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Research Report

Structural Analysis of Lignocellulose Biomass Using Nuclear Magnetic Resonance

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ABSTRACT A bio-refinery that produces fuels and chemicals from plant biomass has the potential for providing technology for a more sustainable society. The efficient utilization of plants requires an understanding of the chemical composition of the plant structure, such as lignocellulose and metabolic components. This study used high-resolution nuclear magnetic resonance (NMR) to assign chemical shifts for cellulose and other structural components of plant biomass to gain insights into the plant structure. Solid-state ^{13}C-^{13}C NMR using ^{13}C-labeled bacterial cellulose allowed the complete assignment of chemical shifts for carbon atoms in the amorphous cellulose structure. In addition, solution-state three-dimensional NMR provided accurate assignments of chemical shifts for polysaccharides and small components in ^{13}C-labeled poplar cellulose. These results increase our understanding of the complex structure of lignocellulose components, and open the possibility for new applications for processing, conversion, and degradation of lignocellulose.

KEYWORDS Bio-refinery, Lignocellulose, Metabolite, Polysaccharide, Solid-state NMR, Solution-state NMR

1. Introduction

Plants have been used in various industrial ways throughout history. The importance of lignocellulose-based bio-refineries have been studied recently in an effort to replace oil-based resources and contribute to a more sustainable energy source.(1) An increase in the utilization of bio-resources requires efficient processing, conversion, and degradation of lignocellulosic materials. Plant biomass has a complex structure involving cellulose, hemicellulose, and lignin, which are connected in complex ways in the cell wall (**Fig. 1**).(2) Cellulose is composed of D-glucose units that condense through β (1-4)-glyosidic bonds, and consists of crystalline and amorphous regions. Hemicellulose is composed mainly of xylose units, and one main hemicellulose is xylan, which is partially acetylated with sugar residues (e.g., arabinose). Lignin consists of polymeric phenolic units based on syringyl, guaiacyl, and *p*-hydroxyphenyl moieties. Understanding the chemical structures of these plant materials is needed for their efficient utilization. Gaining knowledge about structures of plant tissue has been accomplished using X-ray diffraction,(3) infrared spectroscopy,(4) microscopy,(5) mass spectrometry,(6) and nuclear magnetic resonance (NMR).(7)

NMR is an effective tool for analyzing biomolecular complexes of metabolite components.(8) Various solid and solution-state NMR techniques have been used to detect and identify these polymeric components. Solid-state NMR has the advantage of enabling detection of macromolecules in intact plants. Studies involving beech wood, pine,(9) and wheat straw(10) have been reported using intact tissue. However, assigning all of the signals in a sold-state NMR spectrum is difficult because of its low resolution.(11) Solution-state NMR, in contrast, can detect and identify many polymeric components in plant samples dissolved in dimethylsulfoxide (DMSO)(12) and DMSO/pyridine(13) systems. Polymeric components have been characterized using high-resolution, solution-state two-dimensional (2D) NMR that provided detailed information about metabolites and lignocellulose components,(13,14) and can be used to provide partial assignments of chemical shifts in the spectra of biomolecular components. But completely assigning each signal in the spectra is difficult, because of overlapping chemical shifts. Therefore, a recent study demonstrated that plant compounds labelled with a stable isotope allowed assignment of signals from multidimensional NMR.(15)

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The present study describes several approaches for analyzing the cellulose structure and the chemical composition of plants using high-resolution NMR. An overview of our attempt is summarized in **Fig. 2**. Important steps are the ^{13}C-labeling and the multidimensional NMR used for assigning the signals in the spectra of biomolecule components. In solid-state NMR, the assignment of each carbon atom (C1-C6) within the bacterial cellulose was examined after preparation with ^{13}C-labeling. Although NMR assignments of ^{13}C-labeled crystalline cellulose were completed for the ^{13}C atoms, not all carbons in the amorphous cellulose were assigned. Based on the chemical shifts, differences in the cellulosic structure due to different pretreatments were identified. In solution-state 2D HSQC and 3D HCCH total correlation spectroscopy (TOCSY) NMR, an attempt was made to assign the detailed spectra of polymeric components from ^{13}C-labeled poplar dissolved in DMSO/pyridine. In addition, intact tissue was analyzed by high-resolution magic angle spinning (HR-MAS) NMR 2D HSQC[16] and 3D HCCH correlation spectroscopy (COSY)[17] for identification of low-molecular-weight components.

