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# Validation of lignocellulosic biomass carbohydrates determination *via* acid hydrolysis

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ABSTRACT

This work studied the two-step acid hydrolysis for determining carbohydrates in lignocellulosic biomass. Estimation of sugar loss based on acid hydrolyzed sugar standards or analysis of sugar derivatives was investigated. Four model substrates (starch, holocellulose, filter paper and cotton) and three levels of acid/material ratios (7.8, 10.3 and 15.4, v/w) were studied to demonstrate the range of test artifacts. The method for carbohydrates estimation based on acid hydrolyzed sugar standards having the most satisfactory carbohydrate recovery and relative standard deviation. Raw material and the acid/material ratio both had significant effect on carbohydrate hydrolysis, suggesting the acid to have impacts beyond a catalyst in the hydrolysis. Following optimal procedures, we were able to reach a carbohydrate recovery of 96% with a relative standard deviation less than 3%. The carbohydrates recovery lower than 100% was likely due to the incomplete hydrolysis of substrates, which was supported by scanning electron microscope (SEM) images.



## 1. Introduction

Carbohydrate is important for food, feed, pulp and paper, biorefinery, plant physiology and agronomic research (Brehm, Seeley, Daniels, & D'Alessio, 2003; Haynes & Francis, 1993; Jenkins, Baxter, Miles, & Miles, 1998; TAPPI, 2009; Van Soest, Robertson, & Lewis, 1991; Weinmann, 1947). Lignocellulosic biomass, a rich resource of carbohydrates (Hu & Ragauskas, 2012), is widely used for food, feed, fibers, materials, fuels and chemicals (Cheremisinoff, Cheremisinoff, & Ellerbusch, 1980, chap. 1; Fernando, Adhikari, Chandrapal, & Murali, 2006; Roman-Leshkov, Barrett, Liu, & Dumesic, 2007). Determining carbohydrates in lignocellulosic biomass is vital and fundamental for all these areas.

Measuring carbohydrate in lignocellulosic biomass usually includes sugar release and sugar analysis. A two-step sulfuric acid hydrolysis is a widely used technique for releasing sugars from biomass (Saeman, Moore, Mitchell, & Millett, 1954; Sluiter et al., 2011; TAPPI, 2009): biomass is firstly treated with 72%w sulfuric acid at low temperature and then hydrolyzed with 4%w sulfuric acid at elevated temperature. Major sugars released during this process are arabinose, galactose, glucose, mannose and xylose (TAPPI, 2009), because plants primarily consist of them in polymeric form.

Sugars released from biomass can be analyzed by colorimetric method (Marsden, Gray, Nippard, & Quinlan, 1982), ultraviolet (UV) spectroscopy (Hu, Wang, Chai, & Kong, 2008), gas chromatography (TAPPI, 2009) and high-performance liquid chromatography (HPLC) (Sluiter et al., 2011). The HPLC analysis is currently popular with advantages including: the ability to detect different sugars simultaneously along with their amounts (Sluiter et al., 2011), relatively simple sample preparation, different columns and detectors can be used (Sluiter et al., 2008, 2011; Zhang & Runge, 2012) making it easily adapted by different labs (Davis, 1998). High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is an HPLC system equipped with anion-exchange column and pulsed amperometric detector (Hughes & Johnson, 1981; Khym & Zill, 1951).

It is known that there is sugar loss during the two-step hydrolysis of carbohydrates because of sugar decomposition (Saeman, Bubl, & Harris, 1945; Saeman et al., 1954). Measured sugars are not the total sugars released from carbohydrates. Thus, the amount of carbohydrates calculated based on these measured sugars is lower than true value. In order to balance the sugar loss, two groups of sugar standards are used for carbohydrates estimation (Sluiter et al., 2011). One group is used to set the standard curve, while another group is typically treated by dilute acid hydrolysis to get a sugar loss rate (Saeman et al., 1945, 1954; Sluiter et al., 2011). Though it is known that the derivatives from sugar decomposition are mainly short-chain fatty acids and furans, *e.g.*, furfural, HMF and levulinic acid (Girisuta, 2007, chap. 1; Hasche, 1945; Xiang,

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Lee, & Torget, 2004), little research has estimated the sugar loss by measuring the derivatives.

