concentrated corn stover hydrolysate showing aliphatic region.

Fig. 3. *(continued)* **(C)** Enhanced ^{13}C-NMR spectrum of pH 10.0 overlimed concentrated corn stover hydrolysate showing aromatic region.

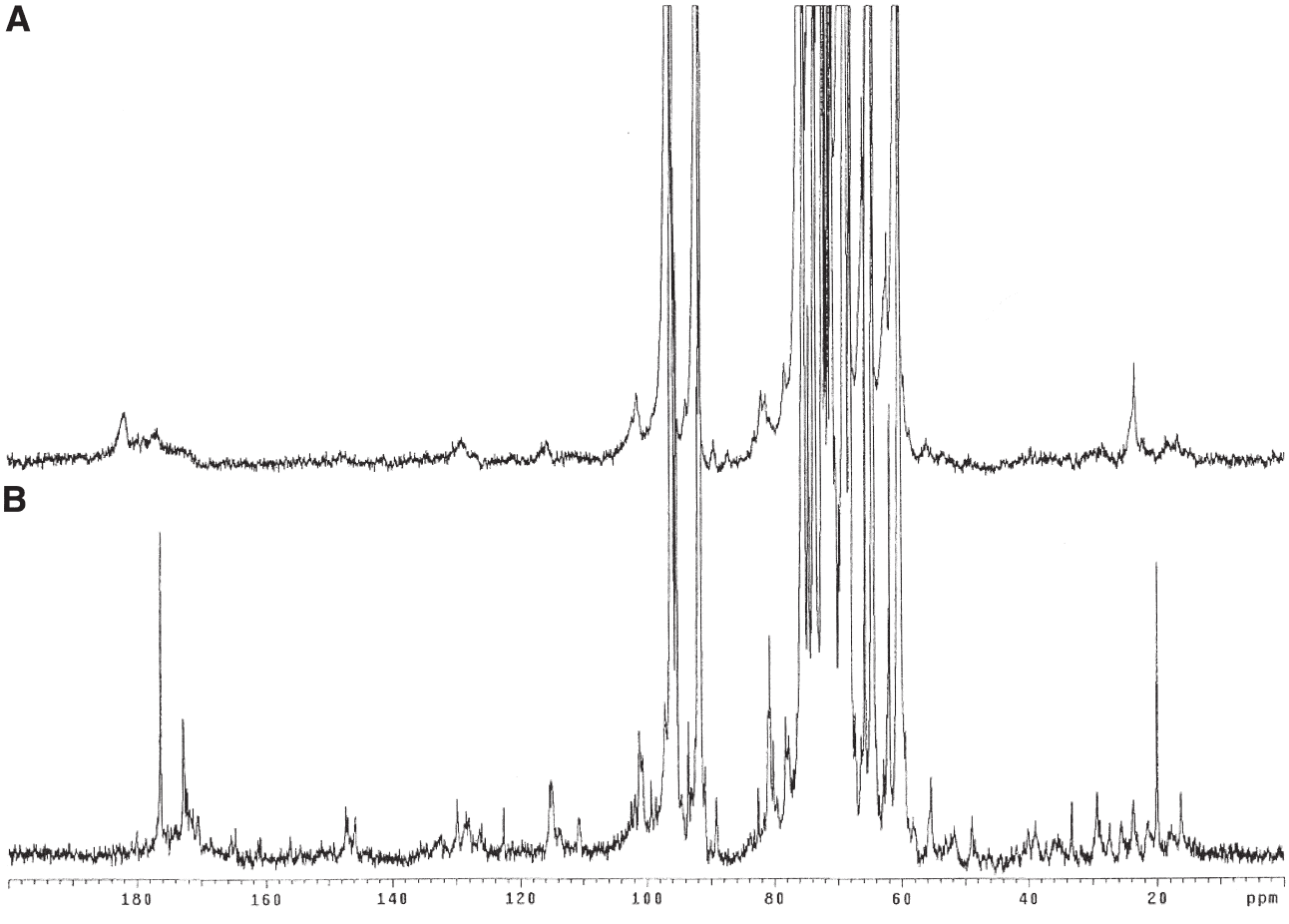

Fig. 4. Comparison of **(A)** overlimed (pH 10.0) and **(B)** nonoverlimed ^{13}C-NMR spectra of concentrated corn fiber hydrolysate.

