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ORIGINAL RESEARCH

Development and validation of time-domain ^{1}H-NMR relaxometry correlation for high-throughput phenotyping method for lipid contents of lignocellulosic feedstocks

Shraddha Maitra[1] | **Bruce Dien**[2] | **Stephen P. Long**[3] | **Vijay Singh**[1]

[1]Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

[2]Bioenergy Research Unit, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA

[3]Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Correspondence
Vijay Singh, Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA.
Email: vsingh@illinois.edu

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Abstract

The bioenergy crops such as energycane, miscanthus, and sorghum are being genetically modified using state of the art synthetic biotechnology techniques to accumulate energy-rich molecules such as triacylglycerides (TAGs) in their vegetative cells to enhance their utility for biofuel production. During the initial genetic developmental phase, many hundreds of transgenic phenotypes are produced. The efficiency of the production pipeline requires early and minimally destructive determination of oil content in individuals. Current screening methods require time-intensive sample preparation and extraction with chemical solvents for each plant tissue. A rapid screen will also be needed for developing industrial extraction as these crops become available. In the present study, we have devised a proton relaxation nuclear magnetic resonance (^{1}H-NMR) method for single-step, non-invasive, and chemical-free characterization of in-situ lipids in untreated and pretreated lignocellulosic biomass. The systematic evaluation of NMR relaxation time distribution provided insight into the proton environment associated with the lipids in the biomass. It resolved two distinct lipid-associated subpopulations of proton nuclei that characterize total in-situ lipids into bound and free oil based on their "molecular tumbling" rate. The *T1T2* correlation spectra also facilitated the resolution of the influence of various pretreatment procedures on the chemical composition of molecular and local ^{1}H population in each sample. Furthermore, we show that hydrothermally pretreated biomass is suitable for direct NMR analysis unlike dilute acid and alkaline pretreated biomass which needs an additional step for neutralization.

KEYWORDS

bioenergy crops, biofuel, bound and free oil, non-invasive cellulosic lipid quantification, pretreatment, *T1T2* relaxation time, time-domain ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR)

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1 | INTRODUCTION

Bioenergy crops such as energycane, sugarcane, miscanthus, and sorghum have immense potential for large-scale drop-in biofuel production (Parajuli et al., 2020; Vanhercke et al., 2019; Zale et al., 2016). Various bioprocesses are being developed to co-produce cellulosic bioethanol and value-added bio-products using these bioenergy crops. However, biodiesel production in the United States is still predominantly dependent on soybean and corn, which are also marketed as food for humans and animals. In 2020, the largest feedstock contributing toward biodiesel production was soybean oil (71.6%) followed by corn oil (12.9%) and canola oil (10.6%; EIA, 2021). Hence, to expand the supply of biodiesel without impacting cooking oil supplies, highly productive lignocellulosic biomass crops are being genetically modified to accumulate energy-rich triacylglyceride (TAG) and fatty acids rich in short, unbranched, and unsaturated side chains. To this end, Andrianov et al. (2010) and Sanjaya et al. (2013) have shown a 20- and 25-fold increase in TAG accumulation in *Nicotiana tabacum* and *Arabidopsis thaliana*, respectively (Andrianov et al., 2010; Sanjaya et al., 2013). Zale et al. (2016) reported a 1.5- to 9.5-fold increase in TAG accumulation in the vegetative tissues of sugarcane—a C4 plant highly efficient in converting solar energy into chemical energy (Zale et al., 2016). Recently, a 400-fold increase in TAG accumulation has been successfully achieved in genetically modified sugarcane as compared to the wild-type variety (Parajuli et al., 2020). In the United States, the biomass yield of sugarcane is approximately 180 Metric tons/ha (70% moisture) as compared to soybean which is approximately 2.8 Metric tons/ha. This implies that high biomass transgenic bioenergy crops can yield higher oil per hectare than most oilseed crops provided it accumulates ~20% lipids in their vegetative tissues (Andrianov et al., 2010; Huang et al., 2016; Parajuli et al., 2020; Poltronieri, 2016; Vanhercke et al., 2015; Zale et al., 2016). Research efforts are now underway to engineer energycane to accumulate energy-rich storage chemicals in the form of TAGs in leaf and stem tissues.

The metabolic engineering of bioenergy crops to sequestrate carbon in vegetative tissues to form triacylglycerol is providing a technology to enhance the energy densification of crops. However, these metabolically engineered plants undergo a physiological unproductive cycle of TAG synthesis followed by its degradation. TAG lipases hydrolyze storage TAGs to fatty acids (FAs), diacylglycerides (DAGs), monoglycerides, and glycerol, hence lipid content falls with time (Vanhercke et al., 2017). During the genetic engineering phase, several tens of hundreds of transgenic lines are grown with various combinations of targeted genes. With different biomass tissues (stems, leaves, and roots), plant maturities, genotypes, and agronomic conditions the total number of samples requiring lipid analysis increases rapidly. Currently used traditional methods require the extraction of lipids with organic solvent often followed by chromatography which makes analysis slow, and tedious, hence increases the analysis time. Screening such oil-containing biomass in the initial developmental phase requires a rapid method for lipid quantification and characterization of a huge quantum of samples to prevent the degradation of energy-rich molecules before analysis. Besides, developing methods for bioprocessing to recover lipids to produce biodiesel will require measuring lipids before, after, and possibly during various unit operations for the quality check during biodiesel production (Arora & Singh, 2020). Therefore, it is critical to have rapid, accurate, and convenient analytical methods to characterize and quantify lipids within lignocellulosic biomass with minimal sample size.