Another question raised is the extent of the carbohydrate hydrolysis. Previous research assumed that the carbohydrates were totally hydrolyzed. However, Chandra et al. (2007) showed that the acid insoluble lignin measured by a similar two-step acid hydrolysis could have artificially high value because of residual carbohydrates. Other studies showed that residual carbohydrates in carbohydrate-lignin complex could hardly be totally removed by acid hydrolysis (Guerra et al., 2006; Wu & Argyropoulos, 2003). Additionally, cellulosic substrates prepared from lignocellulosic biomass could have some residual lignin (Nakagame, Chandra, & Saddler, 2010). Besides, the hydrolysis of carbohydrates may be incomplete because inaccessible char could form from cellulosic materials during the first-step of the acid hydrolysis (Ritter, Mitchell, & Seborg, 1933), described by other research as "pseudo-lignin" (Hu, Jung, & Ragauskas, 2012; Kumar et al., 2013; Li, Henriksson, & Gellerstedt, 2005; Sannigrahi, Kim, Jung, & Ragauskas, 2011). However, limited studies have investigated the incomplete acid hydrolysis of carbohydrates during acid hydrolysis conditions used in analysis.

Thus, several questions were raised: (1) can the sugar loss be estimated by measuring the acids and furans derived from sugar decomposition? (2) Can just one group of sugar standards be used for analyzing carbohydrates instead of two groups? (3) Is the release of carbohydrates from lignocellulosic materials complete during the two-step acid hydrolysis? In our study, two-step acid hydrolysis was carried out on the model substrates of lignocellulosic biomass. Different methods were used to estimate the sugar loss, and the effect of raw material and acid/material ratio was also studied. Finally, SEM images of residues after acid hydrolysis were taken to verify the hypothesis that the acid hydrolysis was incomplete.

## 2. Materials and methods

### 2.1. Chemicals and reagents

D(−)-Arabinose, D(+)-galactose, D(+)-glucose (anhydrous), D(+)-mannose, D(+)-xylose (all 99+%), HMF (98%) and levulinic acid (98+%) were purchased from Acros Organics (Geel, Belgium). Furfural (min 98.0%, GC grade) was purchased from TCI America (Portland, OR, US). Sulfuric acid (96.6%, w/w), sodium chlorite (100.1%) and sodium hydroxide (50%, w/w) were purchased from Fisher Scientific (Fair Lawn, NJ, US). Sulfuric acid (72%, w/w) was purchased from Ricca Chemical Company (Arlington, TX, US). Acetic acid (glacial, ≥99.7%) was purchased from EMD Chemicals (Darmstadt, Germany). Syringe filter with 0.2 μm pore size (Whatman, Cat. No. 6789-1302) was purchased from Fisher Scientific (Fair Lawn, NJ, US). Millipore purified water (HPLC grade) was used for preparing HPLC mobile phase. Deionized (DI) water was used in other steps. The 96.6%w/w sulfuric acid was used for preparing the holocelluloses, and the 72% (w/w) sulfuric acid was used during the acid hydrolysis.

### 2.2. Materials

Model substrates of lignocellulosic biomass were used to create a control system, and their labels used in this paper are shown in Table 1. Processed cotton was purchased from Fisher Scientific (Fair Lawn, NJ, US). Filter paper (Whatman, qualitative, grade 1, 90 mm ∅, Cat No. 1001090) was purchased from Sigma–Aldrich (St. Louis, MO, US). Starch (soluble potato, powder) was purchased from J.T. Baker (Phillipsburg, NJ, US).

**Table 1**
Model substrates and their labels used in this study.

| Label | Raw material |
|---|---|
| *#Co* | Cotton, processed |
| *#F* | Filter paper, Whatman, qualitative |
| *#Ho1* | Holocellulose, pretreatment pH = 5.81 during preparation |
| *#Ho3* | Holocellulose, pretreatment pH = 0.51 during preparation |
| *#S* | Starch, soluble, with secondary hydrolysis |
| *#S2* | Starch, soluble, with two-step hydrolysis |

Two holocellulose samples were prepared in lab from hybrid poplar flour. NM-6 hybrid poplar, *Populus maximowiczii* × *nigra*, were harvested from northern Wisconsin after 10 years of age and seasoned for 3 months before use. The tree boles were hand-debarked with a draw knife, chipped in a disk chipper, and screened with accepts set at 2 mm and 8 mm holes. The chips were allowed to oven-dry to approximately 5% moisture and milled with a 2 mm screen (Wiley mill) to create wood powder.

Then, the wood powder was pre-hydrolyzed. For each sample, 84 g (d.b.) flour was put into a 1000 mL glass bottle with a high temperature resistance cap (PYREX). DI water and sulfuric acid solution were added to reach a liquid/solid (v/w) ratio of 8. The pH was 5.81 for sample *#Ho1* and 0.51 for sample *#Ho3*, respectively. The bottle was capped and put into a 20 L stainless steel reactor with heated circulation. The reactor was partially filled with water. It was heated to 165 °C at a rate of 4.5 °C/min, kept at 165 °C for 60 min, and cooled down to 84 °C at a rate of 1.35 °C/min. After pre-hydrolysis, the mixture was neutralized to pH = 7 by 1.2 N NaOH solution. The solid was washed by 2 L DI water twice, then air-dried at room temperature.