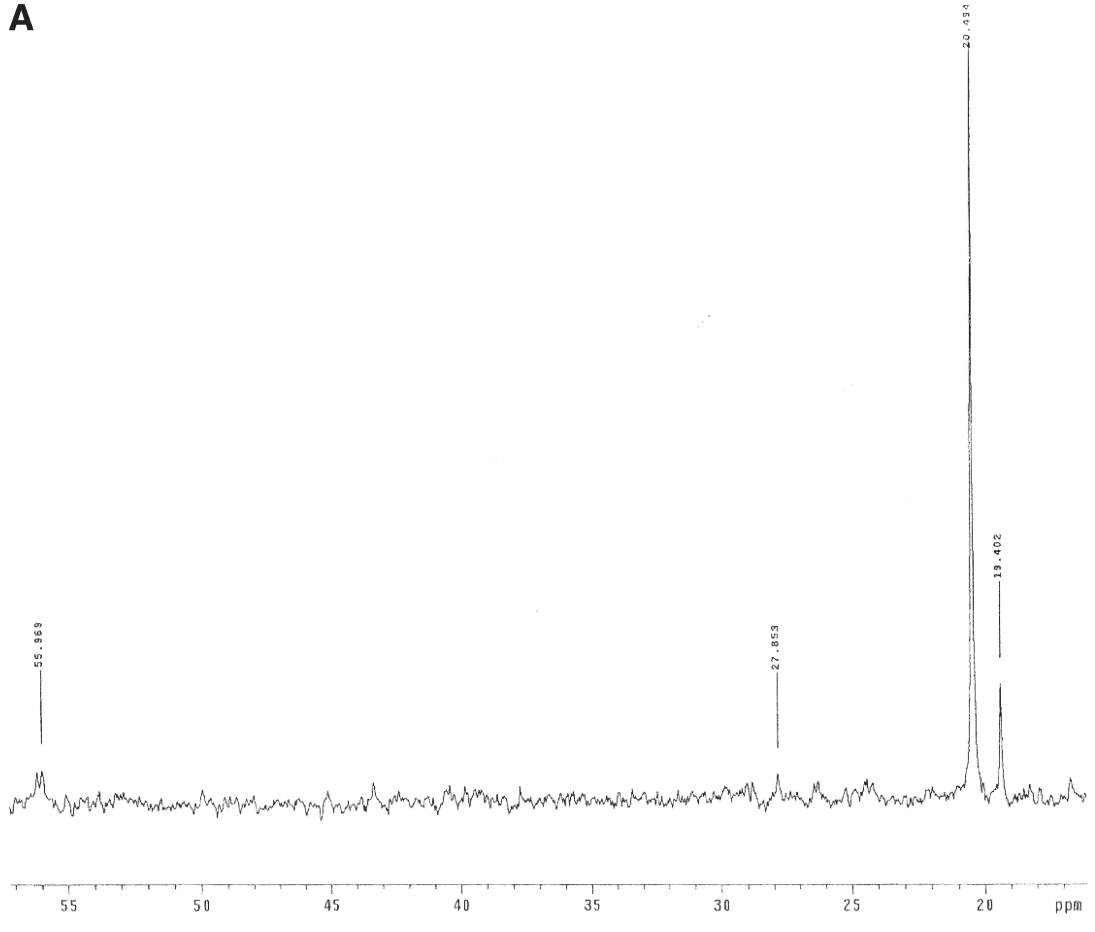

Fig. 5. Enhanced ^{13}C-NMR spectrum of nonoverlimed corn stover hydrolysate analyzed as received (CSHB2) showing **(A)** aliphatic region.

Fig. 5. *(continued)* Enhanced ^{13}C-NMR spectrum of nonoverlimed corn stover hydrolysate analyzed as received (CSHB2) showing **(B)** aromatic region.