Recently, the application of NMR has been expanded to probe lignocellulosic polysaccharide compositions, crystallinity index of cellulose, porosity, and lignin characterization (Foston & Ragauskas, 2010; Haddad et al., 2017; Ibbett et al., 2014; Jeoh et al., 2017; Karuna et al., 2014; Kiemle et al., 2003; Park et al., 2009; Pu et al., 2011; Wu et al., 2017). NMR spectroscopy is also routinely used for quantitative and qualitative analyses of fatty compounds in either pure chemicals or oilseeds (Berman et al., 2013; Knothe & Kenar, 2004; Miyake et al., 1998; Sacco et al., 2000; Zverev et al., 2001). However, the authors are unaware of any reports on the application of td-^{1}H NMR analysis to quantify and characterize in-situ lipids in lignocellulosic biomass including stability and recovery after feedstock preprocessing. This work extends the use of td-^{1}H NMR for the in-situ lipid characterization to non-seed biomass.

The study aims to understand the dynamics of the oil/lipid-associated proton environment in lignocellulosic biomass and consequently develop a powerful analytical method based on ^{1}H-NMR technology to quantify and characterize lipids present in the lignocellulosic biomass into bound and free form. NMR relaxometry spectra were evaluated to investigate the influence of three distinct feedstock pretreatments, that is, two-staged hydrothermal and mechanical, dilute acid, and alkaline on in-situ lipids of transgenic bioenergy crops for their suitability as bioprocess to recover lipids for biodiesel production.

2 | MATERIALS AND METHODS

2.1 | Feedstock and chemicals

2.1.1 | Energycane UFCP82-1655

Energycane UFCP82-1655 bagasse was obtained from the experimental research station located at the University of Florida, Gainesville, Florida, USA. Energycane juice was

extracted and bagasse was dried at 50°C. Dried energycane bagasse was cut into smaller pieces of 1–2 inches with pruning shears and ground in a hammer mill (W-8-H, Schutte-Buffalo Hammermill) equipped with a round hole sieve sized at 2 mm. Ground energycane without vegetative oil served as negative control and backbone material for creating representative biomass.

2.1.2 | Representative/model biomass for NMR studies

Research efforts are still underway for the development of transgenic energycane with elevated levels of vegetative oil. As a proof of concept for the NMR-based analytical method, representative biomass samples containing vegetative oil have been prepared that simulate the oil-producing cane. The ground energycane was soaked in crude corn or crude soybean oil of known concentrations. Crude corn and soybean oil were obtained from One Earth Energy LLC and Incobrasa Industries Limited, respectively. Oil-soaked energycane biomasses were incubated at 32°C for 1–2 months. Representative biomass samples having six different oil concentrations were prepared using corn and soybean oils. The final oil concentrations of the soaked energy cane samples were 0, 0.096, 0.198, 0.309, 0.393, and 0.501 g corn oil per g dry biomass (Figure S1a), and 0, 0.101, 0.216, 0.333, 0.400, and 0.533 g soybean oil per g dry biomass. Pretreatment experiments were performed using the energycane test sample containing 20% crude corn oil per g dry biomass (Figure S1b) unless mentioned otherwise.

2.1.3 | Soybean hull

Soy hull pellets were obtained from Incobrasa Industries Limited. Soybean hulls are obtained as the coproduct of soybean meal production (additional file 1, Figure S1c). It consists of pelletized soybean seed coats and is mixed with external crude soybean oil to provide higher energy values for ruminant animal rations.

2.1.4 | Wild-type sugarcane and transgenic lipidcane 1566

Wild-type sugarcane and transgenic lipidcane 1566 having elevated levels of in-situ oil in vegetative tissues were obtained from the Center of Advanced Bioenergy and Bioproducts (CABBI), University of Illinois at Urbana-Champaign, IL, USA. Wild-type sugarcane and lipidcane stems were processed the same as energycane.

2.2 | Feedstock preprocessing

Pretreated biomass samples were prepared using three methods (two-staged hydrothermal and mechanical, dilute acid, and alkaline) to determine the suitability of pretreated biomass for direct lipid analysis using NMR spectroscopy. All chemicals were of analytical quality.

2.2.1 | Two-staged hydrothermal and mechanical pretreatment

A fluidized sand bath (IFB-51 Industrial Fluidized Bath, Techne Inc.) was used for the liquid hot water pretreatment. Energycane test sample was mixed with deionized water at 20% w/w solid loading and loaded in a capped pipe reactor (316 stainless reactors: 10.478 cm length × 1.905 cm outer diameter × 0.165 cm wall thickness tubing, SS-T12-S-065–20, Swagelok, Chicago Fluid system Technologies; 316 stainless steel caps: SS-1210-C, Swagelok, Chicago Fluid system Technologies). The in-situ reaction temperature during pretreatment was monitored using a thermocouple (Penetration/Immersion Thermocouple Probe Mini Conn [−418 to 1652°F], Mc Master-Carr) inserted into one reactor and connected to a data logger (HH306/306A, Datalogger Thermometer, Omega). After holding the tubes at 180°C for 10 min, the reaction was immediately quenched by submerging the pipe reactors into a cold water bath. Liquid hot water pretreatment was followed by three passes of disk milling (Quaker City grinding mill model 4E, Straub Co.; Kim et al., 2016). The biomass samples were filtered after each pretreatment step and solid residues were oven-dried at 50°C.