A modified acidic chlorite procedure was used to create holocellulose (Leavitt & Danzer, 1993; Lovell, 1945; Sjöström & Alén, 1999, chap. 3). To each 225 ml flask, 3 g (d.b.) pre-hydrolyzed hybrid poplar flour was added. Then, 60 mL DI water, 1.20 g $NaClO_2$ and 0.03 mL acetic acid were added. The pH of the suspension was 4.1 at the beginning. The flask was put into a water bath at 75 °C and shaken regularly. At 6 h, 8.5 h and 11 h, 1.20 g $NaClO_2$ was added, separately. The pH was 3–5 during the reaction. After 2 h, the flask was cooled down by ice water, and 900 mL DI water was added to stop the reaction. The suspension was filtered, and the solid was washed twice by 200 mL DI water, air dried at room temperature and vacuum dried at 45 °C. The yield was 48.02% for sample *#Ho1* and 44.85% for sample *#Ho3*. The amount of $NaClO_2$ used was more than needed during this process compared to literature (Leavitt & Danzer, 1993; Lovell, 1945; Sjöström & Alén, 1999, chap. 3), and created lower holocellulose yields than reported in literature (Leavitt & Danzer, 1993; Lovell, 1945; Sjöström & Alén, 1999, chap. 3). Thus, it is safe to assume the holocelluloses as pure carbohydrates.

Physical properties of these substrates are summarized from literatures as shown in Table 2. Starch is soluble in water, while other

**Table 2**
Properties of model substrates from literatures.

| Materials | Water soluble | Crystallinity index, % | Degree of polymerization (DP) |
|---|---|---|---|
| Soluble starch | Yes | ~32[a] | 10.5–111[e] |
| Holocellulose | No | 36.1–51.5[b] | 3500[f] |
| Filter paper | No | 70.2[c] | 5000–8000[g] |
| Cotton | No | 73.7[d] | 12,000–17,000[h] |

[a] The average of several kinds of potatoes (Tester, Karkalas, & Qi, 2004).
[b] Hybrid *Populus* (Jin & Kamdem, 2009).
[c] Park, Johnson, Ishizawa, Parilla, and Davis (2009).
[d] IFC cotton linter (Park et al., 2009).
[e] Heitmann, Wenzig, and Mersmann (1997).
[f] Sannigrahi, Ragauskas, and Tuskan (2010).
[g] Reported DP for spruce wood cellulose (Lewin, 2007, chap. 11).
[h] Lewin (2007).

materials are insoluble. From starch, holocellulose, filter paper, to cotton, their crystallinity index and their degree of polymerization (DP) both increase.

### 2.3. Acid hydrolysis

Raw materials were treated with 72% sulfuric acid for 60 min at 30 °C in water bath while stirring/crushing with a glass rod. Then, the mixture was diluted to 4% sulfuric acid by DI water, and treated at 121 °C in an autoclave for 60 min. The hydrolysate was neutralized by 1.2 M NaOH solution to pH 6–8. This two-step hydrolysis was carried out on starch (sample *#S2*), holocelluloses (samples *#Ho1* and *#Ho3*), filter paper (sample *#F*) and cotton (sample *#Co*). The soluble starch was assumed to be easily hydrolyzed. Thus, only the second-step hydrolysis was also carried out on starch (sample *#S*).

For each material, the acid/material (v/w) ratio was varied. Cellulosic materials of 0.2 g, 0.3 g, and 0.4 g were treated by 3 mL 72% $H_2SO_4$, resulting in an acid/material (v/w) ratio of 15.4, 10.3 and 7.8, respectively.

### 2.4. Analysis of sugars and derivatives

The hydrolysate was analyzed by the HPAEC-PAD for sugars and HPLC for degradation products. Neutralized samples were diluted by DI water, filtered by 0.2 μm syringe filter, and analyzed on a DIONEX ICS-3000 system. The system was equipped with a DIONEX BioLC CarboPac PA20 analytical column (3× 150 mm), a DIONEX BioLC CarboPac PA20 guard column (3× 30 mm), and an integrated pulsed amperometric detector with disposable gold electrode (DIONEX carbohydrate certified, product No. 060139). The mobile phase was Millipore purified water at a flow rate of 0.35 mL/min with 0.5 M NaOH used as post column eluent. The column compartment was 30 °C (Zhang & Runge, 2012). Acids and furans were also analyzed on the DIONEX ICS-3000 system equipped with a Supelcogel C-610H analytical column (30 cm × 7.8 mm ID), a Supelguard C-610H guard column (5 cm × 4.6 mm ID), and a UV–vis detector set at 205 nm. The eluent was 0.1% w $H_3PO_4$ solution at a flow rate of 0.60 mL/min. The column temperature was 50 °C.

