

Video Article

Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates

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Abstract

The need for renewable, carbon neutral, and sustainable raw materials for industry and society has become one of the most pressing issues for the 21st century. This has rekindled interest in the use of plant products as industrial raw materials for the production of liquid fuels for transportation² and other products such as biocomposite materials⁶. Plant biomass remains one of the greatest untapped reserves on the planet⁴. It is mostly comprised of cell walls that are composed of energy rich polymers including cellulose, various hemicelluloses, and the polyphenol lignin⁵ and thus sometimes termed lignocellulosics. However, plant cell walls have evolved to be recalcitrant to degradation as walls contribute extensively to the strength and structural integrity of the entire plant. Despite its necessary rigidity, the cell wall is a highly dynamic entity that is metabolically active and plays crucial roles in numerous cell activities such as plant growth and differentiation⁵. Due to the various functions of walls, there is an immense structural diversity within the walls of different plant species and cell types within a single plant⁴. Hence, depending of what crop species, crop variety, or plant tissue is used for a biorefinery, the processing steps for depolymerisation by chemical/enzymatic processes and subsequent fermentation of the various sugars to liquid biofuels need to be adjusted and optimized. This fact underpins the need for a thorough characterization of plant biomass feedstocks. **Here we describe a comprehensive analytical methodology that enables the determination of the composition of lignocellulosics and is amenable to a medium to high-throughput analysis (Figure 1).** The method starts of with preparing destarched cell wall material. The resulting lignocellulosics are then split up to determine its monosaccharide composition of the hemicelluloses and other matrix polysaccharides¹, and its content of crystalline cellulose⁷. The protocol for analyzing the lignin components in lignocellulosic biomass is discussed in Part I³.

Video Link

The video component of this article can be found at <http://www.jove.com/video/1837/>

Protocol

1. Cell wall isolation

1. grind roughly 60-70mg of air- or freeze dried plant material with 5.5 mm stainless steel balls in a 2ml sarstedt screw cap tube using a retschmill (1 min, 25 Hz). An alternative, the use of a high-throughput grinding and dispensing robot termed iWall is described in Part I³.
2. remove the steel balls before continuing with the cell wall isolation procedure

The detailed protocol of the preparation of cell wall material is shown in Part I³. For completeness here the written steps of the protocol.

3. add 1.5 ml of 70% aqueous ethanol, and vortex thoroughly
 4. centrifuge at 10,000 rpm for 10 min to pellet the alcohol insoluble residue
 5. aspirate or decant the supernatant
 6. add 1.5 ml of chloroform/methanol (1:1 v/v) solution to the residue and shake tube thoroughly to resuspend the pellet
 7. centrifuge at 10,000 rpm for 10 min and aspirate or decant the supernatant
 8. resuspend pellet in 500 ul of acetone
 9. evaporate the solvent with a stream of air at 35°C until dry
- If needed dried samples can be stored at room-temperature until further processing.
10. To initiate the removal of starch from the sample re-suspend the pellet in 1.5 ml of a 0.1 M sodiumacetate buffer pH 5.0.
 11. cap the sarstedt tubes and heat for 20 min. at 80°C in a heating block.
 12. cool the suspension on ice
 13. add the following agents to the pellet: 35 µl of 0.01% Sodiumazide (NaN₃), 35 µl Amylase (50 µg/1mL H₂O; from Bacillus species, SIGMA); 17 µl Pullulanase (18.7 units from bacillus acidopullulyticus; SIGMA). Cap the tube and vortex thoroughly.
 14. The suspension is incubated over night at 37°C in the shaker. Orienting the tubes horizontally aides improved mixing.

15. heat suspension at 100°C for 10 min in a heating block to terminate digestion.
16. centrifuge (10,000 rpm, 10 min) and discard supernatant containing solubilized starch
17. wash the remaining pellet three times by adding 1.5 ml water, vortexing, centrifugation, and decanting of the washing water.
18. resuspend pellet in 500 µl of acetone
19. evaporate the solvent with a stream of air at 35°C until dry. It may be necessary also to break up the material in the tube with a spatula for better drying.