2. Analysis of ^{13}C-labeled Amorphous Cellulose by Solid-state NMR

Amorphous cellulose, used as the raw material for cellophane or cloth fibers, such as rayon, can be obtained by cellulose regeneration. In the bio-refinery process, the ratio of amorphous cellulose influences the efficiency of enzymatic degradation of cellulose. First of all, the full assignment of ^{13}C-labeled amorphous cellulose pretreated with ionic liquid was attempted, because the ionic liquid was expected to be an effective solvent for cellulose recently.[18] For the solid-state NMR analysis, ^{13}C-labeled bacterial cellulose was prepared from *Acetobacter xylinum*[19] pretreated with 1-ethyl-3-methylimidazolium chloride ([Emin][Cl]). To prepare the NMR samples, *A. xylinum* was cultured in a medium containing $^{13}C_6$-glucose, followed by washing the cellulose obtained with a weakly alkaline solution. The cellulose pellicle was solubilized in [Emin][Cl] at 120°C for 30 min, then regenerated in distilled water. After drying the regenerated cellulose, each sample was packed into a 4-mm ZrO_2 rotor. For solid-state NMR, a DRX-800 spectrometer was used with a magic-angle spinning

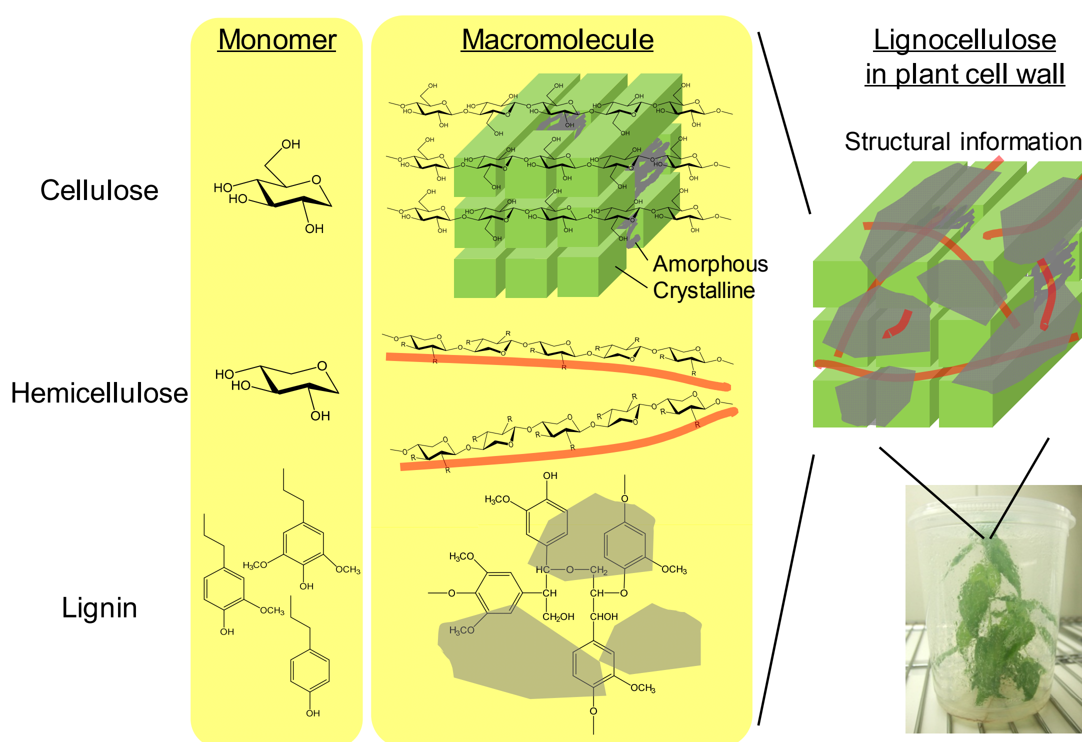

Fig. 1 Lignocellulose of plant cell wall is composed of cellulose, hemicellulose, and lignin. Cellulose is composed of glucose units, hemicellulose is mainly composed of xylose units, and lignin is composed of phenolic units.