### 2.5. Methods for carbohydrates estimation

Three methods for carbohydrates estimation were studied (Fig. 1). In the first method (*Carbohy_1*), the sugar loss was not included. The standard curve was set by a group of normal sugar standards including arabinose, galactose, glucose, mannose and xylose. First, the hexose amount (*Hexose_1*) and the pentose amount (*Pentose_1*) were calculated based on the following reactions:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6$$

$$(C_5H_8O_4)_n + nH_2O \rightarrow nC_5H_{10}O_5$$

Then, the amount of carbohydrates (*Carbohy_1*) was computed by the following formula:

$$Carbohy\_1 = 0.9 \times Hexose\_1 + 0.88 \times Pentose\_1$$

In the second method (*Carbohy_2*), sugar loss was estimated by measuring the derivatives from sugar decomposition. The furfural, HMF and levulinic acid (Girisuta, 2007, chap. 1; Hasche, 1945; Xiang et al., 2004), which were the main derivatives, were analyzed by HPLC. Sugar loss was estimated based on the following reactions:

$$C_5H_{10}O_5 \rightarrow C_5H_4O_2\,(\text{furfural}) + 3H_2O$$

$$C_6H_{12}O_6 \rightarrow C_6H_6O_3\,(\text{HMF}) + 3H_2O$$

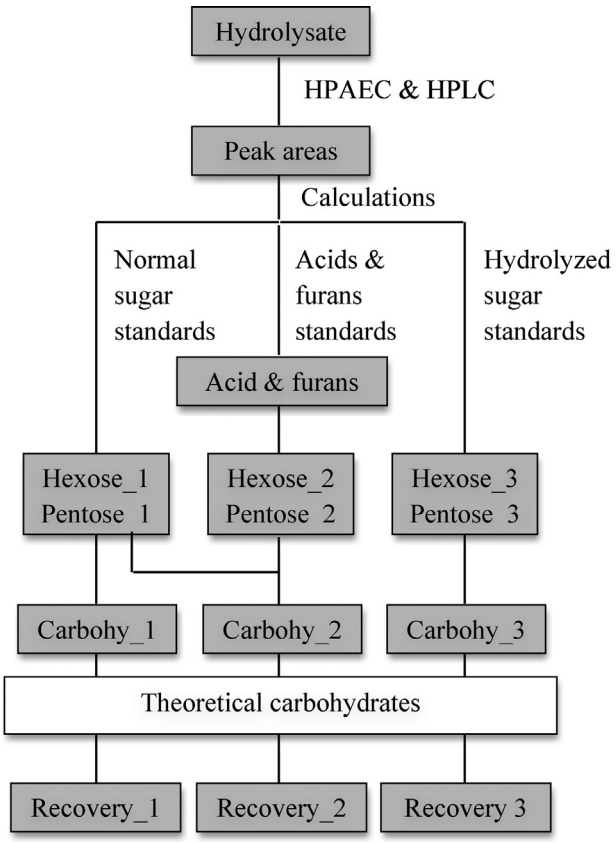


**Fig. 1.** Illustration of the three methods for carbohydrates estimation.

$$C_6H_6O_3\,(\text{HMF}) + 2H_2O \rightarrow C_5H_8O_3\,(\text{levulinicacid}) + CH_2O_2\,(\text{formicacid})$$

The decomposed pentose (*Pentose_2*), and the decomposed hexose (*Hexose_2*) were included in the carbohydrate calculations based on the following formula:

$$Carbohy\_2 = 0.9 \times (Hexose\_1 + Hexose\_2) + 0.88 \times (Pentose\_1 + Pentose\_2)$$

In the third method, the sugar loss was estimated by treating the sugar standards with the second-step hydrolysis. Since the sugar decomposition happened to both the samples and standards, the sugar loss was assumed to be balanced. The *Carbohy_3* was computed by the following formula:

$$Carbohy\_3 = 0.9 \times Hexose\_3 + 0.88 \times Pentose\_3$$

The reason for performing only the dilute acid hydrolysis on the sugar standard was that the sugar decomposition during the primary hydrolysis was much lower than that of the second-step hydrolysis (Saeman et al., 1945, 1954), which could be ignored.