The dried material presents isolated cell wall (lignocellulosics). If needed dried samples can be stored at room-temperature until further processing.

2. Matrix Polysaccharide composition

This method is essentially a modification of the method published by Albersheim 1.

1. To determine the monosaccharide composition of the wall material weigh 2 mg of cell wall material into 2ml starstedt tubes either by hand or in a highthroughput fashion using the iWall, a robotic grinding and weighing robot.
2. Add 20 µl of an Inositol solution (5mg/ml) as an internal standard. For a 2 mg cell wall sample we recommend to add 100 µg.
3. rinse tube walls with 250 µl of acetone to collect the cell wall material on the bottom of the tube, and evaporate the acetone very gentle under airflow.
4. For the weak acid hydrolysis add 250 µl of 2M trifluoroacetic acid (TFA) to each sample. Add TFA carefully to ensure no material is splashed up onto the tube walls.
5. cap tightly and incubate for 90 min at 121°C in a heating block.
6. cool the heating blocks and samples on ice.
7. centrifuge the tubes at 10,000 rpm for 10 min.
8. transfer 100 µl of the acidic supernatant containing the matrix polysaccharide derived monosaccharide to a glass screw cap vials making sure not to disturb the pellet material. The pellet can be used for the crystalline cellulose assay below (see 3.)
9. evaporate the TFA in the glass tube under a gentle stream of air in an evaporation device.
10. add 300 µl 2-Propanol, vortex and evaporate at 25°C (repeat for a total of three times)
11. The first step of the alditol acetate derivatization procedure is to perform a reduction of the monosaccharides to their corresponding alditols. For this purpose add 200 µl of a sodium borohydride solution to each dried sample. Prepare a fresh solution each time using 10mg of Sodium borohydride per 1ml of 1M Ammonium hydroxide.
12. leave glass vial at room temperature for 1.5 hours
13. neutralize the solution by adding 150 µl of glacial acetic acid
14. vortex and evaporate at 25°C .
15. add 250 µl Acetic acid/Methanol (1:9, v/v), vortex and evaporate at 25°C
16. add 250 µl Methanol, and evaporate under stream of air (repeat for a total of three times)
17. For the acetylation of the alditols, add 50 µl of Acetic anhydride and 50 µl of Pyridine, vortex and incubate for 20 min at 121°C in a heating block.
18. cool samples in the block down with ice while wait for temp decrease to approximately room temperature.
19. evaporate the reagents under a gentle stream of air at room temperature. Be careful: alditol acetates are highly volatile.
20. add 200 µl Toluene and evaporate under air (x3)
21. In the final steps the alditol acetates are extracted. First, add 500 µl of ethyl acetate and swirl lightly.
22. add 2 ml of water, cap tubes and vortex.
23. centrifuge tubes at 2,000 RPM for 5 min to obtain clear separate layers (ethyl acetate on top, water on bottom)
24. pipette 50 µl of the ethyl acetate layer into GC/MS vials with inserts.
25. dilute by adding 100 µl of acetone to the GC-vial and cap. The sample volume and dilution volumes can be adjusted to avoid overloading the GC/MS if the sample concentration is too high.

The GC-vial can be stored at 4°C, if the GC/MS analysis does not immediately proceed

26. The samples are injected into a GC that is equipped with a quadrupole MS, but a flame ionization detector is also suitable. A Supelco SP-2380 (30mm X 0.25mm x 0.25 µm film thickness) column is used with a 4min solvent delay and a flow rate of 1.5ml/min. Injected samples are subjected to the following temperature program: Initial hold at 160°C for 2 min; a 20°C/min ramp to 200°C and hold for 5 min; a 20°C/min ramp to 245°C and hold 12 min; spike to 270°C and hold for 5 min before cooling to the initial temperature of 160°C 2.26.) Peaks are identified by mass profiles and/or retention times of standards. Monosaccharides are quantified based on standard curves.