Table 3
Assignment of ^{13}C-NMR Peaks
Based on Model Compounds and Published Literature on Lignin

Chemical shift (ppm)	Assignment
10–20	Saturated hydrocarbons
20.6	CH_3 in acetyl (raw hydrolysate)
23.5	CH_3 in acetyl (overlimed hydrolysate)
22–25	Methyl groups in aliphatic ethers
25–35	C-α and C-β methylenes
48.5	Unknown
55.4–56.0	OCH_3 in sinapyl and guaiacyl
60–109	Carbons in xylose, glucose, arabinose, mannose, galactose, and other sugars
110–111.5	C-2 in coniferyl with α, β-unsaturated
115–116	C-5 in guaiacyl and C3/5 in *p*-hydroxyphenyl
120–124	C-1 cinnamyl alcohols, acids, and esters
125–130	C-2/6 in *p*-hydroxyphenyl
131–132	C-β in cinnamaldehyde and C-1 in coniferyl with α-CO
144–150	C-4 in etherified coniferyl, C-in coniferyl, C-3/5 in sinapyl, C-1 in biphenyls, C-α in cinnamic acids and esters with C-4 etherified or unsubstituted
151–153	C-4 in coniferyl and α-CO and C-3/5 in sinapyl etherified
155–162	C-4 in *p*-coumaryl with C-4 or unsubstituted and CO in acetyl
165–173	CO in aliphatic esters or acetyl, C-6 in uronic acids and esters, C-γ in cinnamic acids and esters
170–172	Aromatic ester
176.6	CO in acetic acid
178	CHO in furfural
181	Acetate in overlimed hydrolysate

(170–172 ppm), which resonate at a higher field than the corresponding aliphatic carbon. There were several peaks between 110 and 165 ppm that were assigned to aromatic carbons. Nimz et al. *(20)* showed that esterified coumaric group and free coumaric acid from milled corn stalk lignin resonated at 171.7 and 167.8 ppm, respectively. Coumaric α carbons resonated at 146 ppm and C1 carbons give signals at 126 ppm. Thus, it appeared from the spectrum that the hydrolysate contains coumarate groups. Nimz et al. *(20)* also showed that in bamboo and wheat straw milled wood lignin (MLW) lignins, CO in primary and secondary acetyls resonated at 170.7 and 170 ppm, respectively. In milled corn stalk lignin, ferrulic acid derivatives gave signals of 127.3 (C1), 111.6 (C2), 148 (C3), 116 (C5), 123.7 (C6), 145 (C-α), 116.6 (C-β), and 169 (CO). All these signals are found in the corn stover hydrolysate. However, syringyl carbon signals were either too weak or were not present in the samples. Thus, it appears that a large fraction of the signals in the hydrolysate derived from coumarate and ferrulic residues.

Aldehyde and ketone carbonyl carbons, which resonate farther downfield in the 190- to 210-ppm region, were absent in this spectrum. This could imply either that the signals were too weak to be detected or that they were completely absent from this hydrolysate.

The spectrum (Fig. 3A) of the overlimed concentrated hydrolysate (CSHB1V10) showed that some functional groups were removed because of the overliming. The most important peaks removed included the aromatic and aliphatic carboxylic groups. At pH 10.0, the intensity of the acetic acid carboxylic carbon peak was considerably reduced, and there was a downfield shift of the peak from 176 to 181 ppm. Similarly, the CH_3 carbon of the acetic acid also underwent a shift from 20 to 23 ppm after the overliming.

There was broadening of the sugar carbon peaks. This signal broadening was attributed to suspended fine particles in the hydrolysate, which were not filtered. It is known that fine solid particles could cause peak broadening in NMR spectra.

In addition to the carboxylic functional groups, several aliphatic groups between 20 and 60 ppm were removed (Fig. 3B). Also interesting to note is the removal of peaks between 100 and 160 ppm, which were assigned to aromatic groups derived from coumaric and ferrulic compounds (Fig. 3C). A comparison of the two spectra (Fig. 4) showed the effect of overliming on the corn stover hydrolysate.

The spectra of CSHB2 and CSHB2V10 (Figs. 5–7) are very consistent with the spectra from the concentrated hydrolysates. Although the peaks in these spectra were weaker than those from the concentrated hydrolysates, the pattern of disappearance and shifting of peaks were very consistent with those of the concentrated hydrolysates.

However, there were some peaks in the CSHB2 that were not detected in the CSHB1 spectrum. Peaks at 19.4, 113, 150, 152, 161, 173.4, 178.5, 180.5, and 180.7 ppm were present in the CSHB2 but absent in the CSHB1 spectrum. The signals at 113, 150, and 178.5 ppm indicate the presence of furfural, which was probably lost during the vacuum evaporation. Although the vacuum concentration removed some compounds, it also increased the concentration of others, which became detectable in the concentrated samples but were undetected in the unconcentrated samples. Several other peaks in the aliphatic region of CSHB2, such as 17.7, 21.4, 23.6, 25.6, 29.3, 33.2, and 48.9 ppm, and in the aromatic region, such as 128.5, 147.2, 170.5, 171.3, 172.1, and 172.7 ppm, were not detected but were present in the CSHB1 spectrum.

