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TITLE:

Estimation of Plant Biomass Lignin Content using Thioglycolic Acid (TGA)

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Summary

Here, we present a modified TGA method for estimation of lignin content in herbaceous plant biomass. This method estimates the lignin content by forming specific thioether bonds with lignin and presents an advantage over the Klason method, as it requires a relatively small sample for lignin content estimation.

Abstract

Lignin is a natural polymer that is the second most abundant polymer on Earth after cellulose. Lignin is mainly deposited in plant secondary cell walls and is an aromatic heteropolymer primarily composed of three monolignols with significant industrial importance. Lignin plays an important role in plant growth and development, as protection from biotic and abiotic stresses, and in the quality of animal fodder, the wood, and industrial lignin products. Accurate estimation of lignin content is essential for both fundamental understanding of the lignin biosynthesis and for industrial applications of biomass. The thioglycolic acid (TGA) method is a highly reliable method of estimating the total lignin content in the plant biomass. This method estimates the lignin content by forming thioethers with the benzyl alcohol groups of lignin, which are soluble in alkaline conditions and insoluble in acidic conditions. The total lignin content is estimated using a standard curve generated from commercial bamboo lignin.

Introduction

Lignin is one of the vital load-bearing components of plant cell walls and the second most abundant polymer on Earth[1]. Chemically, lignin is a crosslinked heteropolymer made up of high molecular weight complex phenolic compounds that form a natural renewable source for aromatic polymers and synthesis of biomaterials[2,3]. This natural polymer plays significant roles in

plant growth, development and survival, mechanical support, cell wall rigidity, water transport, mineral transport, lodging resistance, tissue and organ development, deposition of energy, and protection from biotic and abiotic stresses[4-7]. Lignin is primarily composed of three different monolignols: coniferyl, sinapyl and p-coumaryl alcohols that are derived from the phenyl propanoid pathway[8,9]. The amount of lignin and the composition of monomers vary based on the plant species, the tissue/organ type, and the different stages of plant development[10]. Lignin is broadly classified into softwood, hardwood, and grass lignin based on the source and monolignol composition. Softwood is primarily composed of 95% coniferyl alcohol with 4% p-coumaryl and 1% sinapyl alcohols. Hardwood has coniferyl and sinapyl alcohols in equal proportions, while grass lignin is composed of various proportions of coniferyl, sinapyl and p-coumaryl alcohols[11,12]. The composition of monomers is critical as it determines the lignin strength, decomposition, and degradation of the cell wall as well as determining molecular structure, branching, and crosslinking with other polysaccharides[13,14].

Lignin research is gaining importance in foraging, textile industries, and paper industries and for bioethanol, biofuel, and bio-products due to its low cost and high abundance[15,16]. Various chemical methods (e.g., acetyl bromide, acid detergents, Klason, and permanganate oxidation) along with instrumental methods (e.g., near infrared (NIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and ultraviolet (UV) spectrophotometry) were used for lignin quantification[9,17]. The analysis methods of lignin are generally classified based on electromagnetic radiation, gravimetry, and solubility. The principle behind lignin estimation based on electromagnetic radiation is a chemical property of lignin by which it absorbs light at specific wavelengths. These results were estimated based on the principle that lignin has a stronger UV absorbance than carbohydrates. In 1962, Bolker and Somerville used potassium chloride pellets to estimate lignin content in wood[18]. However, this method has drawbacks in the estimation of lignin content from herbaceous samples due to the presence of non-lignin phenolic compounds and the absence of an appropriate extinction coefficient. In 1970, Fergus and Goring found that the guaiacyl and syringyl compound absorption maxima were at 280 nm and 270 nm, which corrected the extinction coefficient issue of the Bolker and Somerville method[19]. Later, infrared spectroscopy, a highly sensitive technique for characterizing phenolics, was also used for lignin estimation with a small amount of plant biomass samples. This was used was diffuse-reflectance Fourier transform spectrophotometry. This method, however, lacks a proper standard similar to the UV method[20]. Later, the lignin content was estimated by NIRS (near infrared spectroscopy) and NMR (nuclear magnetic resonance spectroscopy). Though, there are disadvantages in these methods, they do not alter the chemical structure of lignin, retaining its purity[20].

The gravimetric Klason method is a direct and the most reliable analytical method for lignin estimation of woody stems. The basis for gravimetric lignin estimation is the hydrolysis/solubilization of non-lignin compounds and the collection of insoluble lignin for gravimetry[21]. In this method, the carbohydrates are removed by hydrolysis of the biomass with concentrated H_2SO_4 to extract lignin residue[20,22]. The lignin content estimated by this method is known as acid insoluble lignin or Klason lignin. Application of the Klason method depends on the plant species, the tissue type and the cell wall type. The presence of variable amounts of non-

lignin components such as tannins, polysaccharides and proteins, results in proportional differences in the estimation of acid insoluble/soluble lignin contents. Hence, the Klason method is only recommended for lignin estimation of high-lignin content biomass such as woody stems[17,23]. Solubility methods such as acetyl bromide (AcBr), acid-insoluble lignin, and thioglycolic acid (TGA) are most commonly used methods for estimation of the lignin content from various plant biomass sources. Kim et al. established two methods for lignin extraction by solubilization. The first method extracts lignin as an insoluble residue by solubilizing cellulose and hemicellulose, while the second method separates lignin in the soluble fraction, leaving cellulose and hemicellulose as the insoluble residue[24].

Similar methods employed in lignin estimation based on the solubility are thioglycolic acid (TGA) and acetyl bromide (AcBr) methods[25]. Both TGA and acetyl bromide methods estimate the lignin content by measuring the absorbance of the solubilized lignin at 280 nm; however, the AcBr method degrades xylans during the process of lignin solubilization and shows a false increase in the lignin content[26]. The thioglycolate (TGA) method is the more reliable method, as it depends on specific bonding with the thioether groups of benzyl alcohol groups of lignin with TGA. The TGA bound lignin is precipitated under acidic conditions using HCl, and the lignin content is estimated using its absorbance at 280 nm[27]. The TGA method has additional advantages of less structural modifications, a soluble form of lignin estimation, less interference from non-lignin components, and precise estimation of lignin due to specific bonding with TGA.

