

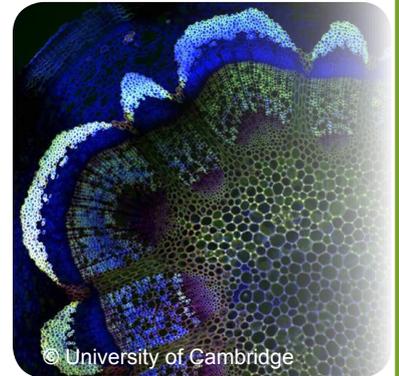
Advanced Biofuels

Activity 2A - Plant material testing

Learning outcomes: By the end of the session students should be able to:

- Describe the main constituents of plant cells.
- Carry out staining for lignin and cellulose in the cell walls.
- Compare the constituents of different plant material and suggest the ideal components of biofuel crops.

Keywords Bioenergy, biofuel, sustainable, renewable, biomass, yield, waste, bioethanol, starch, lignocellulose, lignin, cellulose, cell wall, miscanthus, willow, perennial.



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Stained cross-section of plant stem

Background

Perennial bioenergy crops could offer a more sustainable alternative to biofuels produced from food crops such as sugar beet, wheat or oilseed rape, which are coming under increasing criticism due to their impact on global food security. They don't require much in the way of fertilisers and can be grown on land that is unsuitable for food production. There is a drawback however, in that woody plants, such as miscanthus and willow, convert much of the carbon that they capture into lignocellulosic polymers, which are not a readily fermentable form of carbohydrate.

Lignocellulose is an important component of plants, giving them strength and rigidity. One of the main components of lignocellulose is a polymer called xylan. Xylan in wood and straw is made up of xylose sugar and represents about a third of the sugars that could potentially be used to make bioethanol, but it is locked away. Releasing the energy from lignocellulose is an important challenge to tackle as it will allow the production of fuels from plants in a sustainable way that does not affect the food chain.



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Xylem imaged with a Scanning Electron Microscope

This activity will enable students to visualise the different constituents of plant cells and assess their relative merits as fermentable biofuel feedstocks. Students prepare microscope slides of plant stems with stains that distinguish the components of plant cell structures such as lignin (aniline or phloroglucinol), starch (iodine) and cellulose (Schulze's solution).

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FABIL (fuchsin, aniline blue and iodine in lactophenol) is a reagent which stains and differentiates plant sections. Cytoplasm and nuclei are stained dark blue, cellulose walls a lighter blue and lignin yellow, orange, red or pink, xylem brown and starch black, depending on the nature of the plant material. A variety of alternative stains are available such as phloroglucinol which stains lignin red, Toluidine Blue O which stains lignin and tannins green to blue-green as well as pectins pink to purple, Methylene Blue which stains cellulose blue and the Safranin O-Fast Green technique that stains chromosomes, nuclei, lignin, and cell walls red while the Fast Green stains the cytoplasm and cellulosic cells green. Students should practice with iodine stains as these are less hazardous and most secondary students should be familiar with using iodine to stain for starch. For science fairs and similar events prepare slides in advance and provide microscopes or monitors connected to microscopes to enable the slides to be observed.

Age Range: This experiment is suitable for post-16 students. Restricted use of stains would be suitable for all secondary students.

Duration: 60 minutes.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of cells, microscopy, plant anatomy and transport in plants including the function of xylem and phloem. An understanding of the role of photosynthesis in making the structures and substances in plants will help as will previous experience using stains to identify substances or visualise cells.

What you will need

- Variety of plant material, preferably biofuel feedstocks such as miscanthus, willow and straw
- Variety of stains
- Microscopes
- Glass microscope slides
- Coverslips
- Tweezers
- Scalpel
- Chopping board or white tile
- Petri dish
- Dropping bottles
- Paintbrush
- Absorbent paper
- Mounted needle
- Beaker or sharpsafe
- Gloves

Health and Safety

Students should wear gloves when using stains and take extra care with scalpels. If students have difficulty cutting sections it may be easier if the stems are held in place by inserting into a carrot, potato or polystyrene. If a microtome is available stems could be embedded in wax. Keep the stems moist to soften tissues and ensure the cells do not dry out.

CLEAPSS® laboratory handbook – section 15.5 Plants and seeds (choosing suitable plant material, growing and cultivating plants, sources and suppliers of plants).

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CLEAPSS® Recipe book RB50 (Iodine solution) and RB93 (Stains for plant material). Where possible source prepared solutions. If making up the stains take the following precautions:

Iodine is HARMFUL, see Hazcards 54a and b. Use disposable gloves and eye protection when preparing.

For aniline (phenyl-ammonium) sulfate(VI): see Hazcard 4. Wear eye protection and disposable nitrile gloves when making up the solution. Label the stain HIGHLY FLAMMABLE.

For lactophenol, see Hazcard 38C. Wear goggles and chemical-resistant gloves. Label the stain TOXIC.

Phloroglucinol is an IRRITANT. Ethanol is HIGHLY FLAMMABLE see Hazcards 12, 40 and 47. Label solution HIGHLY FLAMMABLE. Wear eye protection.

Zinc chloride is CORROSIVE see Hazcard 108. Wear eye protection, and chemical-resistant gloves, and carry out the procedure in a fume cupboard. Label the solution CORROSIVE.

The FABIL solution is TOXIC. Wear goggles and chemical-resistant gloves. Label the stain TOXIC.

Toluidine Blue O. Use disposable gloves and eye protection, use a respirator if preparing the solution.

