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bio-protocol http://www.bio-protocol.org/e1494 Vol 5, Iss 11, Jun 05, 2015

Cell Wall Biomass Preparation and Fourier Transform Mid-infrared (FTIR) Spectroscopy to Study Cell Wall Composition

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[Abstract] Plant cell wall biomass is an abundant and renewable organic resource. Of the polymers it encloses, cellulose and hemicellulose are regarded as a raw material for the production of fuels and other products (Klemm *et al.*, 2005; Slavov *et al.*, 2013). Nonetheless, current usage of lignocellulosic biomass is still below its full potential due to a series of limiting factors mainly related to the cell wall recalcitrance to saccharification, a severe constraint to maximum biomass usability in downstream processing (Pauly and Keegstra, 2008).

As a strategy to optimise bio-energy and bio-refining applications, an increasing amount of effort is being put into the advancement of our knowledge concerning the cell wall compositional roots of recalcitrance. Fourier transform mid-infrared spectroscopy (FTIR) represents a very useful tool on this enterprise, as it allows for a high-throughput, non-destructive and low unit cost procedure for the examination of cell wall biomass (Allison *et al.*, 2009; Carpita and McCann, 2015). Furthermore, the use of Attenuated Total Reflection (ATR) in conjunction with infrared spectroscopy (IR) enables cell wall biomass samples to be examined in solid state without extensive preparation. Nonetheless, the analysis of purified cell wall preparations instead of the intact plant biomass is highly recommended, as it minimises or even eradicates interference from biomass components which are not part of the cell wall. Further information regarding the fundamentals of FTIR may be found elsewhere (Smith, 2011).

Datasets generated from FTIR spectroscopy can be extensive and complex. In these situations, data-driven modelling techniques are often used as exploratory approaches to identify the most distinctive features of the collected spectra. Here we suggest the use of Principal Component Analysis (PCA), a frequently employed method to transform a large set of variables into a smaller set of new variables (principal components), effectively reducing dataset dimensionality.

When the aim is a complete and detailed biomass characterisation, the FTIR-PCA method here described does not exclude the need for parallel wet gravimetric and analytical procedures. However, it does lead to a rapid identification of the major compositional shifts across large sets of samples; thus contributing to steer research pathways, minimise time-draining analytical procedures and reduce overall research costs.

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Materials and Reagents

1. Lignocellulosic biomass
   Note: Depending on the aims of the researcher, lignocellulosic biomass from different species, organs or tissues may be used, providing it is conveniently prepared as indicated in the Procedure section below. For an example of the application of this protocol, please refer to da Costa et al. (2014).
2. Deionised H_2O
3. 70% (v/v) aqueous ethanol (molecular biology grade)
4. Chloroform/methanol (1:1 v/v) (molecular biology grade)
5. 100% acetone (molecular biology grade)
6. Type-I porcine α-amylase (Sigma-Aldrich, catalog number: A6255) (saline suspension; 29 mg protein/ml; 1714 units/mg protein)
7. 0.1 M sodium acetate buffer (see Recipes)
8. 0.1 M ammonium formate buffer (see Recipes)
9. 0.001 M sodium azide (see Recipes)

Equipment

1. Laboratory safety equipment (gloves, eye protection and safety mask are recommended particularly during sodium azide handling)
2. Freezer
3. pH meter
4. Freeze dryer (Edwards Pirani 501 Super Modulyo, Edwards Ltd.)
5. Analytical mill (Ika, A11 basic)
6. Sieves (pore sizes: 0.18 and 0.85 mm)
7. Plastic centrifuge tubes (50 ml, with screw cap) (Greiner Bio-One GmbH)
8. Vortex mixer
9. Shaking incubator
10. Centrifuge
11. Fume hood
12. Block heater
13. FTIR spectrometer (Equinox 55, Bruker Optik) equipped with a Golden Gate ATR accessory (Specac)