The recovery was calculated according to the following formula:

$$\text{Recovery}(\%) = \frac{\text{measured carbohydrates}}{\text{theoretical carbohydrates}} \times 100\%$$

Data were analyzed with JMP Pro 10 (SAS Institute Inc., Cary, NC, US), and the charts were reproduced with Microsoft Excel.

### 2.6. Scanning electron microscope (SEM)

The hydrolysate was centrifugalized at 4000 RPM for 30 min. Then, the solid residues were washed three times by 45 mL DI water

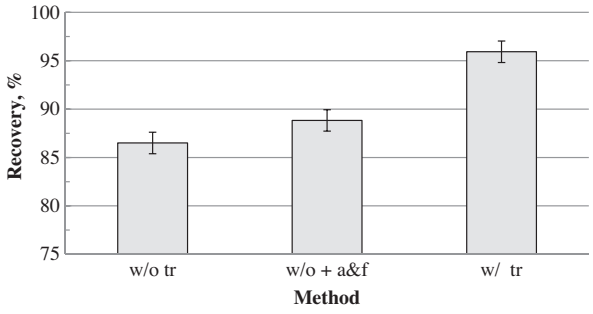


**Fig. 2.** Carbohydrate recovery (%) of different methods (95% CI). (w/o tr, method based on normal sugar standards without hydrolysis; w/o + a&f, method based on normal sugar standards with sugar loss estimated by measuring acids and furans; w/tr, method based on acid hydrolyzed sugar standards; CI, confidence interval).

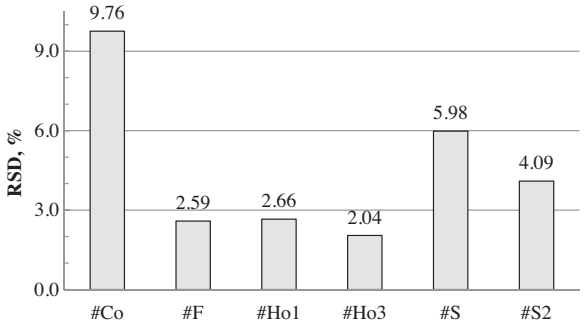


**Fig. 3.** Relative standard deviation (RSD) of different raw materials. (*#Co*, cotton; *#F*, filter paper; *#Ho1*, holocellulose #1; *#Ho3*, holocellulose #3; *#S*, soluble starch with the second-step hydrolysis; *#S2*, soluble starch with two-step hydrolysis).

with 10 min ultrasonic agitation, and centrifugalized at 4000 RPM for 30 min. The solid residues were re-suspended in 10 mL DI water with 5 min ultrasonic agitation. A drop of the suspension was put on a clean glass plate, dried in oven at 40 °C, and coated with gold:palladium using a SeeVac (Pittsburgh, PA) Auto Conductance IV vacuum sputter-coater to improve the conductivity. A Hitachi S-570 $LaB_6$ Scanning Electron Microscopy operated at 10.0 kV was used to take images.

## 3. Results and discussion

### 3.1. Effect of method for carbohydrates estimation

Sugar loss estimation based on measuring sugar derivatives or using just one group of acid hydrolyzed standards, was compared with the method without estimation of sugar loss. Fig. 2 shows the average recovery of each method with 95% confidence interval, calculated by JMP Pro 10 using a means table from an analysis of variance, which allows individual effects to be inspected.

The recovery (*Recovery_1*) based on normal standards was lower than 100%, as expected, as the sugar loss was not included. The recovery (*Recovery_2*) with sugar loss estimated by measuring acids and furans was also lower than 100%, though improved over the first method. Two reasons are hypothesized for this. One reason was that the sugar loss was only partially included. In this study, only furfural, HMF and levulinic acid were included, because the reaction paths from sugars to these chemicals were relatively clear. Other acids, *e.g.*, formic acid and acetic acid, could form from sugars through multiple paths, making correction to the original carbohydrate difficult. Another reason was that some polysaccharides and oligosaccharides (Sasaki, Fang, Fukushima, Adschiri, & Arai, 2000; Sasaki et al., 1998) were not converted into monosaccharides. They would be soluble but would not be detected.

The recovery (*Recovery_3*) based on acid hydrolyzed sugar standards was 95.9%, which is close to 100% as expected. Additionally, the average recovery of samples *#Ho1* and *#Ho3*, the close models of real biomass, was also close to 100%. Thus, this method is recommended for measuring carbohydrates in lignocellulosic biomass.

However, the recovery is still lower than 100%. This was hypothesized because the hydrolysis of carbohydrates was incomplete. Most previous studies assumed the hydrolysis was complete, which was because these studies used real biomass, thus, carbohydrates residues would mix with insoluble lignin and could hardly be observed.