This TGA method is modified based on the kind of plant biomass sample used for lignin content estimation. Here, we modified and adapted the rapid TGA method of rice straws[27] to cotton tissues to estimate the lignin content. Briefly, the dried powdered plant samples were subjected to protein solubilization buffer and methanol extraction to remove proteins and the alcohol soluble fraction. The alcohol insoluble residue was treated with TGA and precipitated lignin under acidic conditions. A lignin standard curve was generated using commercial bamboo lignin and a regression line ($y = mx+c$) was calculated. The x value uses average absorbance values of lignin at 280 nm, while m and c values were entered from the regression line to calculate unknown lignin concentration in cotton plant biomass samples. This method is divided into five phases: 1) preparation of plant samples; 2) washing the samples with water and methanol; 3) treatment of the pellet with TGA and acid to precipitate lignin; 4) precipitation of lignin; and 5) the standard curve preparation and lignin content estimation of the sample. The first two phases are primarily focused on the plant material preparation followed by water, PSB (protein solubilization buffer) and methanol extractions to obtain the alcohol insoluble material. Then, it was treated with TGA (thioglycolic acid) and HCl to form a complex with lignin in the third phase. At the end, HCl was used to precipitate lignin, which was dissolved in sodium hydroxide to measure its absorbance at 280 nm[28].

PROTOCOL:

1. Preparation of plant samples

1.1 Collect two-month-old cotton plants from the greenhouse (**Figure 1A**).

1.2 Flip plant pots gently to separate soil and roots with intact lateral roots by loosening the soil around the plant (**Figure 1B**).

1.3 Wash the collected plants thoroughly in trays filled with water to remove all the dirt (for root samples) (**Figure 1C**).

1.4 Use paper towels to dry separated root, stem, and leaf tissues, and label them (**Figure 1D**). Air dry for 2 days at room temperature to prevent any fungal contamination (**Figure 1E**).

1.5 Transfer sample tissues to labelled containers/aluminum foils and incubate in a temperature-controlled incubator at 49 °C for 7 to 10 days (**Figure 1F**).

NOTE: Higher temperatures may alter the lignin structure. Alternatively, a freeze dryer can be used to dry samples for 1 to 2 days without causing any chemical changes to the plant biomass.

1.6 Use a blade to cut the incubator dried tissue into 5 mm size pieces or alternatively employ a biomass grinder to grind the plant tissues (**Figure 1G, Figure 1H**).

NOTE: The biomass grinder/blade must be cleaned after each sample was cut/ground.

1.7 Transfer the cut tissue/biomass grounded plant material into grinding vials and grind into fine powder of 1 mm size using a freezer mill or cryogenic grinder with liquid N_2.

1.8 Grind samples for three cycles at the rate of 10 CPS (each cycle span of 2 min) into a uniform powder (**Figure 1I, 1J, 1K**).

NOTE: The experiment can be paused at this point and samples can be stored at room temperature in airtight containers for long-term storage.

2. Washing samples with water, PSB, and methanol

2.1 Measure and record the weight of all empty 2 mL microfuge tubes used for lignin content estimation in the lab notebook.

2.2 Transfer 20 mg of the ground sample powder to the pre-weighed tube. Weigh the tube with tissue and tissue powder and record these weights in the lab notebook.

2.3 Incubate all 2 mL microfuge tubes (with open lids) with 20 mg of tissue powder in a heat block or oven at 60 °C for 1 hour.

2.4 After incubation, cool samples for 10 min at room temperature (RT).

2.5 Add 1.8 mL of water to each microfuge tube and mix by vortexing. Then, centrifuge at 25,200 x g (15,000 rpm) for 10 min at RT and discard the supernatant (**Figure 2**).

2.6 Add 1.8 mL of Protein Solubilization buffer (PSB) (**Table 1**) to each retained pellet and mix by vortexing. Centrifuge at 25,200 x g (15,000 rpm) for 10 min at RT and discard the supernatant.

2.7 Repeat step 2.6 again for each sample.

2.8 Add 1.8 mL of water to each pellet, mix by vortexing, and centrifuge at 25,200 x g (15,000 rpm) for 10 min. After centrifugation, save the pellet and discard the supernatant.

2.9 To the retained pellet, add 1.8 mL of methanol and incubate in a 60 °C heat block for 20 min. Then, centrifuge at 25,200 x g (15,000 rpm) for 10 min at RT. After centrifugation, discard the supernatant and retain the pellet (**Figure 2**).

2.10 Repeat step 2.9 again for each sample.

2.11 Air dry the pellet at RT or proceed immediately by vacuum drying. Vacuum dry using vacuum drier at 30 °C for 2 to 3 hours or until the pellet is completely dry.

NOTE: The experiment can be paused at this point by air drying over-night or continue by vacuum drying.

2.12 After drying, weigh sample tubes with the dried pellet and record the weight next to the respective empty tube weight in the lab notebook. Estimate the pellet weight by subtracting the two values. These weights will be used for lignin estimation at the end of lignin extraction process.

2.13 At this point of lignin extraction, include the commercial bamboo lignin for generation of the lignin standard curve. Measure commercial bamboo lignin into separate tubes ranging from 0.5 mg to 5 mg in 0.5 mg increments (0.5 mg, 1 mg, 1.5 mg, 2 mg, 3 mg, 3.5 mg, 4 mg, 4.5 mg, and 5.0 mg). Measure each concentration three times for three technical replicates.

NOTE: From here on, the standards measured in the above step were processed in the same way as samples that were dried.

3. Treatment of pellet with TGA and acid to precipitate lignin

3.1 Subject processed samples from the above step, along with measured standards, to TGA (thioglycolic acid) treatment.

3.2 Add 1 mL of 3 N HCl (**Table 1**) and 100 µL of TGA to each pellet.

3.3 Vortex and incubate in an 80 °C preheated heat block for 3 hours in a fume hood (**Figure 2**).

NOTE: The heating step at 80 °C must be monitored. High pressure buildup may open the lids and can lead to chemical spills. Screw cap tubes are recommended but 2 mL tubes can be loosely capped during this step as an alternative to prevent such spills.