Software

1. Bruker OPUS IR spectroscopy software (version 5.0; Bruker Optik)

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Procedure

A. Biomass preparation-organic solvent wash

1. Pre-freeze and freeze-dry lignocellulosic biomass (time may vary depending on sample source (for whole *Miscanthus* spp. tillers the samples were freeze-dried for an excess of 7 days to ensure complete dryness).
2. Grind tissues to a particle size in the range 0.18-0.85 mm (suggested equipment: IKA A11 Handheld Analytical Mill; sieves with mesh sizes of 80 and 20 µm).
3. Weigh approximately 1 g of the ground plant biomass into a 50 ml plastic centrifuge tube.
4. Add 30 ml 70% (v/v) aqueous ethanol, mix thoroughly using a vortex mixer and leave in a shaking incubator set at 40 °C/150 rpm for 12 h.
5. Centrifuge at 900 *x g* for 10 min and discard the supernatant by decantation or aspiration.
6. Add 30 ml 70% (v/v) aqueous ethanol, mix using a vortex mixer, but this time incubate the samples for 30 min at 40 °C/150 rpm.
7. Centrifuge at 900 *x g* for 10 min and discard the supernatant.
8. Repeat steps A6-7.
9. Add 20 ml of the chloroform/methanol (1:1 v/v) solution, mix to re-suspend the pellet and leave in a shaking incubator for 30 min at 25 °C and 150 rpm.
10. Centrifuge at 900 *x g* for 10 min and discard the supernatant after the chloroform/methanol wash.
11. Repeat steps A9-10 twice.
12. Add 15 ml of acetone, mix to re-suspend the pellet and leave in a shaking incubator for 30 min at 25 °C and 150 rpm.
13. Centrifuge at 900 *x g* for 10 min and discard the supernatant after the acetone wash.
14. Repeat steps A12-13 twice.
15. Let the organic-washed biomass samples dry overnight in a fume hood (alternatively they can be left in an oven set at 35 °C for approximately 16 h).

B. Biomass preparation-starch removal

1. Re-suspend the dry, organic solvent-extracted biomass in 15 ml of 0.1 M sodium acetate buffer (pH 5.0).
2. Heat for 20 min at 80 °C in a heating block to induce starch gelatinisation.
3. Cool suspensions on ice for 15 min.
4. Centrifuge at 900 *x g* for 10 min and discard the supernatant.
5. Wash the pellet twice with 30 ml H_2O, with centrifugation (900 *x g* for 10 min) and supernatant removal after each wash.

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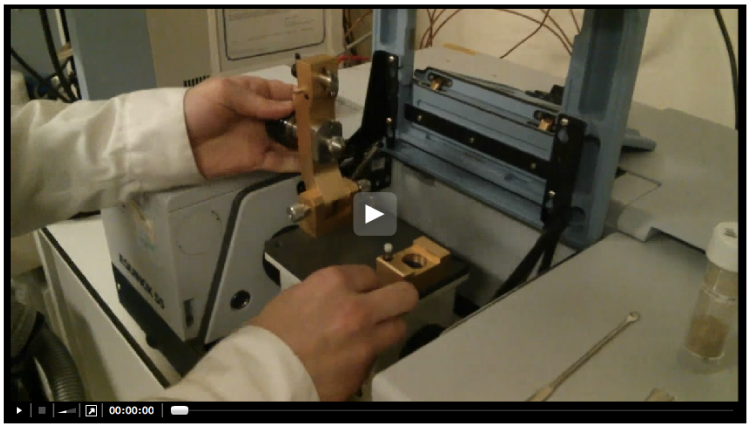
6. Add to the pellet the following reagents: 10 ml 0.1 M ammonium formate buffer (pH 6.0), 10 µl type-I porcine α-amylase (47 units per 100 mg cell wall) and 500 µl 0.001 M sodium azide solution.
7. Leave in a shaking incubator for 48 h at 25 °C/110 rpm.
8. Terminate the digestion by heating to 95 °C/15 min.
9. Cool samples on ice for 15 min.
10. Centrifuge at 900 *x g* for 10 min and discard the supernatant.
11. Wash the pellet three times with 30 ml H_2O and twice with 20 ml acetone, with centrifugation (900 *x g* for 10 min) and supernatant removal after each wash.
12. Let the prepared cell wall material samples dry overnight in a fume hood (alternatively they can be left in an oven set at 35 °C for approximately 16 h; the samples are stable for months at room temperature if kept in a sealed container and protected from direct light).

C. Biomass examination-Fourier transform mid-infrared spectroscopy

1. Without further preparation, place approximately 10 mg of the dry cell wall powder onto the Golden Gate ATR crystal.
2. Press the sample into optimal contact with the ATR crystal using the anvil of the Golden Gate ATR accessory.
3. Collect spectra in duplicate for each biomass sample

   Note: Each spectrum is collected by ATR in a mid-infrared range of 4,000-600 cm^{-1} and consists of the average over 32 scans at a resolution of 4 cm^{-1}. Spectra were corrected for background absorbance by subtraction of the spectrum of the empty ATR crystal.
4. Recover the sample using a spatula or a razor blade (Video 1).

Video 1. Collection of FTIR spectra from cell wall biomass



5. Clean the contact areas with a laboratory tissue wipe and ethanol between samples.
6. Repeat steps C1-5 for all samples.

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7. Convert each collected spectrum into an individual text file containing the spectral two dimensional Cartesian coordinates (*x*, *y*) (Figure 1) in two separate columns (we used the Bruker OPUS IR spectroscopy software). Subsequently, matrices containing the raw data may be created and the underlying relationships between the spectra may be investigated using numerical or statistical computing software (such as R or MATLAB). As an example, we used PCA to identify the features most distinctive of spectra collected from *Miscanthus* spp. leaf and stem cell wall biomass (Figure 2).