After the overliming, the spectra from the concentrated and unconcentrated hydrolysates were almost identical. This indicates that compounds that were removed by vacuum evaporation also reacted with the $Ca(OH)_2$. Thus, the concentration of the hydrolysate removed compounds that would have otherwise reacted with the $Ca(OH)_2$. The toxicity of the hydrolysates was mostly preserved after the concentration.

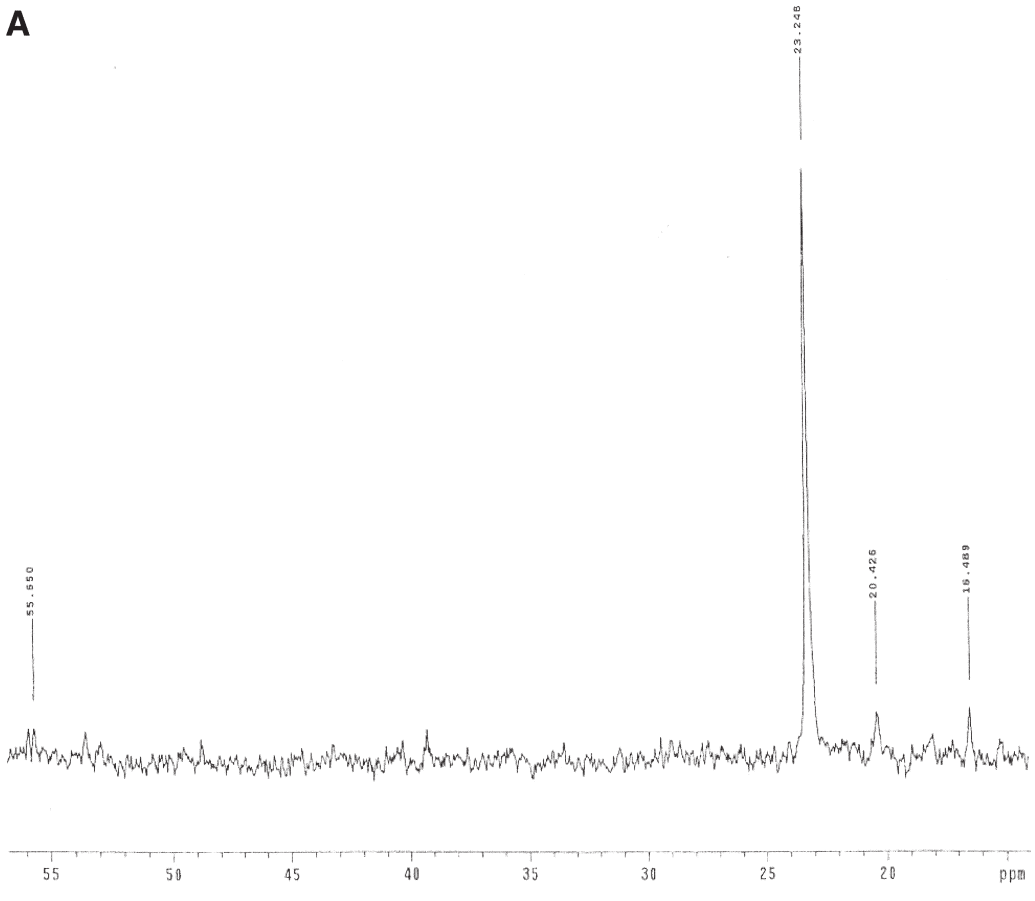

Fig. 6. Enhanced ^{13}C-NMR spectrum of pH 10.0 overlimed corn stover hydrolysate analyzed as received (CSHB2V10) showing **(A)** aliphatic region.