3.4 After incubation, cool tubes at RT for 10-15 min and centrifuge at 25,200 x g (15,000 rpm) for 10 min at RT.

NOTE: Waste generated from acid and organic solvents must be separated and stored in glass containers with ventilated caps. Use separate glass containers for the collection of acid and TGA waste.

3.5 After centrifugation, discard the supernatant and retain the pellet. Add 1 mL of water, mix by vortexing, and centrifuge at 25,200 x g (15,000 rpm) for 10 min at RT.

3.6 After centrifugation, discard the supernatant and mix the pellet in 1 N NaOH for 24 h at 37 °C shaker/thermal mixer at low speed (**Figure 2**).

NOTE: This incubation time can be reduced to 1 hour[7].

3.7 After incubation, centrifuge the 2 mL microfuge tubes at 25,200 x g (15,000 rpm) for 10 min at RT. Retain the supernatant for the following step.

NOTE: The procedure involves use of strong acids and other chemicals that are corrosive in nature. Hence, wearing proper PPE is recommended throughout the process of lignin estimation. TGA has a strong unpleasant smell and is corrosive in nature. Hence, it is recommended to use only in the hood.

4. Precipitation of lignin

4.1 Transfer the supernatant to a fresh 2 mL microfuge tube and add 200 µL of concentrated HCl. Incubate at 4 °C for 4 hours or overnight (**Figure 2**).

NOTE: The extraction process can be paused at this point by extending the refrigeration step to overnight.

4.2 Centrifuge at 25,200 x g (15,000 rpm) for 10 min at RT and dissolve the pellet in 1 mL of 1 N NaOH.

4.3 Incubate in the shaker at RT for 10 min to suspend the pellet completely in NaOH.

4.4 Finally, measure the absorbance of samples at 280 nm using a spectrophotometer and compare with the standard lignin curve.

4.5 Measure the unknown concentration of lignin by using the calibration curve regression line values, and absorbances of extracted samples at 280 nm.

5. Standard curve preparation and lignin estimation in the sample

5.1 Process lignin standards in the same way as experimental samples from TGA treatment.

5.2 Measure commercial bamboo lignin standards in 0.5 mg increments starting from 0.5 mg, 1 mg, 1.5 mg, 2 mg, 2.5 mg, 3.0 mg, 3.5 mg, 4.0 mg, 4.5 mg and 5 mg. Then, process by TGA, HCl, dissolve in 1 N NaOH followed by measuring absorbance at 280 nm (**Figure 3A**).

5.3 Use values of lignin concentration and absorbance readings to generate a scattered plot of standard lignin curve (**Figure 3B**).

5.4 Use the regression line, y = mx+c generated in the scattered plot, for the estimation of unknown lignin content of prepared samples using "x" values from average absorbances of extracted samples at 280 nm and "m" and "c" values from lignin standard curve regression line.

5.5 Divide the lignin content in the resultant y value by total weight of the vacuum/air dried plant biomass sample after methanol extraction in mg (approximately 15 mg) to obtain lignin concentration per mg. Then, multiply this value by 100 to calculate lignin percentage per mg.

REPRESENTATIVE RESULTS

Two different cotton experimental lines were compared for differences in their lignin contents in different tissues. The extracted lignin content of each sample was measured at 280 nm and recorded its respective absorbance values. The average absorbance values of each biological replicate were compared against the regression line of the lignin standard curve (**Table 2**, **Figure 3C**). The regression line, y = mx + c, is used to calculate the unknown lignin content of the extracted experimental lines, sample 1 and sample 2. The results of average OD values were substituted in "x" while "m" and "c" values were plugged from the regression line of lignin standard curve to obtain lignin concentration "y" in mg (**Table 3**, **Figure 3B**). In the next step, to calculate per 1 mg of lignin content, divide the "y" value by the weight of the sample (15 mg) after methanol extraction. In the following step, to calculate per gram (= 1,000 mg) the y/15 value was multiplied by 1,000. To get % of lignin we divide y/15 value by 1,000 and multiply by 100. The average of lignin % for three biological replicates (of each line, sample 1 and sample 2) was compared between the two experimental lines sample 1 (11.7%) and sample 2 (10.3%). The lignin values were consistent among biological replicates suggesting that the TGA method is a reliable method and highly specific to measure the lignin content. Comparison studies were also made between different tissue types (root, stem and leaves) of two experimental lines of cotton, and both lines showed relatively lower lignin content in leaves (3.4%) compared to stems (9.4% to 9.9%) and roots (9.4% to 9.2%) (**Table 4**, **Figure 4**).

Figure 1: **Preparation of plant biomass sample**

(**A**) Collected cotton plant material from green house. (**B**) Gently flipped pots to separate roots. (**C**) Thoroughly washed in water to remove all the dirt. (**D**) Separated root, stem and leaf tissues. (**E**) Air-dried tissue for 2 days after separating the tissue. (**F**) Air dried tissue is transferred to the incubator at 49 °C for 10 days. (**G**) Biomass grinder was used to grind plant biomass samples. (**H**) Ground plant biomass samples of root, stem and leaf. (**I**) Ground samples are loaded into the grinding vials, placed in the freezer mill chamber, grounded in the freezer mill at a rate of 10 CPS for 3 cycles. (**J**) Grinded vials showing finely ground tissue powder after grinding in the freezer mill. (**K**) Resultant of finely ground tissue powder of root, stem and leaf after using freezer mill for grinding.

Figure 2: **Critical steps involved in TGA mediated lignin extraction**

Flow chart of critical steps involved in lignin extraction from plant biomass to lignin content estimation using TGA method: 1. Preparation of plant samples by sufficient drying and grinding into fine powder using freezer mill; 2. 20 mg of tissue powder was subjected to PSB, methanol and water washes and dried; 3. Using TGA and acid, lignin was precipitated; 4. Preparation of lignin standard curve using commercial bamboo lignin; 5. Estimation of lignin content.

Figure 3: **Standard curve preparation and lignin estimation in the sample**

(**A**) Table showing different concentrations of commercial bamboo lignin used for generating lignin standard curve from absorbance readings at 280 nm. (**B**) Scattered plot generated with Excel program using the values from table A. (**C**) Bar graphs representing the estimated root tissue lignin contents of sample 1 and sample 2.

Table 1: Preparation of solutions used in the protocol.