### 3.2. Variability of the third method

A good method should have both high accuracy and precision. The variability of the third method was also studied in addition to accuracy (as discussed previously in Section 3.1). The relative standard deviation (RSD) for each material is shown in Fig. 3. An acceptable RSD should be less than 5%, such as sample *#F* and *#S2*. The RSD of sample *#Co* was very large, possibly because cotton was difficult to mix with the 72% sulfuric acid during the first-step hydrolysis. The sample *#S* has a RSD a little larger than 5%. This was because the starch was not mixed well with 4% sulfuric acid before the bottle was put into the autoclave. However, these two RSDs cannot determine whether this method is acceptable for measuring carbohydrates or not, because cotton and starch were not the close models of real lignocellulosic biomass. The closest models of real lignocellulosic biomass were samples *#Ho1* and *#Ho3*. The RSDs of them were all smaller than 3%, which suggests the third method provided precise results. Thus, this method was identified as the preferred method for measuring carbohydrates.

### 3.3. Raw material effect

Raw material is an important variable that might affect the carbohydrate analysis (Milne, Chum, Agblevor, & Johnson, 1992). The content of cellulose and hemicellulose greatly varies among grass, hardwood and softwood (Saeman et al., 1945), though all of them are lignocellulosic biomass. The carbohydrate content can even vary significantly for the same species or even the same pot of sample (Milne et al., 1992). The physical structure, *e.g.*, pore size and degree of crystallinity, also varies greatly. There is no fixed pattern of hydrolysis behavior among different lignocellulosic biomass.

In order to reach a systemic understanding, a series of model substrates including cotton, filter paper, holocellulose and starch were studied (Table 2). Cotton, which is insoluble in water with high crystallinity and high DP, was assumed difficult to be hydrolyzed. Filter paper was expected to be a close model of real biomass, but more difficult to be hydrolyzed than real biomass. Holocellulose was thought to be the closest model of real lignocellulosic biomass. Starch, which is soluble with low crystallinity and low DP, was assumed easy to be hydrolyzed. Thus, cotton and starch were taken as the "upper" and "lower" limits of lignocellulosic biomass during acid hydrolysis. Additionally, all these materials were pure carbohydrates allowing the calculation of recoveries.

Fig. 4 shows the average carbohydrate recovery of each raw material using an analysis of variance to create a means plot to inspect only the impact of a single effect. The recovery increases from cotton to starch, indicating that the raw material had a significant impact on the recoveries. Based on their physical properties, we hypothesized that the carbohydrate recovery of real lignocellulosic biomass should be lower than that of starch but higher than that of cotton. The recoveries of samples *#Ho1*, *#Ho3* and *#F*, which are close models of real biomass, verified this hypothesis. The authors suggest that the carbohydrate recovery in real

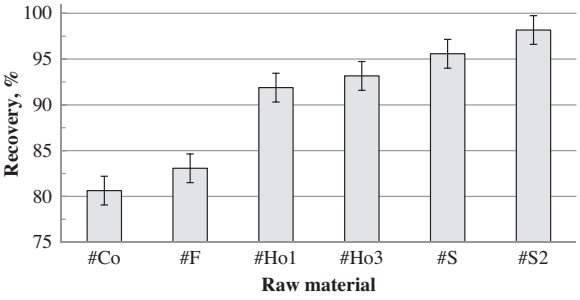


**Fig. 4.** The effect of raw material on carbohydrate recovery (95% CI).

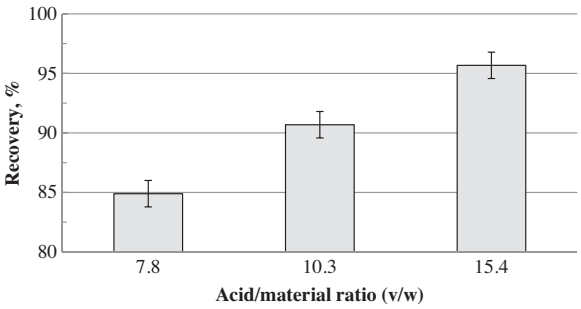


**Fig. 5.** The effect of acid/material ratio on carbohydrate recovery (95% CI).

lignocellulosic biomass will be close to the average recovery of the two holocellulose samples, which was 96% when the third method was used. This was consistent with previous discussion.

The increasing recoveries also indicated that the hydrolysis was incomplete. If the hydrolysis was already complete under present conditions, the raw material type would have no significant effect on the average carbohydrate recovery, because the carbohydrate recovery of each raw material would be ~100%. In our study, a higher carbohydrate recovery indicated that the material was more approaching complete hydrolysis.