2.2.2 | Alkaline pretreatment

The energycane test sample was mixed with 1 N NaOH solution to obtain 20% w/w solid loading in stainless steel reactors (same set as used for the hydrothermal pretreatment). The pretreatment reactors were heated in a fluidized sand bath (same as used for the hydrothermal pretreatment) at 100°C for 30 min (Maryana et al., 2014). The pretreated biomass was cooled and thoroughly washed with deionized water to remove NaOH. The washed sample was oven-dried at 50°C.

2.2.3 | Dilute acid pretreatment

A low severity dilute acid pretreatment was performed as outlined by Sindhu et al. (2011) with slight modifications. The energycane test sample was mixed with 2.0% w/w H_2SO_4

solution to obtain 20% w/w solid loading in autoclavable glass reactors. The mixture was heated at 121°C for 60 min under 15 psi pressure. The sample was cooled and thoroughly washed with deionized water to remove the acid and dried at 50°C.

2.3 | Time domain-^{1}H-nuclear magnetic resonance (NMR) spectroscopy

A time-domain one-dimensional benchtop nuclear magnetic resonance system (Minispec mq20, Bruker) equipped with an 18 mm thermostat ^{1}H-probe operating at 0.47 T/20 MHz was used for the analysis of *T1T2* relaxation times and quantification of total lipid contents. NMR analysis of biomass samples was performed at a constant temperature of 40°C. The moisture contents of all the biomass samples were kept consistent and below 2%w/w to abate the contribution of proton signals from water molecules. For the consistency of analysis, 1 g of dry biomass was used for all analyses.

2.3.1 | *T1T2* relaxometry spectra analysis

The spin–spin or transverse (*T2*) relaxation times for the biomass samples were obtained using the Carr–Purcell–Meiboom–Gill (CPMG) application (Atta-ur-Rahman et al., 2016) with a 180° pulse separator of 2.00 ms over 800 spin-echoes and fitted to a bi-exponential equation of order two. A full decay was obtained for *T2* relaxation time. The spin-lattice or longitudinal (*T1*) relaxation time was analyzed using the inversion recovery method (Loening et al., 2003). The inverse recovery method was started after 2–5 ms (to avoid receiver artifacts) and run over a duration of 800 ms for each data point. A set of 10 spin-ecohoes were obtained for each sample and fitted to a bi-exponential equation of order two. The CONTIN algorithm software provided by Bruker was used to obtain continuous distributions of *T1* and *T2* values. Analysis time for *T1* and *T2* relaxometry of each biomass sample was 5 and 2 min, respectively.

2.3.2 | Non-invasive quantification of total lipids

Calibration curve was established for each type of biomass, that is, energycane test sample and soy hull and transgenic lignocellulosic biomass with enhanced lipid content (lipidcane 1566) for absolute quantification of total lipids. A regression value of above 99.5% (R^2 = 0.995) was obtained.

2.4 | Organic solvent extraction and quantification of oil

The total lipid content of untreated and pretreated biomass was extracted using the organic solvent method reported by Huang et al. (2017). Briefly, 1.00 g of the dry biomass sample was mixed with 10 ml isopropanol and 15 ml hexane in a 50-ml screw-top tube and homogenized 2× 1 min with a homogenizer (LabGen 700, Cole Parmer) at a speed of 5000 rpm. The homogenized mixture was agitated with a wrist action shaker (HB-1000 Hybridizer, UVP LLC) at room temperature for 10 min. The slurry was mixed with 16 ml of (6.7%, w/v) sodium sulfate solution agitated for 10 min and centrifuged at 200 rpm for 20 min. The top phase was collected in a new pre-weighed screw-capped tube and the solvent was evaporated by passing over a gentle stream of nitrogen. Once the solvent was removed, the recovered oil was weighed on an analytical balance. Gravimetric oil measurements were compared with the values obtained using NMR spectroscopy.

2.5 | Statistical analysis

All the samples were analyzed in triplicate. Regression analysis between NMR intensity and total lipid content was performed to determine the accuracy of the NMR calibration. ANOVA (analysis of variance) was performed using R statistical software (i386 3.6.2) to compare lipid contents determined by NMR spectroscopy and the standard organic extraction/gravimetric method with a significance threshold of $p \leq 0.05$.

3 | RESULTS

3.1 | T1T2 relaxometry correlation exhibits two distinct proton subpopulations

^{1}H-NMR relaxation time distributions of energycane bagasse (control), energycane bagasse soaked in various amounts of crude corn oil, transgenic lipidcane 1566 bagasse, wild-type sugarcane bagasse, and soyhulls were analyzed. The samples exhibited two distinct subpopulations of proton nuclei, one with shorter relaxation time and one with longer relaxation time for each *T1* and *T2* relaxation time (Table 1). Shorter relaxation time arises from proton nuclei with restricted movement and longer relaxation time from those with a higher degree of freedom of movement. The existence of restricted oil/lipid movement can be attributed to either entrapment of oil molecules within the porous structure of lignocellulosic biomass. A background proton signal was obtained in dry biomass without oil, that is, control energycane biomass. *T1*

TABLE 1 *T1* (spin–lattice) and *T2* (spin–spin) relaxation times for various concentrations of energycane-oil mixtures, energycane test sample, transgenic lipidcane 1566 bagasse, and soy hull. Shorter and longer relaxation times correspond to lesser and higher molecular fluidities and hence, relative fractions of bound and free oil/lipid in the biomass sample