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(MAS) triple resonance probe. The 1D ^{13}C cross-polarization MAS (CP-MAS) and 2D ^{13}C-^{13}C refocused incredible natural abundance double quantum transfer experiment (INADEQUATE) NMR spectra were obtained at 12 kHz MAS, and each spectrum was analyzed using NMRPipe.

To determine the assignments of each carbon atom (C1-C6) within cellulose, 2D ^{13}C-^{13}C refocused INADEQUATE spectroscopy was attempted (**Fig. 3**).[20] The ^{13}C-labeling provided clear identification of cross correlations with an increased signal-to-noise ratio. Starting with the C1 chemical shifts obtained from a previous study, all chemical shifts could be determined using the same overlapping positions for each cross peak (**Table 1**). The C2 and C3 chemical shifts were expected to result from intermolecular hydrogen bonding and the interactions between cellulose chains. Hydrogen atoms linked to C2 and C3 were involved in intermolecular hydrogen bonding. Differentiating C2 and C3 atoms enables analysis of the interactions among cellulose chains that are important in cellulose degradation. In addition, assignment of C4 chemical shifts revealed differences in the carbon atoms of different cellulose structures, such as cellulose I, cellulose II, and amorphous cellulose. Cellulose II appears to contain molecular chains in antiparallel alignment against those in cellulose I. Untreated bacterial cellulose could be differentiated from cellulose I and amorphous cellulose, because, upon pretreatment with [Emin][Cl], it formed amorphous cellulose.

3. Assignment of NMR Spectra to Obtain the Structure of Pretreated Cellulose

The C4 chemical shifts were used to help determine the cellulose structure. Ratios of cellulose structural states after various pretreatment conditions were examined using solid-state NMR. To prepare samples of cellulose with various structures, two types of ionic liquids were used, 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]) and 1-ethyl-3-methylimidazolium diethylphosphate ([Emim][DEP]). The pretreated ^{13}C-labeled cellulose was dissolved in each ionic liquid and incubated at