Table showing the preparation of different solutions used in the protocol.

Table 2: Lignin standard curve prepared from 0.5 mg to 3.5 mg of industrial bamboo lignin.

Scattered graph with regression line showing m and c values.

Table 3: Lignin template used for calculation of unknown lignin content using absorbance readings of samples at 280 nm (as x) and standard curve regression line 'm' and 'c' values from the standard curve.

Table 4: Lignin content from different tissues (root, stem and leaves) of cotton plant at post flowering stage.

DISCUSSION

Lignin plays a significant role in plant growth and development and recently has been extensively studied for biofuel, bioenergy and bioproduct applications. Lignin is rich in aromatic compounds that are stored in all vascular plant secondary cell walls. It has several industrial applications such as wood panel products, bio dispersants, flocculants, polyurethane foams and in resins of circuit boards[29,30 31]. Most of the lignin generated from paper and pulp industries is released as waste

or burned for heat production. Thus, if efficiently processed, lignin can be utilized as an alternative to both fossil fuel based products[32,33] and bioelectricity production[34]. Hence, precise estimation of lignin content and composition are critical for industrial applications as the composition varies based on the plant species as well as plant organ type. The major limitation for lignin estimation is the difference arising from the method selected for the estimation of lignin content[35]. The estimation differences among different methods are primarily due to the contamination with other non-lignin components, variation in the solubility, addition of new groups to lignin, xylan degradation/contamination, native structural changes and loss of some lignin fraction during the elimination of other components. Further, the majority of lignin protocols are originally developed based on wood chemistry[27]. Hence, there is a critical need for establishing lignin protocols for herbaceous samples as more crop/plant species are targeted for biofuels and bio products. The TGA method estimates pure lignin content based on specific bonding with TGA. Therefore, the lignin estimation by TGA yields lower lignin content when compared to Klason and acetyl bromide methods[35,36]. This is because of the specific bonding of lignin with TGA as well as loss of some lignin content during lignin precipitation (insoluble part).

The lignin content estimated using TGA method is reproducible and consistent. The results obtained in this study were consistent among the biological replicates and showed a significant difference between two lines, suggesting the reliability of TGA method for lignin estimation. For data reproducibility and precise estimation of lignin content, it is important to follow the steps and take following precautions. Inclusion of positive controls in different concentrations, ranging from 0.5 mg to 5 mg in three replicates, and processing them along with samples from the TGA step will avoid experimental errors and results in precise estimation of the lignin content. The standard curve must be generated for each set of samples and regression line statistic R^2 must fall in the range of 97% to 99%. Th exact weight of the empty tube and dried methanol extracted tissue is critical for exact lignin content estimation. Additionally, various factors such as specific stage of plants, growing conditions, genotypes, type of tissue and the age of the plant will affect the lignin content[30,37,38]. Hence, it is important to grow all the experimental lines in the same environment and harvest the same type of tissues at the same time. Results of the current study showed an expected trend of lower lignin content in the leaves, higher lignin content in stems and roots, and demonstrated the applicability of this method to various plant tissues. Further, less variation among biological replicates suggested that TGA can estimate reproducible lignin content in all plant tissues.

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DISCLOSURE:
The authors declare that they have no conflict of interest.

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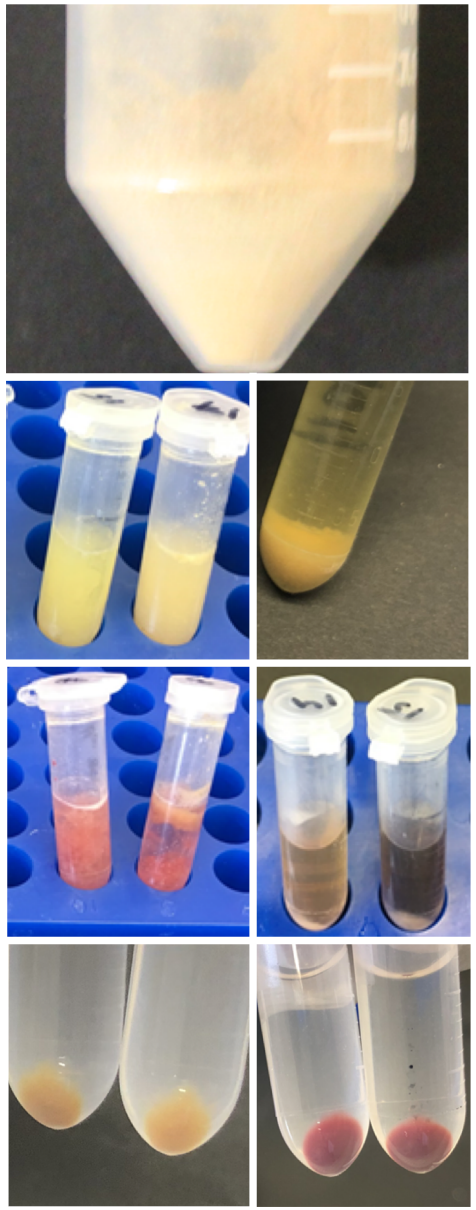
Figure 1