Biomass (g oil/ g dry biomass)	*T1* (ms) (1)	*T1* (ms) (2)	*T2* (ms) (1)	*T2* (ms) (2)
0 (Control)	6 ± 1	80 ± 3	0	0
0.096	25 ± 5	150 ± 10	46 ± 3	192 ± 4
0.198	39 ± 6	200 ± 10	60 ± 1	234 ± 3
0.309	44 ± 6	220 ± 10	73 ± 1	265 ± 3
0.393	48 ± 6	230 ± 10	78 ± 1	277 ± 3
0.501	53 ± 6	240 ± 10	81 ± 1	283 ± 2
Energycane (test sample)[a]	37 ± 6	200 ± 10	60 ± 2	235 ± 3
Wild-type sugarcane	8 ± 1	136 ± 2	20 ± 10	0
Lipidcane 1566	18 ± 4	125 ± 5	11 ± 3	54 ± 9
Soy hull	17 ± 3	108 ± 8	14 ± 2	94 ± 3

Note: Average ± standard deviation.

[a]Ground energycane bagasse with ~20% crude corn oil per g dry biomass. Energycane test sample has been used for all the pretreatment studies.

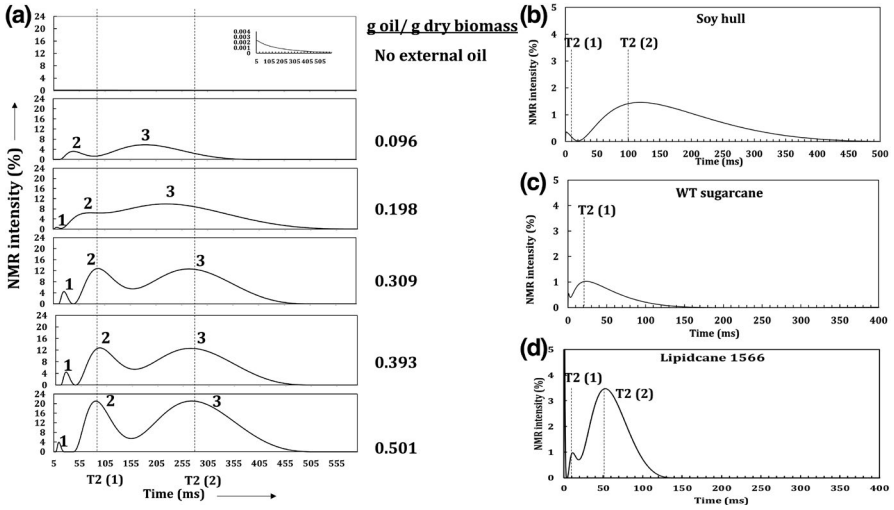

FIGURE 1 T2 relaxometry spectra loss for (a) model energycane bagasse mixed with different amounts of crude corn oil (biomass in Set 2), (b) Soy hull, (c) Wild-type sugarcane bagasse (control), and (d) transgenic lipidcane 1566 bagasse. Peak 1 in all Set 2 biomass samples corresponds to the background signal from biomass fibers (addition of external oil increased the molecular fluidity of fiber molecules which in turn increased the relaxation time)

analysis of control biomass read *T1* (1) 6 ± 1 ms and *T1* (2) 80 ± 3 ms, which can be attributed to the spin of proton nuclei of biomolecules including proteins, carbohydrates, phospholipids, and any remaining water molecules. However, the control with a zero *T2* value indicates highly constraint proton nuclei having minimal interaction with the local environment (i.e. a very solid surface with constrained molecular movement).

The influence of surface chemistry on *T1T2* relaxometry correlation spectra of oil was investigated by comparing them for two distinct sets of biomasses. Set 1 included soy hull and transgenic lipidcane 1566 bagasse that naturally contains oil (wild-type sugarcane bagasse served as a control for Set 1), and Set 2 included ground energycane bagasse samples soaked in varying amounts of corn oil (Table 1). Ground energycane bagasse without externally added oil served as the control for Set 2. Interestingly, biomass samples from Set 1 and Set 2 presented contrasting results. Biomass with externally added oil had a higher magnitude of *T2* relaxation times as compared to *T1* relaxation times. Although the magnitude of *T2* was greater than *T1* for the biomass samples in Set 2, they followed the principle of NMR physics, that is, $T2 \leq 2\,T1$, thereby confirming the validity of NMR relaxometry correlation analysis. The significant effect of externally added oil on *T2* relaxation time as compared to *T1* relaxation time implies that the dephasing of the spinning electrons is influenced by the local environment present within the sample. Therefore, samples need to be consistent (for moisture content and sample weight) during the analysis.

The analysis of *T2* relaxation spectra of biomass samples from Set 2 reveals two important trends: (1) the NMR intensity increases with an increase in oil concentration due to the addition of oil-associated proton nuclei to the sample and (2) higher concentration of free oil/lipid content increases the fluidity of proton nuclei, which shifts the relaxation time toward higher magnitude (Figure 1a). This implies that variation in the magnitude of NMR intensity and relaxation time is directly associated with the concentration of oil in

the biomass provided moisture content is kept minimal and uniform among samples. The lower rate of increase in the magnitude of the relaxation time associated with bound oil upon doubling the oil concentration indicates that added oil gets entrapped in microporous structures or interacts weakly with the chemical structure of biomass.