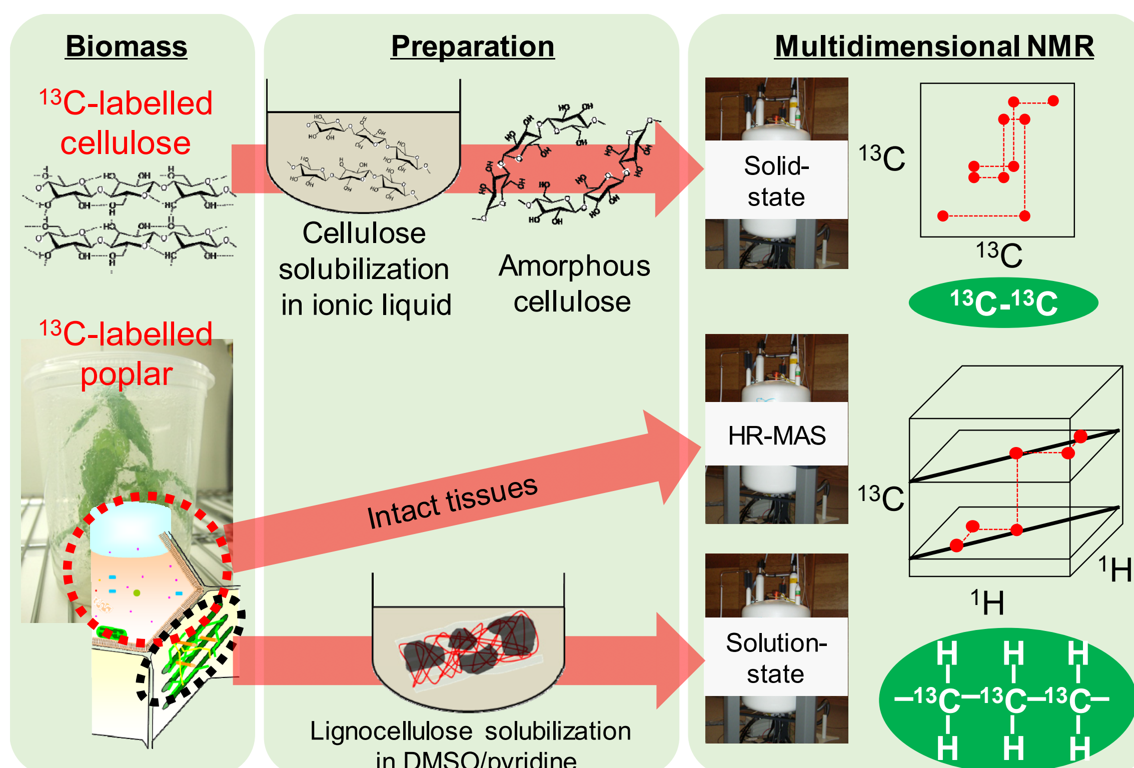

Fig. 2 Overview of NMR analysis used in this study: Solubilization of lignocellulose and multi-dimensional NMR was applied for analyzing lignocellulose components; the preparation of ^{13}C-amorphous cellulose via regeneration from an ionic liquid; metabolites of intact tissues of ^{13}C-poplar were analyzed by high-resolution magic angle spinning (HR-MAS).

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120°C for 30 min. Then, the cellulose was regenerated with distilled water and dried. After drying, each sample was packed into a 4-mm ZrO_2 rotor. Solid-state NMR spectra were obtained using a DRX-800 spectrometer and the spectra obtained were analyzed using NMRPipe. The 1D ^{13}C-CP-MAS spectra indicated that cellulose pretreated with [Emim][Cl] was almost amorphous, while that pretreated with [Emim][DEP] and [Emim][Ac] formed a mixture of cellulose II and amorphous cellulose. Pretreatment with [Emim][Ac] gave results similar to that for [Emim][DEP] (**Fig. 4**).[20] Complete assignment of each cellulosic carbon atom (C1-C6) is expected to allow characterization of different cellulose structures after various pretreatment methods. Peak tracking using ^{13}C backbone assignments of crystalline and amorphous cellulose enabled evaluation of the chemical and physical properties of cellulose.

4. Analysis of ^{13}C-poplar Low-molecular-weight Components without Extraction

Using ^{13}C-labeling, HR-MAS NMR spectra were generated for the analysis of plant metabolites. As the next trial, poplar was selected for this analysis because it is a main source of lignocellulose. A ^{13}C-labeled poplar plant was grown in a medium containing $^{13}C_6$-glucose during $^{13}CO_2$ incubation as a carbon source.[21] The poplar plant was allowed to grow until it reached a height of approximately 10 cm, and then intact ^{13}C-poplar tissue and D_2O were placed in a 4-mm ZrO_2 rotor. The HR-MAS spectra

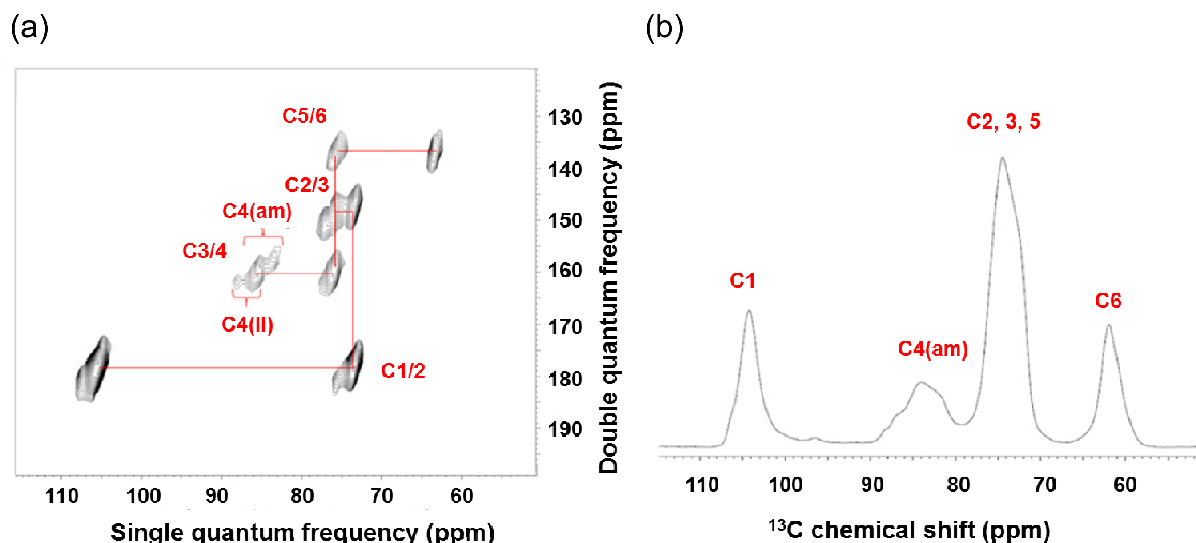

Fig. 3 (a) Solid-state ^{13}C-^{13}C refocused INADEQUATE spectrum of cellulose pretreated with [Emin][Cl]. Solid lines indicate connections between neighboring carbons and represent the amorphous cellulose signal. A small amount of cellulose II was detected at a chemical shift of 88.2 ppm in the C3/4 region. (b) Solid-state ^{13}C NMR spectra of cellulose pretreated with ionic liquids [Emin][Cl].