Click here to access/download;Figure;Lignin figure 1 revised.pdf

Figure 2



1. Preparation of plant samples

2. Preparation of alcohol insoluble material

3. Precipitation of lignin using TGA and acid

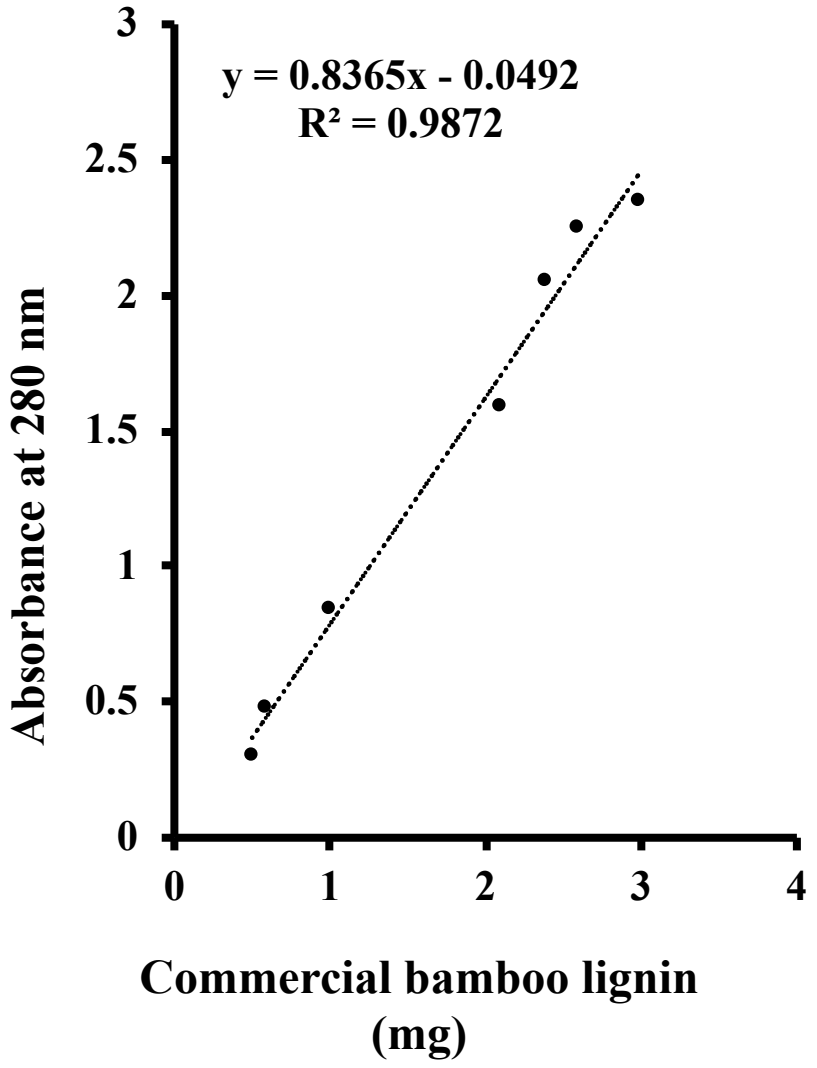
4. Preparation of lignin standard curve

5. Estimation of lignin

A

Commercial bamboo lignin (mg)	Absorbance at 280 nm
0.5	0.31
0.6	0.48
1.0	0.84
2.1	1.59
2.4	2.05
2.6	2.25
3.0	2.35

B



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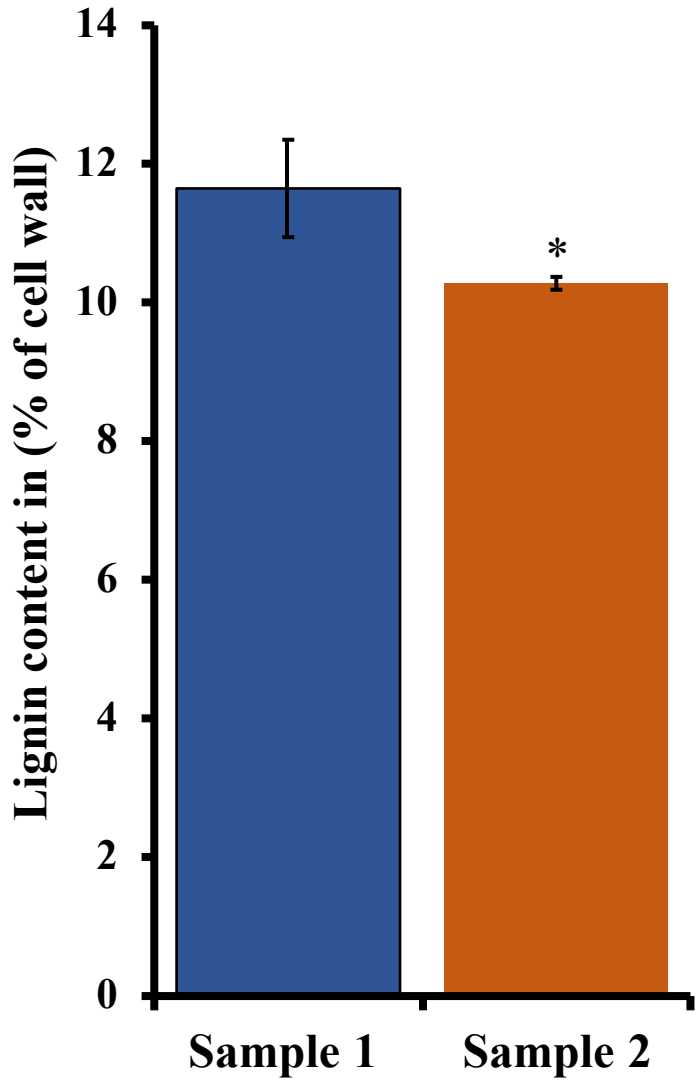
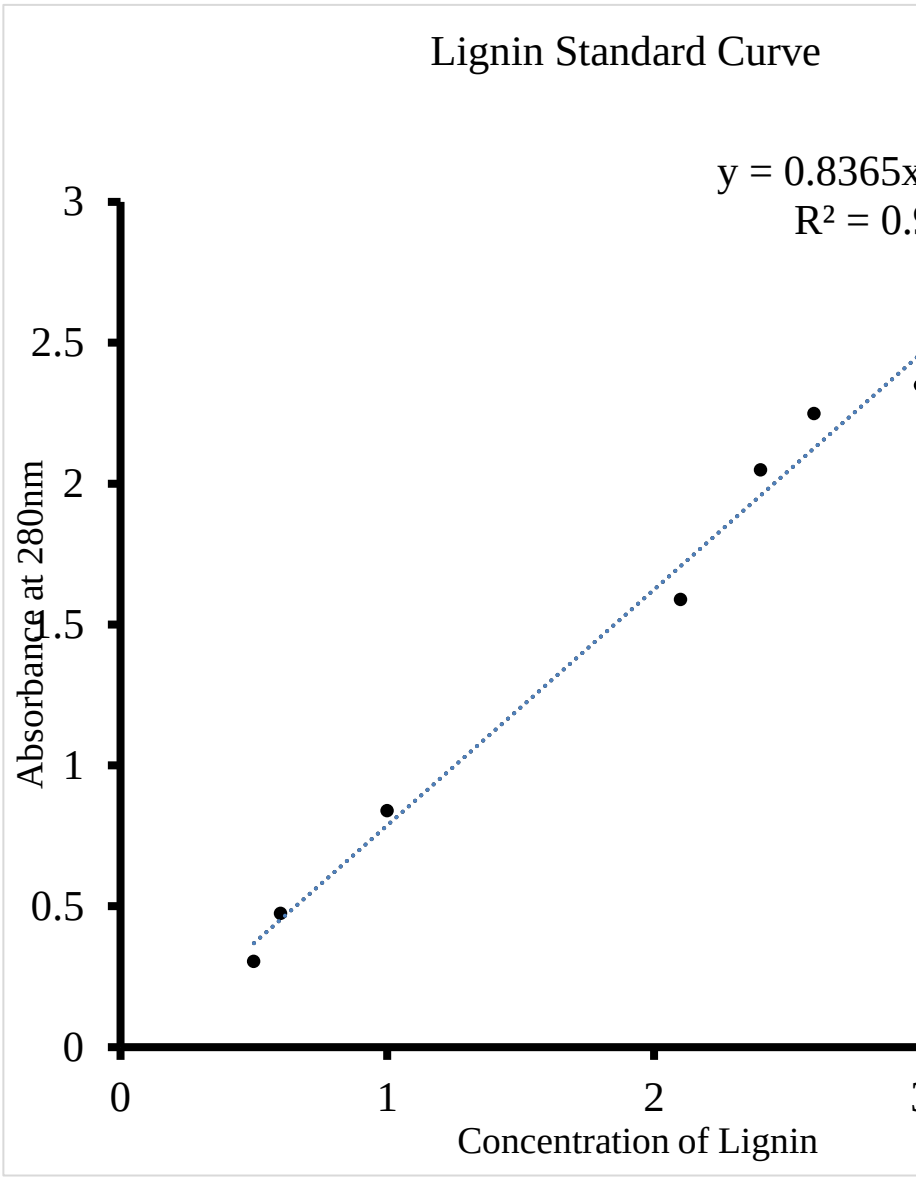
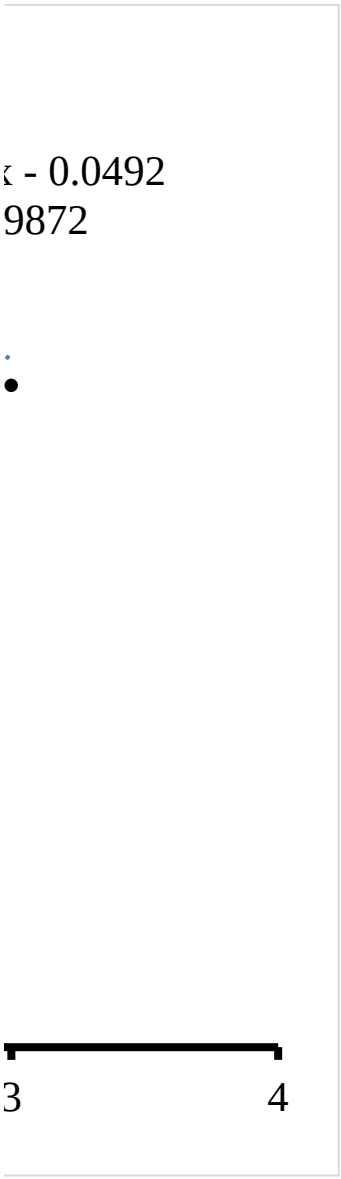