3.2 | Quantification of total lipids in lignocellulosic biomass

Having confirmed the presence of two distinct proton subpopulation corresponding to bound and free oil in the lignocellulosic samples, NMR was calibrated for the quantification of total lipids. Since the NMR signal is directly related to the percentage of hydrogen in the oil sample, therefore, to improve the accuracy of the total lipid analysis in transgenic lines, lipid extracted from lipidcane 1566 was used for NMR calibration. NMR measurements were highly correlated with oil contents ($R^2 \geq 99.5\%$; Figure S2a–c). For validation, oil contents measured by NMR were compared to values obtained using the classical organic solvent extraction method.

Lignocellulosic biomass is a complex matrix. It is necessary to minimize the background signal of proton nuclei from various membrane and phospholipids present in the biomass sample. This was accomplished by extracting lipid biomolecules from energycane bagasse with organic solvents such as hexane, isopropanol, and ethanol. The lipid-extracted energycane bagasse was used as a biomass matrix for calibration. It was mixed with different concentrations of crude corn oil, crude soybean oil, and extracted lipids of lipidcane 1566 for NMR calibration. Figure 2 compares the total lipid contents measured by NMR versus extraction with organic solvent extraction for an energycane test sample (with known oil concentration), soy hull pellets, and lipidcane 1566. For the energycane test sample made up using 0.20 g crude corn oil per gram dry biomass, the td-^{1}H-NMR method predicted 0.182 g oil per g dry biomass. Soy hull pellets and lipidcane 1566 contained 0.017 and 0.0289 g oil per g of dry biomass as measured using NMR spectroscopy, respectively. The total lipid contents measured using both methods were not statistically different ($p \geq 0.05$).

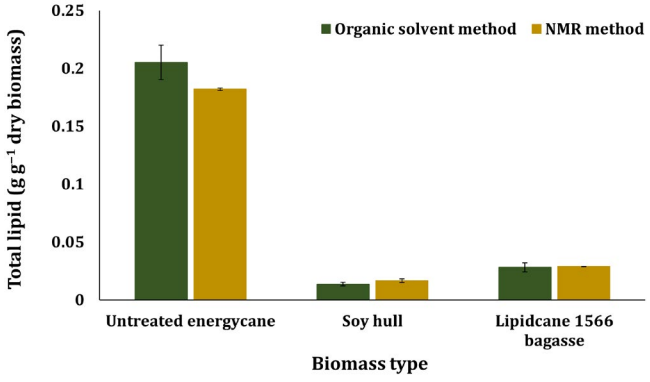

FIGURE 2 Validation of total oil/lipid content measured by td-^{1}H-NMR using hexane extraction. The measured values from both the methods were not significantly different, that is, $p \geq 0.05$

3.3 | Lipid recovery study and assessment of pretreated biomass using td ^{1}H-NMR

To evaluate the accuracy of the NMR-based quantification and interpretation of relaxometry correlation for biomass after bioprocessing, energycane bagasse with known lipid content (20% per g dry biomass) was pretreated for lipid recovery using two-staged hydrothermal and mechanical milling, dilute acid, and alkaline procedures. Total lipid content in pretreated biomass samples measured with td ^{1}H-NMR was compared with the lipid measurements obtained using the organic solvent extraction method (Figure 3). Comparable numbers for total oil content per g dry biomass were obtained for the biomass samples processed with two-staged hydrothermal and mechanical, and alkaline pretreatment in contrast to results for the dilute acid pretreated biomass. A higher lipid measurement was observed for biomass pretreated with dilute acid. NMR analysis also exhibited a higher total lipid content for control energycane biomass (without oil) after dilute acid pretreatment (Table S1). Data in Table 2 present the percent variance in the measured total oil content of untreated and pretreated biomass between ^{1}H-NMR and the conventional organic solvent method. The percent variance between the two methods is least when the pH of the sample after pretreatment is close to neutral, that is, 7. The dilute acid pretreated sample (pH 1–2) showed the maximum variance in measurement, that is, 74.6%. The presence of excess H^+ ions in the dilute acid pretreated samples appears to be interfering with the lipid quantification using NMR spectroscopy.

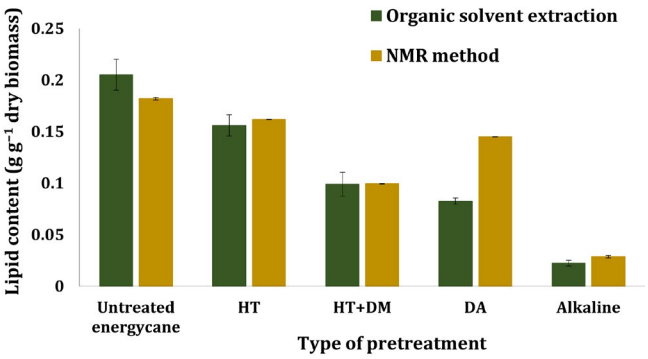

FIGURE 3 Comparison of total lipid content measured in biomass samples pretreated with various feedstock preprocessing namely hydrothermal (HT), hydrothermal + disk milling (HT + DM), dilute acid (DA), and alkaline using organic solvent extraction and ^{1}H-NMR spectroscopy

TABLE 2 Comparison of organic solvent extraction and NMR spectroscopy method for total oil content in biomass samples before and after feedstock preprocessing

Feedstock	pH of sample	Organic solvent extraction	NMR spectroscopy	Percent variance
Untreated (test sample)	6.6–7.2	0.205 ± 0.015	0.182 ± 0.001	11.2
HT	6.1–6.8	0.156 ± 0.011	0.168 ± 0.002	7.6
HT + DM	6.1–6.8	0.099 ± 0.011	0.099 ± 0.0002	0.4
DA[a]	1.0–2.0	0.083 ± 0.003	0.145 ± 0.0003	74.6*
Alkaline[a]	12.5–13.5	0.023 ± 0.004	0.028 ± 0.001	21.7

Note: Average ± standard deviation.