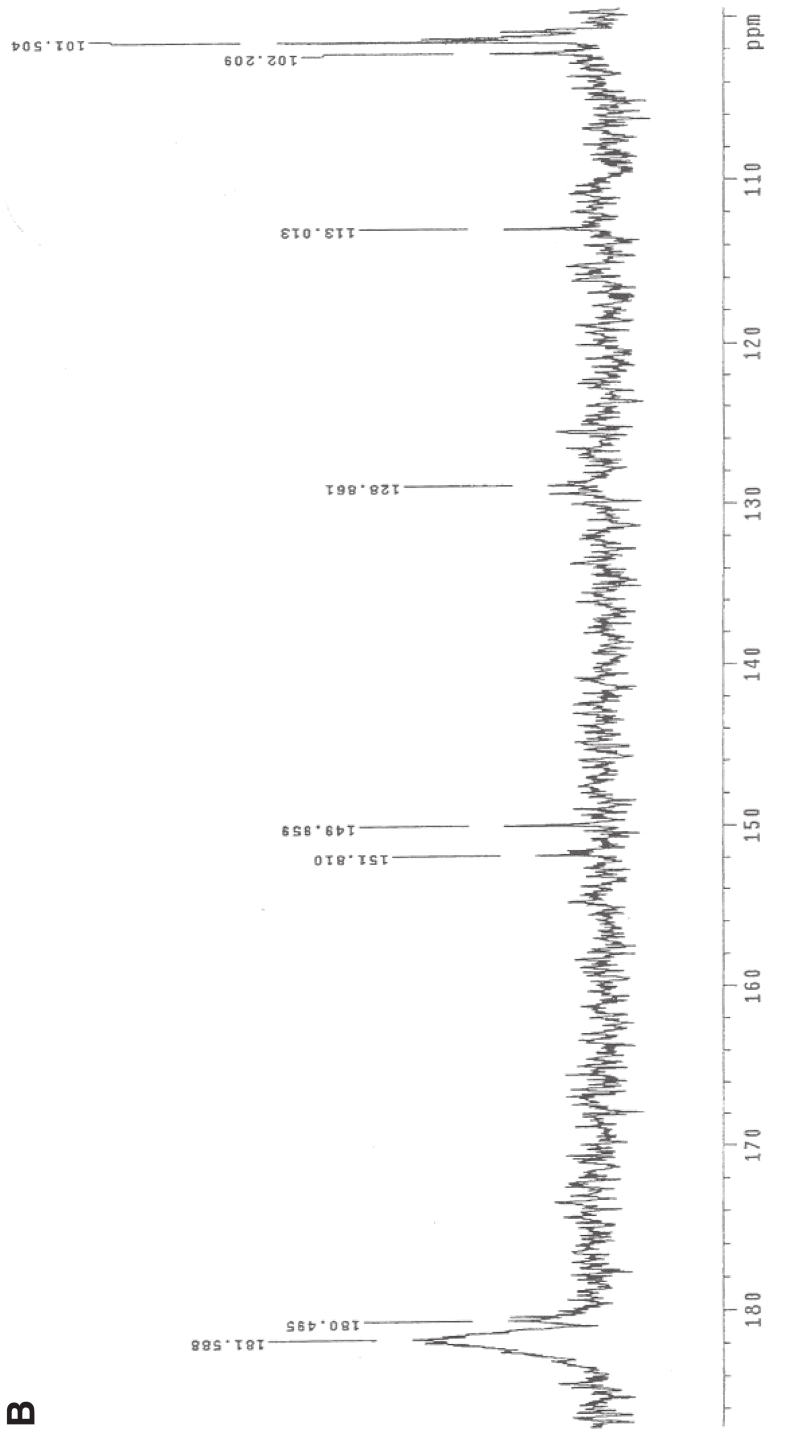

Fig. 6. *(continued)* Enhanced ^{13}C-NMR spectrum of pH 10.0 overlimed corn stover hydrolysate analyzed as received (CSHB2V10) showing **(B)** aromatic region.