3. Crystalline Cellulose Content

This method is essentially described by Updegraff⁸. There are a number of starting materials for this procedure: Isolated cell wall material (see 1) or wall material that has already been treated with 2M TFA (see 2.8) either the remaining pellet immediately after the acid treatment (see 2.8) or a TFA pellet, that has been washed with 2-propanol and dried.

1. Add to the TFA pellet in the screw capped glass tube 1 ml of Updegraff reagent (Acetic acid: nitric acid: water, 8:1:2 v/v).
2. Cap tube tightly, vortex, and heat in a heating block at 100°C for 30 min. As a result of this treatment only crystalline cellulose remains insoluble in the pellet.
3. Cool samples in the block on ice to room-temperature or cooler
4. centrifuge samples at 10,000 rpm for 15 min
5. Discard supernatant ensuring that the pellet is not disturbed and no material from the pellet is removed. For this purpose leave approx. 150 µl of supernatant in the tube.
6. add 1.5 ml of water, shake, centrifuge, and discard supernatant as done above
7. repeat washing procedure 3 additional times using 1.5 ml of acetone
8. Air dry pellet very gently with air, or let dry on bench overnight
9. the pellet (crystalline cellulose) is now completely hydrolyzed into glucose by what is called a Saeman hydrolysis. For this purpose add 175 µl 72% Sulfuric acid to the sarstedt tube
10. incubate at room temperature for 30 min, vortex and incubate for another 15 min
11. add 825 µl water and vortex

12. centrifuge samples at 10,000 rpm for 5 min. There might be some brown insoluble material, lignin, remaining in the tube.
13. The glucose content of the supernatant is assayed using the colorimetric anthrone assay. This assay is performed in a 96 well polystyrene microtiter plate.
14. For the standard curve use a 1mg/ml glucose stock (stored at 0°C) and create duplicate 0, 2, 4, 6, 8, and 10 ug standards by pipetting 0, 2, 4, 6, 8, and 10 ul into separate appropriate well. Fill each well up to 100 ul with water.
15. add 10 µl of each sample supernatant and 90 ml of water into separate cells but on the same microtiter plate as the standard.
16. add 200 µl of freshly prepared Anthrone Reagent (Anthrone dissolved in concentrated sulphuric acid , 2 mg anthrone/ ml sulphuric acid)
17. heat plate for 30 min at 80°C in an oven (aluminum heat spreader). Glucose containing samples turn from yellow into blue-green.
18. let the plate cool to room-temperature and shake thoroughly.
19. read absorption of plate at 625 nm using a microtiter plate reader.
20. Glucose (and hence crystalline cellulose content) is calculated based on the absorbance compared to the standard curve established on the same plate.

4. Representative Results

An example of a wall analysis is presented in Figure 2. In this case poplar stem (wood) was analyzed by the various procedures outlined in the protocol section. The matrix polysaccharide composition is highlighted by an example chromatogram identifying the typical sugar present in plant cell walls, fucose, rhamnose, xylose, arabinose, galactose, mannose and glucose (and the internal standard inositol). The main hemicellulosic component of poplar is xylan as demonstrated by the high xylose content. However, the abundance of these sugars will vary depending on the feedstock used⁴. The glucose in this analysis is derived from the hemicellulose xyloglucan and amorphous cellulose. Due to the analysis the data can be presented as mol% or ug/ mg wall material (or dry weight). The content of crystalline cellulose is self-explanatory, one can expect values of between 20-50% of the wall dry weight. Based on the results presented here and in Part I³ the lignocellulosic composition of poplar wood is 21% lignin, 30% hemicelluloses, and 41% crystalline cellulose. The remainder would be ash.