Abbreviations: DA, dilute acid pretreatment; HT, hydrothermal pretreatment at 180°C; HT + DM, hydrothermal pretreatment at 180°C followed by disk milling.

[a]The pH of the sample was measured after washing.

*Significant difference ($p \leq 0.05$).

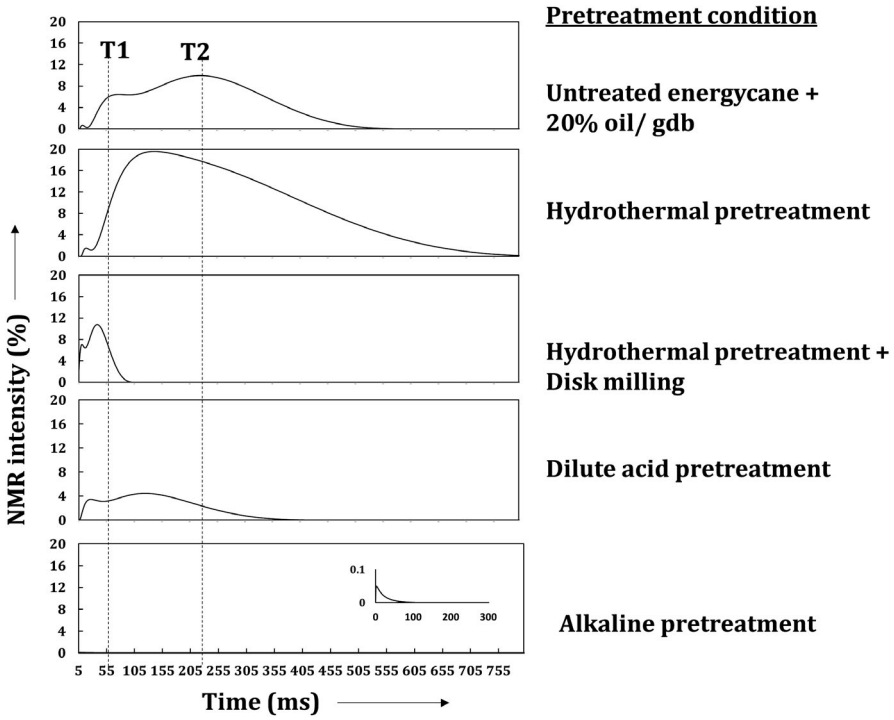

FIGURE 4 Lipid-associated *T2* relaxometry spectra loss due to various physical and chemical feedstock preprocessing. All the pretreatment procedures were carried out with representative/model biomass

A decline in total lipid content subsequent to each type of feedstock preprocessing was observed in pretreated biomass residues (Figure 3). The decline in lipid content can be ascribed to either recovery or degradation of lipids during pretreatment and, hence was further investigated using the NMR relaxometry study.

3.4 | Investigating the stability of oil during feedstock processing

T2 relaxometry spectra in Figure 4 correlate the change in the fluidity/degree of freedom of proton nuclei after various pretreatment procedures, that is, relaxation time distribution and NMR intensity, with stability and recovery of total lipid from lignocellulosic biomass. A reduction in the magnitude of *T2* relaxation time of the pretreated sample is directly correlated with the recovery of corresponding bound and free oil from the biomass upon pretreatment, as described previously (Table 1). The in-situ lignocellulosic lipid retained its stability on two-staged hydrothermal and mechanical pretreatment, and dilute acid pretreatment protocols while alkali pretreated biomass demonstrates the complete degradation of oil (Figure 4). On the other hand, an increase in the magnitude of NMR intensity in hydrothermally pretreated biomass indicates the enrichment of lipid due to pretreatment.

The biomass pretreated with two-staged hydrothermal and mechanical pretreatment showed promising results for recovery of oil, and therefore was studied in more detail. Analysis of total oil recovery (Figure 5b) and *T1T2* relaxometry correlation spectra (Figures 4 and 5a; Table S2) of pretreated biomass provided insight into the percent recovery

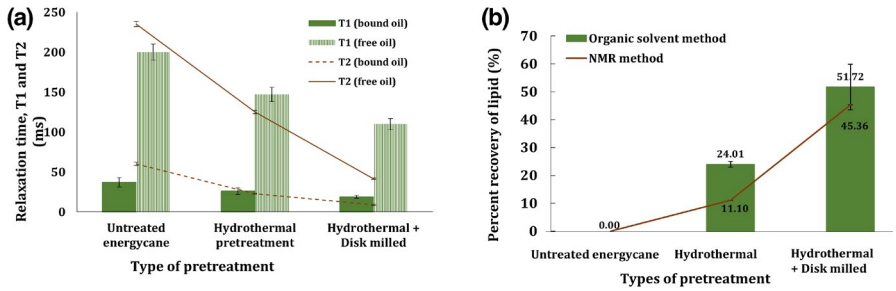

FIGURE 5 (a) NMR relaxometry spectra demonstrate the release of the bound and free oil with each step of two-staged hydrothermal and mechanical pretreatment. (b) Assessment of an average percentage recovery of oil from the lignocellulosic sample after each step of pretreatment using organic solvent extraction and ^{1}H-NMR spectroscopy