Solution	Stocks needed	Preparation
Protein solubilization buffer (PSB)	1 M Tris HCl pH 8.8 and 0.5 M EDTA pH 8.0	To prepare 100 mL of working solution of PSB with final concentration of 50 mM Tris, 0.5 mM EDTA and 10 % SDS, add 5 mL of 1 M Tris, 1 mL of EDTA and 10 g of SDS to 80 mL sterile water, mix, dissolve and make up the final volume to 100 mL with sterile water. Autoclave at 121 ^{0}C, 15 psi pressure, for 30 min.
1 M Tris HCl		To prepare 100 mL of 1 M Tris by adding 12.1 g of Tris HCl (molecular weight = 121.14 g) in 80 mL of water. Mix Tris HCl by stirring on a magnetic stirrer, adjust the pH with NaOH to 8.8 and make up the volume to 100 mL with sterile water and autoclave at 121 ^{0}C, 15 psi pressure, for 30 min.
0.5 M EDTA (Ethylenediamine tetraaceticacid)		To prepare 100 mL of 0.5 M EDTA add 18.6 g of EDTA in 70 mL water. Adjust the pH to 8.0 (EDTA completely dissolves at pH 8.0) using sodium hydroxide pellets and make up the volume to 100 mL. Autoclave the solution at 121 ^{0}C, 15 psi pressure, for 30 min.
3 N Hydrochloric acid (HCL)		To prepare 100 mL of 3 N HCl, add 26 mL of concentrated HCL to 74 mL of sterile water.
4 % Sodium hydroxide (NaOH)		Prepare 1 N sodium hydroxide solution by adding 4 g of sodium hydroxide in 90 mL of sterile water, dissolve, make up the volume to 100 mL and autoclave at 121 ^{0}C, 15 psi pressure, for 30 min.