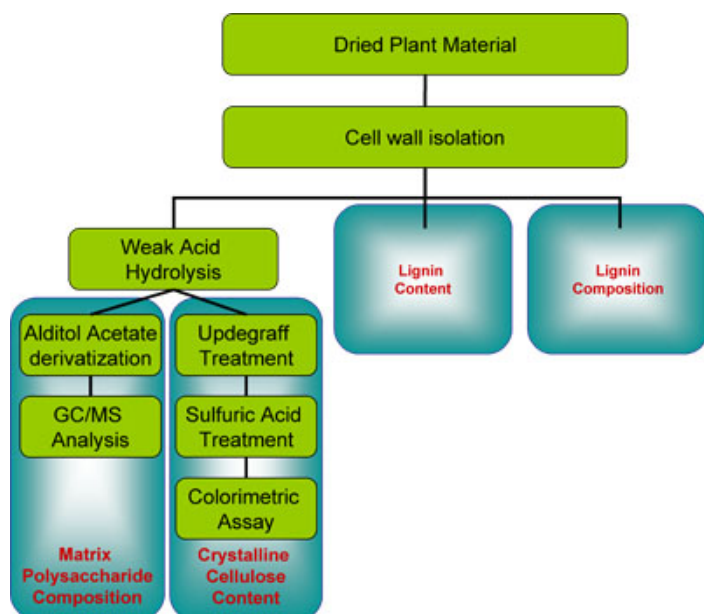


Figure 1: Overview of lignocellulosic analysis. Cell walls (lignocellulosics) are isolated from crude dried plant material. The wall material is then weighted into aliquots and subdivided for the various assays. Matrix polysaccharide composition is established after treating the wall material with a weak acid (2M TFA), derivatizing the resulting solubilized monosaccharides to their alditol acetates, and analysis by GC-MS. The residue of the weak acid treatment is washed with the so-called Updegraff reagent leaving only insoluble crystalline cellulose behind. The cellulose is solubilized by sulfuric acid and quantified by a colorimetric assay determining the glucose content. In parallel, the content and composition of lignin can be determined as described in Part I³.

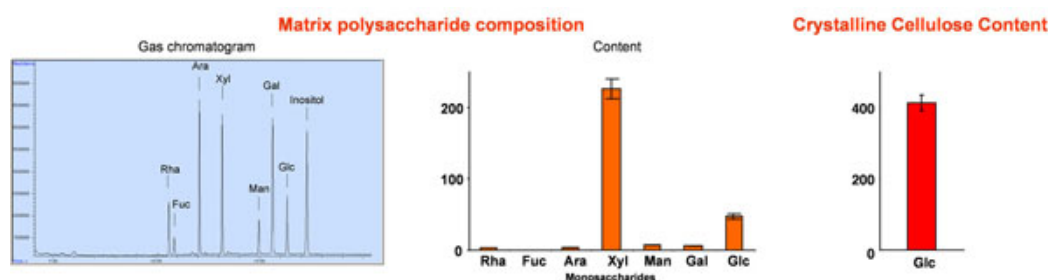


Figure 2: Comprehensive lignocellulosic analysis of poplar wood. Wood chips from poplar (*Populus tremuloides*) were subjected to the described protocol.

Upper left: Matrix polysaccharide composition; Fuc fucose; Rha rhamnose; Ara arabinose; Xyl xylose; Man mannose; Gal galactose; Glc glucose; inositol internal standard.

Discussion



The described methods enable a rapid quantitative assessment of the composition of lignocellulosic plant biomass. The method allows the determination of the composition of such materials including the sugar composition of the matrix polysaccharides namely the hemicelluloses, the crystalline cellulose content. The throughput of the various analytical methods per person varies. Using the protocols described here, 20 samples can be processed for matrix polysaccharide compositions and 30 for crystalline cellulose content. Due to the quantitative nature of the data optimal feedstock crops, variety or genotypes can be assessed in terms of their suitability for biofuel production.

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