Note that these data were the average recoveries of all three methods. The effect of sugar loss may not be excluded. Thus, the recoveries of different raw materials based only on the third method were calculated, which were 88.9%, 89.4%, 95.9%, 96.3%, 99.9% and 105.2% for samples *#Co*, *#F*, *#Ho1*, *#Ho3*, *#S* and *#S2*, respectively. The recoveries also increased from cotton to starch, indicating that the hydrolysis was incomplete. For example, if the hydrolysis of sample *#S* was complete, then the hydrolysis of all other materials except starch was incomplete, and the recovery of sample *#S2* was likely over-estimated.

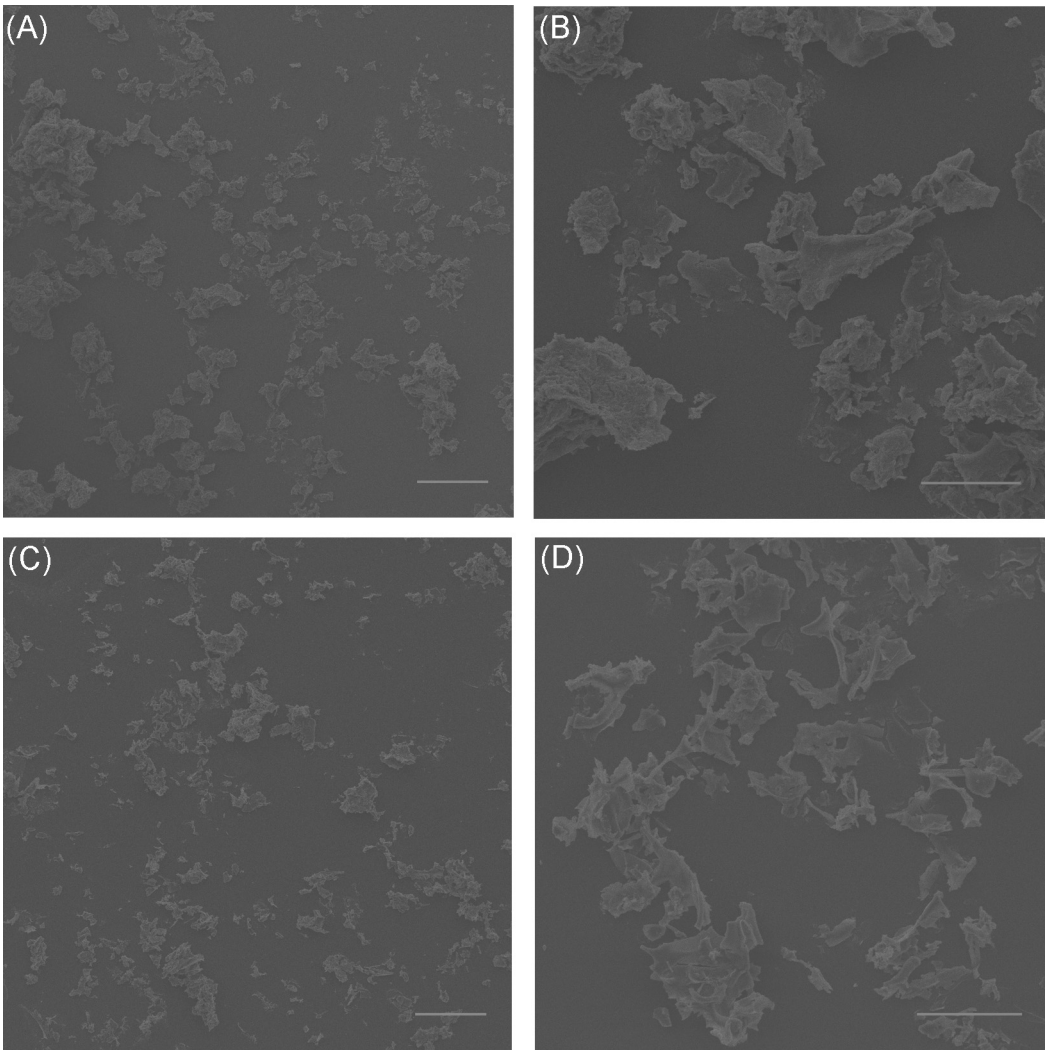


**Fig. 6.** SEM images of hydrolysate solid residues. (A: *#F*, X100; B: *#F*, X500; C: *#Ho1*, X100; D: *#Ho1*, X500; scale bar: 150 μm for A and C, 50 μm for B and D).