Concentration of industrial lignin	Av.Absorbance at 280nm
0.5	0.30533
0.6	0.47567
1	0.84
2.1	1.59
2.4	2.05
2.6	2.25
3	2.35

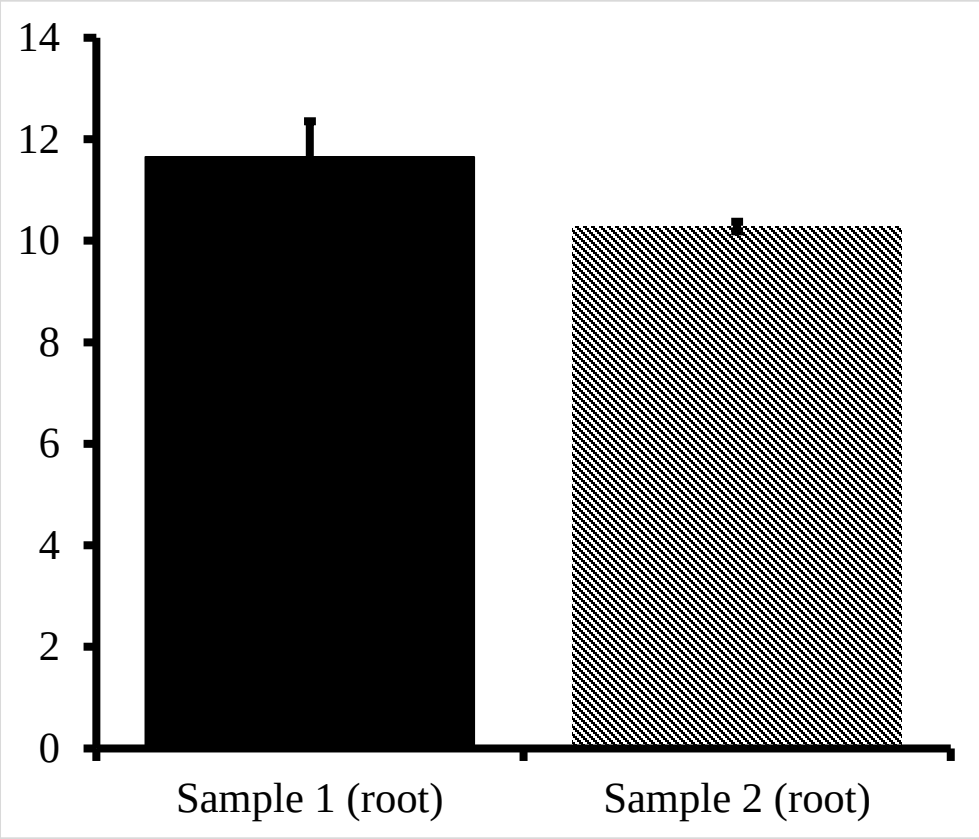



- 0.0492
9872
3
4

Sample nun	Replicates	Absorbance at 280nm
		Average of three technical replicat
7	R1	2.255666667
8	R2	2.617
9	R3	1.379666667
10	R1	2.050666667
11	R2	1.875
12	R3	1.784

Lignin (mg) y = mx-c	**Cell wall (mg)**	**mg.lig/mg cell wa**
x is average OD, y is lignin content		**per 1 mg**
1.837665167	15.5	0.118559043
2.1399205	20.9	0.102388541
1.104891167	8.6	0.128475717
1.666182667	15.9	0.104791363
1.5192375	14.9	0.101962248
1.443116	14.2	0.101627887

Line	Root lignin cont	Standard error
Sample 1 (root)	11.64744336	0.702423008
Sample 2 (root)	10.27938328	0.092709398

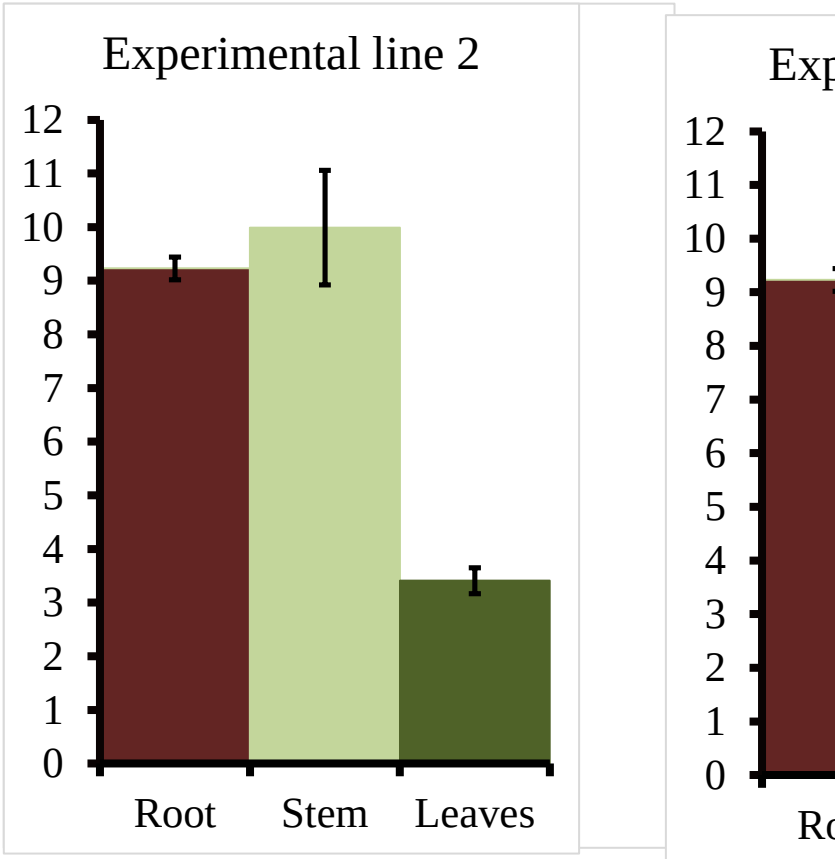


mg.lignin/g	% Lignin	Average %	Std. dev	Std. error	
118.559	11.8559043	11.64744336	1.07515701	0.702423	ttest
102.3885	10.23885407				0.215
128.4757	12.84757171				
104.7914	10.47913627	10.27938328	0.14190475	0.092709	
101.9622	10.19622483				
101.6279	10.16278873				

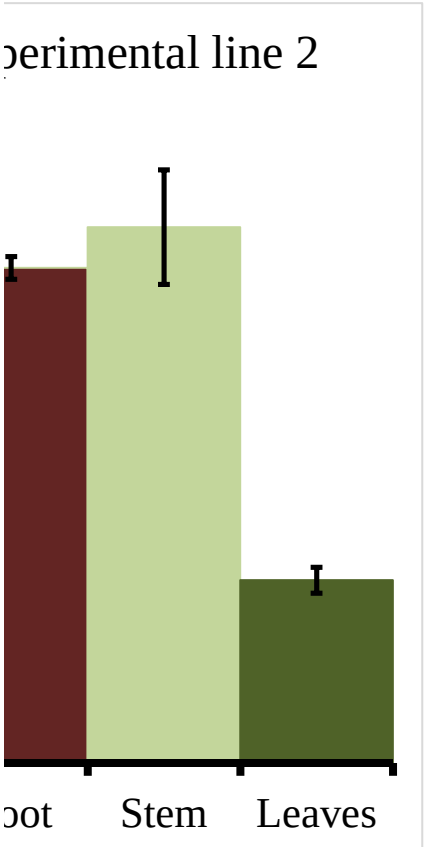
	Differe	percent decreas		Sample lines
0.785	1.368	0.117	11.75	1 and 2