Table 1 Assignment of peaks in solid-state ^{13}C NMR spectra of cellulose (ppm). In cellulose regenerated from [Emim][Cl], chemical shifts in parentheses were obtained from 1D CP-MAS data.

Samples	C1	C2	C3	C4	C5	C6	Experiment
Untreated	105.3			89.2 84.0		65.6 62.5	1D CP-MAS
Regenerated from [Emim][Cl]	105.0	72.8	75.1	85.0	74.8	62.7 (62.7)	Refocused INADEQUATE (1D CP-MAS)
	104.8 (105.0)	72.6	75.4	84.5 83.1 82.8 (84.8)			

for ^{13}C-poplar without extraction were obtained using DRX-400 and DRX-500 spectrometers equipped with Z-axis high-resolution magic angle spinning probes. The MAS rotational speed was maintained at a constant 4000 Hz. The 2D ^{1}H-^{13}C-HSQC and 3D HCCH-COSY spectra were obtained using a DRX-500 spectrometer and DRX-400 spectrometer, respectively. Each NMR spectrum was processed using NMRPipe. Signals from each metabolite were assigned using the ^{1}H and ^{13}C chemical shift database SpinAssign.[22-24]

The HR-MAS 2D ^{1}H-^{13}C-HSQC spectrum is shown in **Fig. 5**.[25] Although each signal was broadened in the ^{1}H dimension by residual ^{1}H-^{1}H dipolar interactions, chemical shift dispersion could be resolved in intact tissues. Using the SpinAssign database,[22-24] a number of peaks could be assigned to particular metabolites, such as amino acids, ethanol, malate, choline, ethanolamine, and glucose. Multiple candidate metabolites also were observed in the database during the process of matching chemical shift data for each signal. In general, solution-state NMR requires extraction of plants, and depends on the solvents used for extraction. However, the HR-MAS technique demonstrated here did not have this limitation and was used to analyze intact tissue to gain information about metabolites such as lipids, leucine, ethanol, and isoleucine.

5. Polysaccharide Analysis of ^{13}C-poplar Dissolved in DMSO/pyridine Solvent

Polysaccharides are polymeric carbohydrates in which monosaccharides are connected through glyosidic linkages. Freeze-dried ^{13}C poplar was pretreated according to an above method, and the crushed sample was ball-milled. The milled sample was extracted with ethanol and distilled water, and then dissolved in DMSO-d_6/pyridine-d_5 (4:1), shaken, and centrifuged. Solution NMR spectra were obtained from the soluble matter in the sample using a DRU-700 spectrometer. **Figure 6** shows the anomeric and aliphatic regions assigned using solution-state 2D ^{1}H-^{13}C-HSQC NMR (each peak corresponds to a value in **Table 2**).[25] The signals detected in the polysaccharide anomeric region of the ^{1}H-^{13}C-HSQC spectrum were assigned. However, only