of bound and free oil during the pretreatment processes. The relaxometry study indicates that hydrothermal pretreatment at 180°C reduced approximately 50% of the oil-associated fluidity (degree of freedom of molecular movement) of the biomass sample when compared to untreated biomass. Coupling hydrothermal pretreatment with disk milling process reduced the fluidity of pretreated biomass by 80%. The mechanical refining by disk milling extracted a considerable amount of oil from the lignocellulosic biomass (reduced the oil-associated fluidity of biomass by approximately 65%). The extent of bound and free oil recovered from lignocellulosic biomass at each step of two-staged pretreatment is interpreted from Figure 5a. Free oil in biomass is more accessible than bound oil and, hence was easier to extract. Coupled hydrothermal treatment and disk milling recovered approximately 50% of the available lipids. The stability of lipids cannot be directly measured because it does not track the fate of the lipids in the liquid phase. However, the NMR relaxometry spectra and corresponding quantification of oil (Figures 3–5) suggest that hydrothermal pretreatment at 180°C followed by disk milling maintains the stability and quality of oil in biomass. Perhaps it is just enough to state that it can be used to measure residual lipids remaining with the pretreated solids for the hydrothermal–mechanical pretreatment, which is the major interest.

4 | DISCUSSION

4.1 | Characterization of bound and free oil/lipid in lignocellulosic biomass

In ^{1}H NMR relaxometry, the times taken by proton nuclei to return to equilibrium following a pulse of energy are measured. In most of the liquid, the relaxation time is inversely proportional to the viscosity. The correlation between viscosity and relaxation time can be described by the equation:

$$\tau_c \ll 1/\nu_o, \quad (1)$$

where τ_c and ν_o represent correlation time associated with Brownian motion of protons in the sample and the Larmor frequency, respectively. *T1* relaxation takes place along fluctuation in the magnetic field, most effectively at the Larmor frequency (ν_o) indicating that *T1* relaxation is field-dependent, while *T2* relaxation is induced by fluctuation in both external or internal fields, mainly due to molecular motion (Pfeifer, 1994). The diagram in Figure 6a illustrates the correlation of relaxation time and viscosity of the liquid sample (adapted from Bloembergen et al., 1947). Similarly, in solids, the ^{1}H-NMR relaxometry spectra correlate with the degree of freedom of proton nuclei in the sample and facilitate the resolution of different subpopulations of proton nuclei based on their "molecular tumbling" rates. Typically, in solid samples, the motion of the molecules is restricted and exhibits a shorter relaxation time. The magnitude of relaxation times of solids and liquids can vary by a factor of 10 or 100 s depending on the viscosity of the liquid (Purcell et al., 1948). A solid sample containing fluidized elements, for instance, unbound oil or moisture, exhibits multiple distinct relaxation times based on the molecular tumbling rate of each subpopulation of proton nuclei. Therefore, in the present study, it is convenient to assign the populations of proton nuclei associated with oil in the biomass sample exhibiting short and long relaxation time as bound and free oil, respectively, in as far as sample moisture was kept ≤2% (Zheng et al., 2017). As depicted in the schematic diagram (Figure 6b), the shorter relaxation times were assigned to the proton nuclei associated with bound oil; similarly, longer relaxation times were assigned to the proton nuclei associated with free oil in the biomass sample.

In agreement with the above explanation, the correlation fitted well for biomass samples in Sets 1 and 2 (Figure 1a–d). Wild-type sugarcane bagasse (control) had only one *T2* relaxation time with a lower NMR intensity generated by the plant fibers. The comparison of NMR intensities of soy hull and lipidcane 1566 suggested the later had a higher lipid content (proven correct on lipid quantification). Moreover, *T2* relaxation times of transgenic lipidcane 1566 with in-situ oil

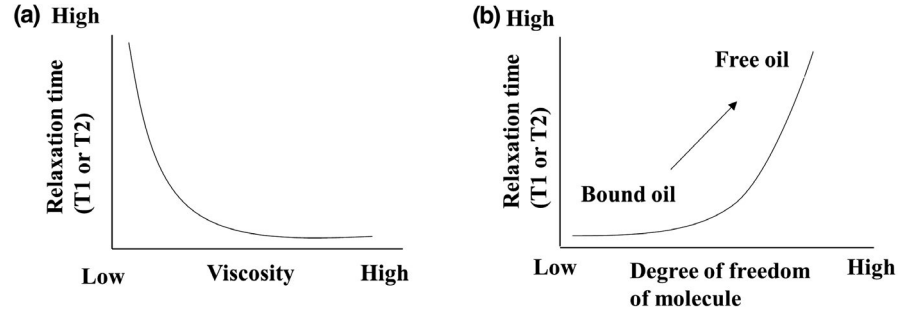

FIGURE 6 Illustration of one-dimensional NMR relaxometry correlation spectra. (a) Typically relaxation time (*T1T2*) of an object varies inversely proportional to the viscosity of that object, that is, highly viscid liquids have shorter relaxation time (Bloembergen et al., 1947). (b) Objects with lower relaxation time have a lesser degree of freedom of molecules, that is, tightly packed molecules than the objects with longer relaxation time. In the present study with ^{1}H-NMR, the subpopulation of proton nuclei with shorter and longer relaxation time (*T1* or *T2*) in each biomass sample are associated with tightly packed (bond oil) and relatively free (free oil) proton nuclei within the sample, respectively

exhibited a higher magnitude of free oil which is in agreement with observations by Parajuli et al. (2020) describing hyperaccumulation of lipids in the form of droplets inside the vegetative tissues of transgenic lipidcane 1566 (Parajuli et al., 2020).