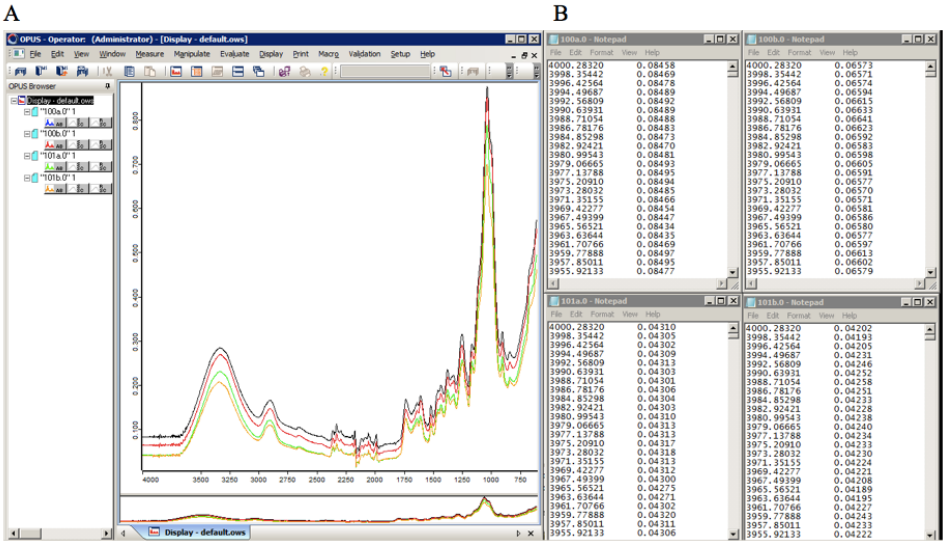

Figure 1. FTIR spectra of cell wall biomass (*Miscanthus* spp.), plotted using Opus IR spectroscopy software (A), and after being converted into text files containing their Cartesian coordinates in two separate columns (B)

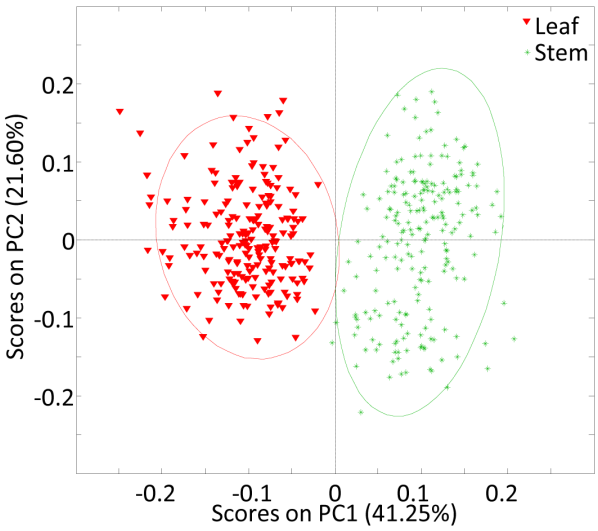

Figure 2. Plot of principal component one (PC1) and principal component two (PC2) scores for *Miscanthus* spp. leaf and stem cell wall spectra generated by FTIR (da Costa *et al.*, 2014)

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Recipes

1. 0.1 M sodium acetate buffer (pH 5.0)
   Prepare 0.2 M acetic acid (A) by mixing 11.55 ml glacial acetic acid in 500 ml H_2O and adjusting to 1 L with H_2O
   Prepare 0.2 M sodium acetate solution (B) by dissolving 27.21 g sodium acetate trihydrate in 800 ml H_2O and adjust to 1 L with H_2O
   Mix 14.8 ml of A, 35.2 ml of B and 50 ml of H_2O
   Confirm pH=5.0 with a pH meter (if needed, the pH may be adjusted with 10 M sodium acetate or with glacial acetic acid)
2. 0.1 M ammonium formate buffer (pH 6.0)
   Dissolve 6,306 mg ammonium formate in 500 ml H_2O and adjust pH to 6.0 with formic acid
   Make up to 1 L with H_2O
   Confirm pH=6.0 with a pH meter (if needed, the pH may be adjusted with formic acid)
3. 0.001 M sodium azide
   Dissolve 10 mg sodium azide in 145 ml H_2O

Acknowledgments

The methods here described were employed in da Costa *et al.* (2014) for the analysis of *Miscanthus* spp. cell wall samples. Portions of this procedure were modified from various sources: organic solvent washing and starch gelatinization from Foster *et al.* (2010); starch removal from Persson *et al.* (2007) and Kong *et al.* (2011); and Fourier transform mid-infrared spectroscopy from Allison *et al.* (2009). The original work was supported by the European Regional Development Funding through the Welsh Government for BEACON Grant number 8056 to R. M. F. da Costa, G. G. Allison and M. Bosch.

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