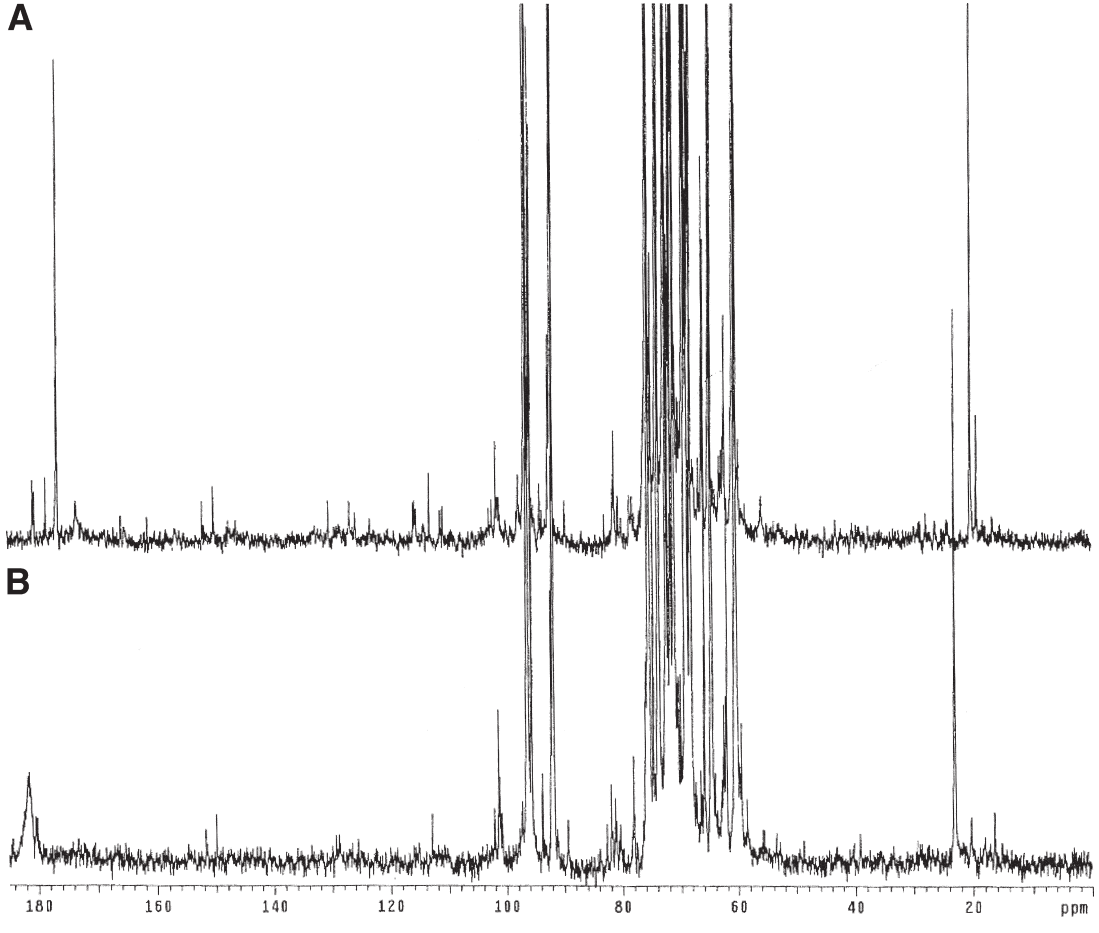

Fig. 7. Comparison of **(A)** overlimed (pH 10.0) and **(B)** nonoverlimed spectra of corn stover hydrolysate analyzed as received.

Decreasing the pH from 10.0 to 7.0 using sulfuric acid did not appear to have any detectable effect on the ^{13}C-NMR spectra of the hydrolysates (pH 7.0 spectra not shown). Thus, it appears that the most important role in reducing the pH of the overlimed hydrolysate is to make it more suitable for fermentation. Any secondary chemical reaction appears to be minimal in terms of removal of toxic components.

It is clear from these spectra that during the overliming of corn stover hydrolysate both aromatic and aliphatic carboxylic functional groups are removed in addition to other aliphatic and aromatic groups. The carboxylic functional groups could be either esters or carboxylic acids. Confirmation of the true identity of functional groups requires more rigorous studies. Most of the functional groups removed by overliming appeared to be lignin derived.

Analysis of Overlimed Precipitate

The ^{13}C-NMR spectra of the deuterated acetone extract of the overlimed corn stover hydrolysate precipitate are shown in Fig. 8A–C. Assignment of the peaks in the spectra was based on model compounds and published grass lignin data *(19–23)*. The acetone extract showed saturated hydrocarbon peaks upfield between 10 and 20 ppm. There was also a methyl ether peak at 24.22 ppm. Methyl conjugated peaks were also present between 28 and 34 ppm. C-α and C-β methylene peaks, which occur between 25 and 43 ppm, were present in the spectra. The methoxyl peak at 56 ppm was extremely weak, showing that very little methoxylated compounds were removed by overliming. There were a number of intense peaks between 51 and 67 ppm, which were not observed in the filtrate because of the occurrence of the intense sugar peaks in the same region. Since there were no sugars in this fraction, the only intense peak in this area was that due to the solvent (acetone).