4.2 | td-NMR for lipid quantification in lignocellulosic biomass bioprocesses

The NMR signal is directly related to the percentage of hydrogen in the oil sample, which varies significantly among different oils depending on their fatty acid profile (Luthria et al., 2004). The difference in the calibration curves for crude corn and soybean oil (Supplementary, Figure S2a–b) showed that corn and soybean oils have a significantly different composition of fatty acids (corn oil is richer in monosaturated fatty acids). Therefore, to improve the accuracy of the total lipid analysis in transgenic lines of lignocellulosic biomass, lipid extracted from lipidcane 1566 was used for NMR calibration. Lipid quantification of biomass samples having different oil compositions was measured accurately using the corresponding calibration.

Furthermore, the transgenic biomass containing lipids needs to be extensively processed to recover them to be used for biodiesel production. The present study showed that td-NMR can be used to measure the residual lipids in the fiber after each bioprocess. However, a higher NMR reading for lipid quantification in dilute acid pretreated biomass was observed. This can be attributed to an increase in the concentration of hydronium ions during acid pretreatment, although the residual biomass was washed twice (Lloyd & Wyman, 2004; Speight, 2018). NMR analysis also exhibited higher total lipid content for control energycane biomass (without oil) after dilute acid pretreatment (Table S1). The acidic pH of the sample confirmed increased H^{+} molecules after pretreatment. The observation is in agreement with the reports by other research groups that acid treatment interferes with the overall charge of the slurry and needs a neutralization process (Ariunbaatar et al., 2014; Nazari et al., 2018). On the other hand, the alkaline pretreated biomass had a soapy feel and frothed when washed with deionized water (Figure S3), which is expected from alkali catalyzed saponification of the oil to the soap (Brown, 1916). The main chemical reactions during alkaline pretreatment involve solvation and saponification, resulting in the swelling of the biomass, making the cellular parts susceptible to react with the external agent (Chen & Wang, 2017). The inconsistency in relaxometry analysis of alkali pretreated biomass revealed degradation of oil due to saponification reaction (Figure 4; Table S2). The observation suggests that alkaline pretreatment is unsuitable for the recovery of lipids from transgenic biomass.

The NMR relaxometry for assessment of water-associated proton nuclei for their mobility in biomass structure has been successfully established. Foston and Ragauskas (2010) used a combination of ^{1}H and ^{2}H NMR techniques to demonstrate the pore expansion of lignocellulosic fibril bundle on acid pretreatment of *Populus* (Foston & Ragauskas, 2010). Jeoh et al. (2017) performed 2D ^{1}H-NMR on SO_2 catalyzed thermal pretreated Spruce biomass to establish the microstructure of the water environment within pretreated biomass (Jeoh et al., 2017). In both cases, the moisture content of the biomass was maintained ≥10%, and improvement in the porosity was studied by correlating the increase in *T2* relaxation time of water-associated proton nuclei as it suggests more space for the water molecules in the porous biomass to have a higher molecular tumbling. The present study extends the use of NMR relaxometry for the assessment of oil-associated proton nuclei. However, in contrast, for analysis of oil in lignocellulosic biomass using ^{1}H-NMR, it is necessary to minimize the contribution of water molecules by keeping the moisture content to a minimum, and the recovery of oil resulted in a decrease in the magnitude of *T2* relaxation time. The relaxometry

correlation holds with the quantification of oil in pretreated biomass. The understanding of NMR spectroscopy and relaxometry facilitates a quick and non-destructive assessment of the effectiveness of bioprocessing such as porosity (Foston & Ragauskas, 2010; Jeoh et al., 2017), recovery and quality of sugars (Kiemle et al., 2003), and total lipids (this study) in transgenic lines. Further investigations with lipidcane (genetically modified sugarcane with hyperaccumulation of in-situ oil) are in progress.

5 | CONCLUSION

This study establishes the use of td ^{1}H-NMR as a robust and convenient method for qualitative and quantitative analyses of in-situ lipid content of lignocellulose. The method is non-destructive, fast, and amendable to high sample throughput. The evaluation of *T1T2* relaxometry correlation spectra of lignocellulosic in-situ lipids separated total lipid content into bound and free fractions based on the degree of freedom of different proton nuclei subpopulations. The study presents the interpretation of relaxometry spectra of the lignocellulosic biomass for the qualitative reasoning of the consequences of various physical and chemical feedstock preprocessing on the stability and recovery of total lipid content, thereby determining the suitability of three prevalent feedstock preprocessing protocols for lignocellulosic biomass containing oil in a single step. As a further application, this method is used to monitor changes in the total lipid content, and the fraction of bound and free lipid after processing with alkali, dilute acid, and hot water/disc milling. The adaption of time-domain ^{1}H-NMR spectroscopy for chemical-free and rapid quantification of vegetative oil/total lipids in lignocellulosic biomass represents a significant and novel contribution to the present analytical methods and to the best of our knowledge, this is the first report of this kind. It can significantly speed the development of new energy crops such as energycane, miscanthus, and sorghum that are being genetically modified to accumulate oilseed like energy-rich molecules for conversion to fuels and bioproducts.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Shraddha Maitra https://orcid.org/0000-0003-3129-3566
Stephen P. Long https://orcid.org/0000-0002-8501-7164
Vijay Singh https://orcid.org/0000-0003-4349-8681

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SUPPORTING INFORMATION

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