This study also inspired the carbohydrate hydrolysis after acid pretreatment, which is considered as a promising pretreatment for biorefinery (Sun & Cheng, 2002). The two holocellulose samples were prepared under near identical conditions, with the only difference being the pH during pre-hydrolysis, indicating different extent of pretreatment. Consequently, the sample *#Ho3* which was pre-hydrolyzed in harsher conditions had a higher average recovery of 93.2% (Fig. 4), while the sample *#Ho1* which was pre-hydrolyzed in milder conditions had a lower average recovery of 91.9%. This suggests that biomass with a harsher acid pretreatment could be more easily hydrolyzed.

### 3.4. Acid/material ratio effect

Saeman et al. (1945) used 5 ml 72% sulfuric acid to treat 0.35 g cellulose during the two-step hydrolysis. Saeman et al. (1954) also used 3 ml 72% sulfuric acid to treat 0.3 g cellulose. Hoebler, Barry, David, and Delort-Laval (1989) used 1.25 mL 72% sulfuric acid to treat 0.1 g cellulose. The acid/material (v/w) ratios in these studies were 14.3, 10.0 and 12.5, respectively, among which the 10.0 was the commonly used one (Saeman et al., 1954; Sluiter et al., 2011; TAPPI, 2009). Though acid/material ratio (v/w) varying from 10.0 to 14.3 has been reported, the effect of the acid/material ratio on carbohydrate analysis was less studied.

In our work, the effect of acid/material ratio with an even wider range (7.8, 10.3 and 15.4, v/w) was studied. The average carbohydrate recovery of each acid/material ratio is shown in Fig. 5, using the means plot from an analysis of variance. The data indicate the recovery increased with increasing acid/material ratio. This was believed to be because a higher acid/material ratio resulted in better dissolution of the lignocellulose matrix which led to higher reactivity in the secondary hydrolysis. When the acid/material ratio was high, the average amount of acid around a particular biomass particle was high, improving solubility and accessibility of this particle. Thus, this particle was more easily to be completely hydrolyzed.

The effect of acid/material ratio also implied that the hydrolysis was incomplete. If the hydrolysis were already complete, the acid/material ratio would have had little effect on the recovery. In other words, the significant positive effect of acid/material ratio would not show. In a case that the hydrolysis is complete, a higher acid/material ratio would even possibly have had a negative effect, resulting in a lower recovery because of sugar decomposition.

### 3.5. SEM

SEM images were taken to verify the hypothesis that the acid hydrolysis of carbohydrates was incomplete. The carbohydrate recovery data already indicated that the hydrolysis of carbohydrates could be incomplete. Additionally, there were solid particles, which were visible, in the hydrolysate of cotton, filter paper and holocellulose. SEM images of the hydrolysate solid residues from a filter paper sample and a holocellulose sample are showed in Fig. 6. Comparing the size and surface look of our residues with those reported by Sannigrahi et al. (2011) and Du, Ren, Xu, and Zhou (2012), we can conclude that our solid residues are highly likely to be non-hydrolyzed carbohydrates. Inspecting the images, it may be seen the residues generally had dimensions of 30–50 μm, while the residues after acid hydrolysis reported by Du, Ren, Xu, and Zhou (2012) had dimension of 100–250 μm. Our residues had a smaller size likely because the conditions used in our study were harsher than those of Du et al. (2012). Additionally, the size of filter paper residues was larger than that of holocellulose, which was consistent with that the carbohydrate recovery of sample *#F* was lower than that of sample *#Ho1*. The incomplete hydrolysis was possibly due to "pseudo-lignin" formation during acid hydrolysis. According to previous studies (Li et al., 2005, 2007), pseudo-lignin which can resist acid hydrolysis could form and cover the surface of carbohydrate particles. Thus, these particles survived during acid hydrolysis and were left as solid residues.

## 4. Conclusions

Determination of carbohydrates in lignocellulosic biomass was studied with model substrates. Three methods for carbohydrates estimation were compared, and the effect of raw material and acid/material ratio was investigated. The method with only one group of sugar standards treated with dilute acid hydrolysis was recommended for carbohydrate determination of lignocellulosic biomass. Raw material can affect the carbohydrate recovery a lot. Thus, the conditions for hydrolysis should be modified for specific material. A higher acid/material ratio would result in a higher carbohydrate recovery. A larger acid volume is recommended because it would allow a more representative biomass sample. Following optimal procedures, we were able to reach a carbohydrate recovery of 96% with a relative standard deviation (RSD) less than 3%. The carbohydrate recovery of less than 100% was theorized to be due to incomplete acid hydrolysis of carbohydrates, which should be considered as an artifact of this test methodology.

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