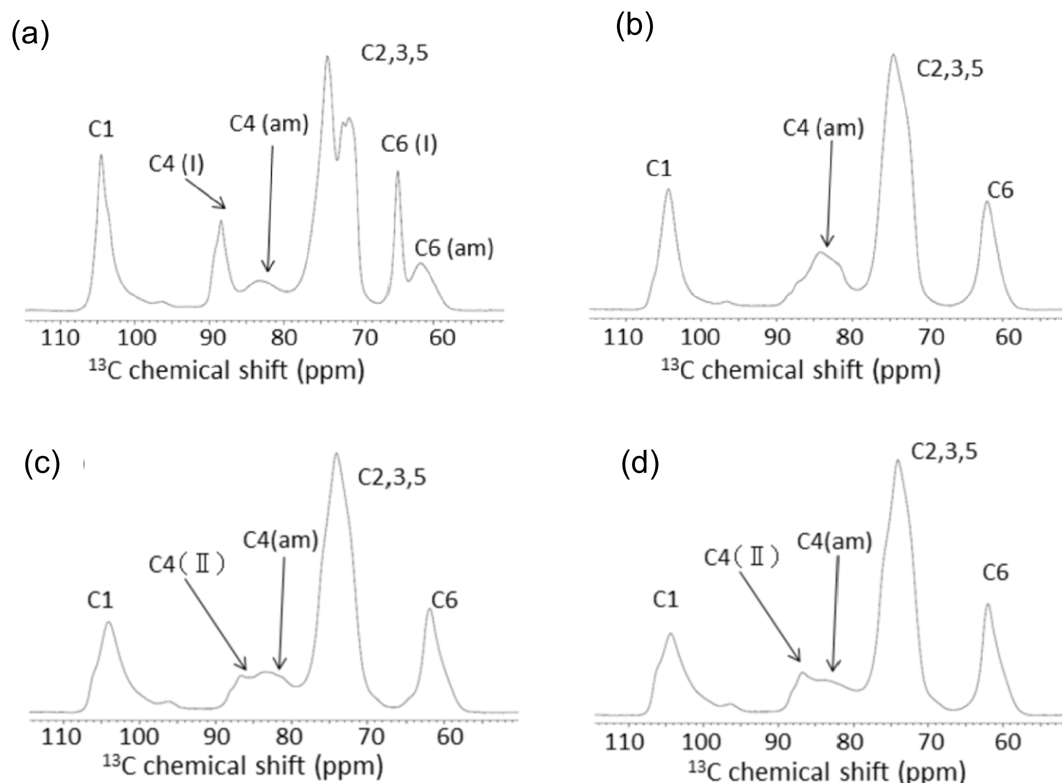

Fig. 4 Solid-state ^{13}C NMR spectra of (a) untreated cellulose, and cellulose pretreated with (b) [Emim][Cl], (c) [Emim][DEP], and (d) [Emim][Ac] at 120°C for 30 min. C1-C6 represent the carbon backbone of cellulose.

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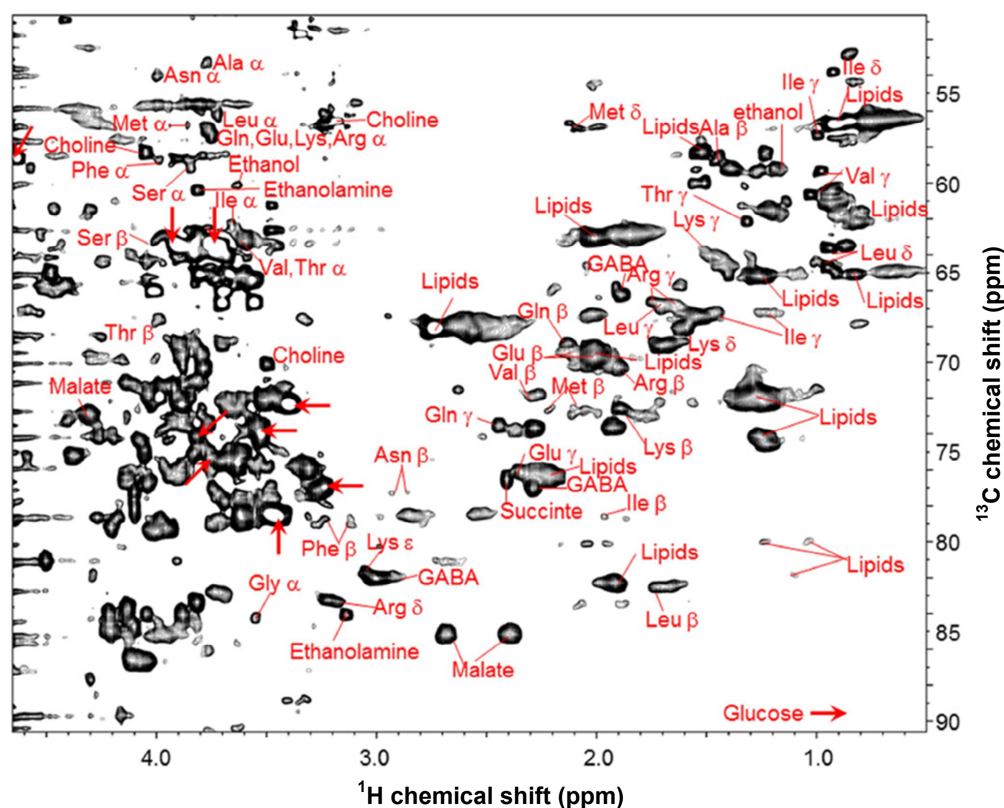