The most intense peak in the spectra besides the solvent was at 79.39 ppm. In the original corn stover hydrolysate and the overlimed sample, this peak was not observed, probably because of interference from the sugar carbons. The signals at 99.2, 108.7, 109.4, 110.1, 110.9, 116.4, 116.7, 123.8, 130.9, 143–155, and 175.2 ppm suggest that *p*-hydroxyphenyl or cinnamyl alcohols, acids, and esters were removed by the overliming process. Nimz et al. *(20)* showed that coumaric acid esters in ball-milled corn stalk lignin resonated at 171.7 ppm, whereas coumaric acid carbon resonated at 167.8 ppm. Coumaric acid aromatic carbons in ball-milled corn stalk lignin resonated at 111, 115, 116, 131, 123, and 126 ppm. Thus, the peaks in the solid residue clearly show that coumaric and ferrulic acid residues were removed during the overliming reaction. These data complement those obtained for the filtrate. However, it is not clear from these data whether these compounds were bonded to the calcium salt or adsorbed on the precipitate. Some of the peaks that disappeared after overliming were not observed in the spectrum. This suggests that some of the compounds could not be extracted with acetone after reacting with the $Ca(OH)_2$.

Fig. 8. **(A)** Enhanced ^{13}C-NMR spectrum of acetone-extracted solid precipitate from overliming of corn stover hydrolysate showing aliphatic region.

Fig. 8. *(continued)* **(B)** Enhanced ^{13}C-NMR spectrum of acetone-extracted solid precipitate from overliming of corn stover hydrolysate.

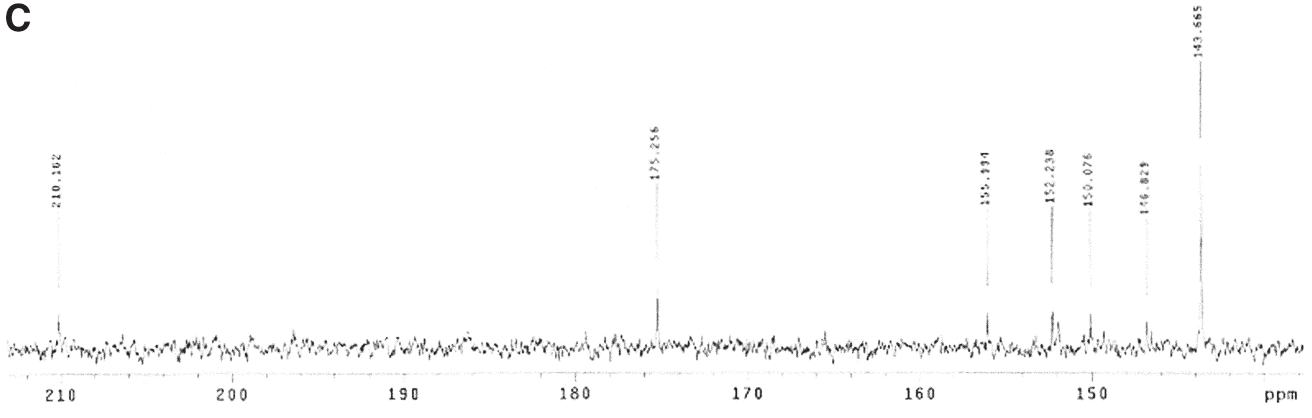

Fig. 8. *(continued)* **(C)** Enhanced ^{13}C-NMR spectrum of acetone-extracted solid precipitate from overliming of corn stover hydrolysate.

Conclusion

The use of ^{13}C-NMR as an effective tool for the identification of functional groups in corn stover hydrolysate was demonstrated. The spectra show that most of the inhibitory compounds are lignin derived and are mostly ferrulic or coumaric acid derived. There was hardly any signal from the syringyl groups. Apart from furfural, there were hardly any ketone or aldehyde functional groups in the hydrolysate.

The overliming removed most of the lignin-derived compounds. Some of the functional groups removed during overliming were extractable from the precipitate using acetone. These extracts confirmed the presence of coumaric and ferrulic compounds. However, not all compounds that were removed by the $Ca(OH)_2$ were detected in the acetone extract of the precipitate.

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