Table 4

Sample number	Plant and tissue	Empty tube weight	Tube with tissue after washes	Tissue weight after washes	Absorbance at 280 nm		
1	Cotton Ex	1177.5	1194.8	17.3	1.995	1.993	1.993
2	Cotton Ex	1176.4	1194	17.6	2.072	2.074	2.069
3	Cotton Ex	1175.3	1190.9	15.6	1.849	1.836	1.848
4	Cotton Ex	1176.3	1191.4	15.1	1.763	1.769	1.75
5	Cotton Ex	1178.8	1194.3	15.5	1.773	1.773	1.774
6	Cotton Ex	1176.1	1195.1	19	2.203	2.213	2.219
7	Cotton Ex	1176.2	1188.1	11.9	0.587	0.587	0.585
8	Cotton Ex	1175.6	1187.5	11.9	0.46	0.453	0.45
9	Cotton Ex	1170.8	1185.5	14.7	0.723	0.74	0.721
10	Cotton Ex	1177.4	1197.4	20	2.262	2.266	2.268
11	Cotton Ex	1176.1	1191.6	15.5	1.723	1.724	1.719
12	Cotton Ex	1175.3	1190.8	15.5	1.819	1.82	1.818
13	Cotton Ex	1173.6	1189.6	16	1.696	1.708	1.702
14	Cotton Ex	1172.2	1191.7	19.5	2.443	2.443	2.444
15	Cotton Ex	1172.3	1191.7	19.4	2.642	2.65	2.661
16	Cotton Ex	1179.2	1190.9	11.7	0.543	0.54	0.534
17	Cotton Ex	1172.5	1181.5	9	0.453	0.463	0.455
18	Cotton Ex	1175.5	1184	8.5	0.369	0.383	0.373



Average absorbance	Lignin content, Y=mx-c	Lignin / mg	Lignin per gm
1.993666667	1.618502167	0.093555039	93.55503854
2.071666667	1.683749167	0.095667566	95.66756629
1.844333333	1.493584833	0.095742618	95.74261752
1.760666667	1.423597667	0.094277991	94.27799117
1.773333333	1.434193333	0.092528602	92.52860215
2.211666667	1.800859167	0.094782061	94.7820614
0.586333333	0.441267833	0.037081331	37.08133053
0.454333333	0.330849833	0.027802507	27.802507
0.728	0.559772	0.038079728	38.07972789
2.265333333	1.845751333	0.092287567	92.28756667
1.722	1.391253	0.089758258	89.75825806
1.819	1.4723935	0.094993129	94.99312903
1.702	1.374523	0.085907688	85.9076875
2.443333333	1.994648333	0.102289658	102.2896581
2.651	2.1683615	0.111771211	111.7712113
0.539	0.4016735	0.034331068	34.33106838
0.457	0.3330805	0.037008944	37.00894444
0.375	0.2644875	0.031116176	31.11617647



Lignin percent	Average % lignin content	
9.355503854		
9.566756629		
9.574261752	9.49884	0.1014
9.427799117		
9.252860215		
9.47820614	9.38629	0.09657
3.708133053		
2.7802507		
3.807972789	3.43212	0.46274
9.228756667		
8.975825806		
9.499312903	9.23463	0.21375
8.59076875		
10.22896581		
11.17712113	9.99895	1.06833
3.433106838		
3.700894444		
3.111617647	3.41521	0.2409

Name of the material/Equipment	Company	Catalog Number	Comments/Description
BioSpectrophotometer kinetic	Eppendorf kinetic	6136000010	For measuring absorbance at 280 nm
Centrifuge	Eppendorf	5424	For centrifuging samples
Commercial bamboo lignin	Aldrich	1002171289	Used in the preparation of the standard curve
Distilled water	Fischer Scientific	16690382	Used in the protocol
Falcon tubes	VWR	734-0448	Containers for solutions
Freezer mill	Spex Sample Prep	68-701-15	For fine grinding of plant tissue samples
Heat block/ Thermal mixer	Eppendorf	13527550	For temperature controlled steps during lignin extraction
Hotplate stirrer	Walter	WP1007-HS	Used for preparation of solutions
Hydrochloric acid (HCL)	Sigma	221677	Used in the protocol
Incubator	Fisherbrand	150152633	For thorough drying of plant tissue samples
Measuring scale	Mettler toledo	30243386	For measuring plant tissue weight, standards and microfuge tubes
Methanol (100 %)	Fischer Scientific	67-56-1	Used in the protocol
Microfuge tubes (2 mL)	Microcentrifuge	Z628034-500EA	Containers for extraction of lignin
Plant biomass gerinder	Hanchen	Amazon	Used for crushing dried samples
pH meter	Fisher Scientific	AE150	Measuring pH for solutions prepared for lignin extraction
Temperature controlled incubator/oven	Fisher Scientific	15-015-2633	Used in the protocol

Thioglycolic acid (TGA)	Sigma Aldrich	68-11-1	Used in the protocol
Vacuum dryer	Eppendorf	22820001	Used for drying samples
Vortex mixer	Eppendorf	3340001	For proper mixing of samples

Editorial comments

1. Please upload the revised Figure 3.

Thank you very much. We have uploaded figure 3.

2. Please note that in the PDF, we cannot display Tables with multiple tabs. I suggest you break up Table 2 into multiple Tables files or present Table 2 as a supplemental file.
Thank you very much for the suggestion and we have updated as suggested and uploaded. We have updated manuscript.

Changes to be made by the Author(s) regarding the video:
1. Please revise the title card in the video to be the following: Estimation of Plant Biomass Lignin Content using Thioglycolic Acid (TGA)

Thank you very much for the suggestion and we have updated the title card.