Fig. 5 HR-MAS ^{1}H-^{13}C-HSQC spectrum of ^{13}C-poplar, measured without sample extraction. Peaks in the spectrum corresponding to metabolites were assigned by 3D HCCH-COSY experiments and matched by standard metabolites and SpinAssign. Ala, Alanine; Glu, Glutamic acid; Phe, Phenylalanine; Gly, Glycine; Ile, Isoleucine; Lys, Lysine; Leu, Leucine; Met, Methionine; Asn, Asparagine; Gln, Glutamine; Arg, Arginine; Ser, Serine; Thr, Threonine; Val, Valine; GABA, γ-amino butyric acid.

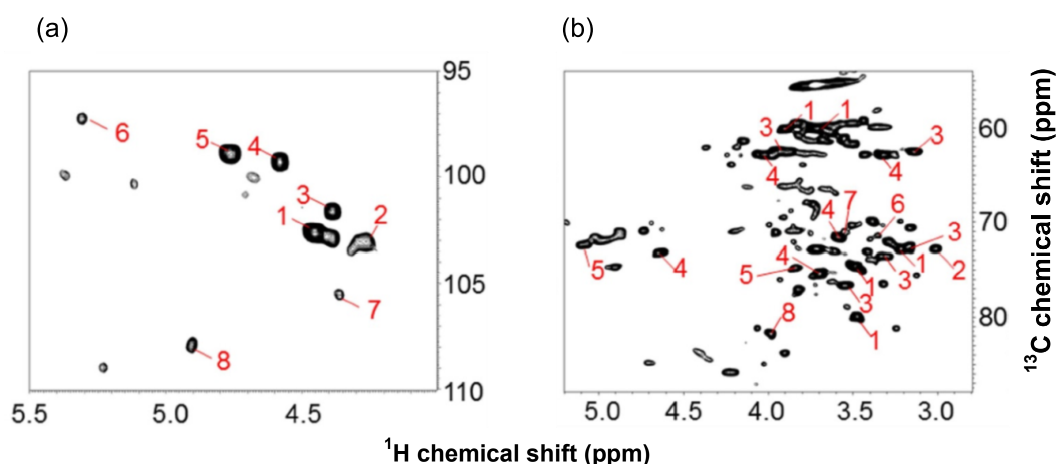

Fig. 6 Solution-state ^{1}H-^{13}C HSQC spectrum of ^{13}C-poplar extracted in DMSO/pyridine. (a) Anomeric region. (b) Aliphatic region. Peaks were assigned by 3D HCCH-TOCSY experiments and matched on the basis of the previous reports. Peak numbers in the figure correspond with those listed in Table 2.

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anomeric carbons could be identified using this approach. To identify other carbon signals, 3D NMR experiments were combined with 2D experiments, similar to HR-MAS. The peaks detected in the anomeric and aliphatic regions were assigned based on 3D HCCH-TOCSY data. **Figure 7** shows ^{1}H-^{1}H planes from the 3D ^{1}H-^{1}H-^{13}C spectrum sliced along the ^{13}C axis of the chemical shifts of (1,4)-*β*-D-glucopyranoside.(25) Based on the connections among C1-C6, the signals for

Table 2 ^{1}H and ^{13}C chemical shift assignments for polysaccharide components from ^{13}C-poplar, based on a combination of the 3D HCCH-TOCSY.

No	Saccharide	Chemical shift (ppm)					
		C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
1	(1, 4)-*β*-D-Glc*p*	102.6/4.45	74.6/3.48	74.6/3/48	79.9/3.48	74.6/3.48	60.1/3.90, 60.1/3.72
2	Unknown polysaccharide G	103.1/4.27	72.8/3.01	ND	ND	ND	ND
3	Unknown polysaccharide X	101.6/4.39	72.6/3.17	73.6/3.34	76.6/3.55	62.5/3.93, 62.9/3.14	
4	2-O-Ac-*β*-D-Xyl*p*	99.3/4.58	73.2/4.64	71.6/3.59	75.4/3.67	62.9/4.02, 62.9/3.32	
5	2-O-Ac-Man*p*	98.9/4.76	72.4/5.10	74.8/3.84	ND	ND	ND
6	4-O-MeGlcA	97.2/5.31	71.4/3.35	ND	ND	ND	ND
7	(1, 4)-*β*-D-Gal*p*	105.5/4.36	71.0/3.55	ND	ND	ND	ND
8	*α*-L-Ara*f*	107.9/4.90	81.8/3.98	ND	ND	ND	

ND: not determined

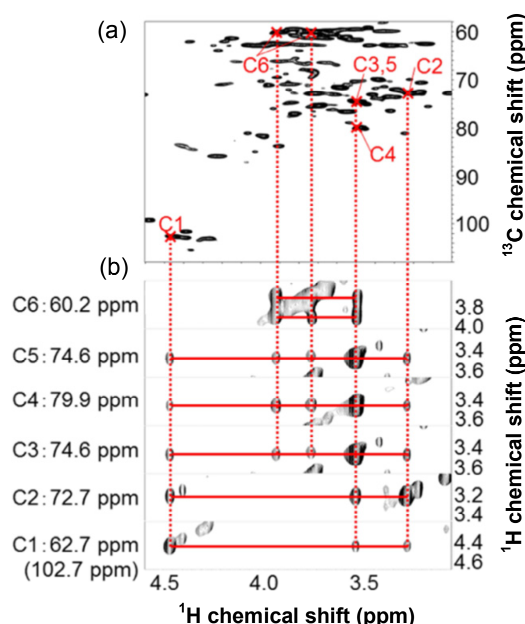

Fig. 7 Analysis of (1, 4)-*β*-D-Glc*p* using solution-state 2D HSQC and 3D HCCH-TOCSY spectra of ^{13}C-poplar extracted in DMSO/pyridine. (a) 2D ^{1}H-^{13}C HSQC spectrum. Crossed marks show C1-6 signals of (1, 4)-*β*-D-Glc*p* assigned by 3D HCCH-TOCSY. (b) 2D ^{1}H-^{1}H planes at 60.2, 74.6, 79.9, 74.6, 72.7 and 62.7 (folded spectrum; 102.7) ppm of ^{13}C, which correspond to the C6, C5, C4, C3, C2, and C1 of (1, 4)-*β*-D-Glc*p*, slicing the 3D ^{1}H-^{1}H-^{13}C spectrum. Red transverse lines connect ^{1}H-^{13}C-^{13}C-^{1}H cross peaks and vertical dashed lines connect corresponding signals between 3D and 2D spectra.

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(1,4)-β-D-Glc*p*, 2-O-acetyl-β-D-xylopyranoside, and 2-O-Ac-β-D-Xyl*p* were completely assigned. In addition, (1,6)-β-D-glucopyranoside, 3-O-acetyl-β-D-xylopyranoside, 2-O-acetyl-β-D-mannopyranoside, 4-O-methyl-α-D-glucuronic acid, β-D-galactopyranoside (β-D-Gal*p*), and α-L-arabinofuranoside could be detected (Table 2). These results demonstrate that a significant number of peaks corresponding to polysaccharide could be identified, especially by combining 2D and 3D NMR spectral data. Although some polysaccharides had only low-intensity signals that were assigned, the results of this study provide an approach that could improve signal sensitivity from small amounts of polymeric components.

6. Conclusions

To gain insights into bio-refining, the supramolecular structure of lignocellulose must be elucidated, even though it is not well understood due to the complex mixture of cellulose, hemicellulose, and lignin it contains. Analyzing solid-state and solution-state NMR data provided an effective approach for identifying biomolecular compounds. By enhancing the NMR signals with ^{13}C-labeling techniques, polymeric components and metabolites of polysaccharides could be assigned completely. Since chemical shifts reflect structural characteristics, the database of chemical shifts accumulated for lignocellulose components allowed construction of a biomass profile. NMR-based technologies can help provide a more comprehensive understanding of cell wall dynamics. In the future, effective utilization of biomass may be enhanced by information about the lignocellulose structure, which can contribute to a more sustainable society.

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Figs. 3, 4 and Table 1
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