Methylene Blue. Use disposable gloves and eye protection, use a dust mask if preparing the solution.

Safranin O. Use disposable gloves and eye protection.

Fast Green. Use disposable gloves and eye protection.

Method

1. Prepare the stains to be used in advance (see below).
2. Carefully using a pair of tweezers and a scalpel, slice a thin transverse section off the stem of the plant. Demonstrate this step to students and emphasise the need to take care and cut sections as thinly as possible. Many sections may be too thick but with practice some sections will be thin enough to use.
3. Place the stem sections in a petri dish of water to keep moist.
4. Repeat steps 2 and 3 to produce a number of sections from each plant stem being investigated.
5. Remove stem section from the petri dish and place on a slide.
6. Remove excess water by carefully touching the edge of the section with absorbent paper.
7. Add 1 to 2 drops of stain to the section. Phloroglucinol should be left for 4 minutes before removing excess stain and adding a drop of HCl. Detailed instructions for other stains are provided below.
8. Remove excess stain with absorbent paper as before.
9. Slowly lower the coverslip onto the section using the mounted needle making sure that air bubbles are removed from the slide.
10. Label the slide and examine under the microscope.

Place any broken or used coverslips and slides in the beaker or sharpsafe.

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Stain preparation and use

The following instructions for stain preparation are taken from the CLEAPSS® Recipe book. The stains should be prepared by a technician in advance of the practical and stored appropriately.

Iodine stain for starch (also known as Lugol's solution) Use 0.01 M iodine (I_2) solution. 8 g of potassium iodide + 2.54 g of I_2 in 100 ml of water, add the I_2 to moistened KI, make up to 100 ml then dilute tenfold. See CLEAPSS® *Recipe Sheet 50*. Starch will turn blue to black.

Aniline (phenyl-ammonium) sulfate stain for lignin Mix 89 ml of ethanol, 10 ml of 0.05 M sulfuric acid and 1 g of phenylammonium sulfate [aniline sulfate(VI)]. Stains lignin yellow

Phloroglucinol for pentoses and lignin Dissolve 5 g of phloroglucinol (benzene-1,3,5-triol) in 75 ml of ethanol and 25 ml of water. Ligneous tissue should be well-flooded and staining continued for about 4 minutes after which 1 drop of concentrated hydrochloric acid should be added. Phloroglucinol stains lignin red.

Schulze's solution (Chlor-zinc-iodide) for cellulose Dissolve by warming 20 g of anhydrous zinc chloride in 8.5 ml of water and allow the mixture to cool. In a separate container, dissolve 1 g of potassium iodide and 0.5 g of iodine in 20 ml of water. Add this solution drop wise to the zinc chloride solution until iodine precipitate persists on agitation. Stains cellulose blue-violet, lignin yellow, cutin and suberin yellow or brown and starch blue.

FABIL for plant tissues Prepare 3 solutions: 0.5 % solution of aniline blue in lactophenol, 0.5% solution of basic fuchsin in lactophenol and a solution containing 0.3 g of iodine and 0.6 g of potassium iodide in 100 ml of lactophenol. When required, mix in the proportions of 4:1:5 and allow to stand overnight. Filter before use. (Cell contents stain blue, cellulose walls stain light blue and lignin stains yellow.)

Toluidine Blue O for lignin, tannins and pectins Use 0.05% aqueous solution. Leave the stain on for 2-4 minutes.

Methylene Blue for cellulose. Use 0.1% aqueous methylene blue. Leave the stain on for 15-20 minutes.

Safranin O-Fast Green technique. Use a 1% solution. Safranin stains chromosomes, nuclei, lignin, and cell walls red while the Fast Green stains the cytoplasm and cellulosic cells green.

Extension activities

Recording the results of the staining procedures is an essential part of histological analysis and the method may well depend on the microscopes and facilities available. The simplest approach is to provide pencils and paper for the students to sketch what they can observe on their prepared slides. Alternatively if images can be taken with a camera or saved on to a computer connected to the microscope they can be analysed later. If there are only a few microscopes available it can be far quicker and easier to process a whole class of results by taking images of the slides for students and then allowing the students to analyse them in their own time.

Quantitative calculations can be carried out such as calculating the lignification index: see Science and Plants for Schools (SAPS) [Student Sheet 16 - What is Wood?](#) for further details.

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Suppliers

Phloroglucinol, basic fuchsin, lactophenol and aniline (also known as cotton blue) solutions are available from [Sigma-Aldrich](#). Iodine solution is available from [Philip Harris Education](#), Hyde Buildings, Hyde, Cheshire, SK14 4SH, tel: 0845120 4520 fax: 0800 138 8881.

[Timstar Laboratory Suppliers Ltd](#), Timstar House, Marshfield Bank, Crewe, Cheshire, CW2 8UY tel: 01270 250459 fax:01270 250601

A general botanical staining kit insert is available from Philip Harris www.philipharris.co.uk/secondary/biology/microbiology/botanical-staining-kit/?itemcode=B8A14126

Further reading and links

Prepare and examine microscopically the transverse section of a dicotyledonous stem, a prescribed biology activity from the Republic of Ireland National Council for Curriculum and Assessment Senior Cycle Leaving Certificate. www.curriculumonline.ie/en/Post-Primary_Curriculum/Senior_Cycle_Curriculum/Leaving_Certificate_Established/Biology/Biology_Support_Materials/Prescribed_Activities/Detailed_Templates/Prepare_and_examine_microscopically_the_transverse_section_of_a_dicotyledonous_stem.html

Testing leaves for starch, Practical Biology. www.practicalbiology.org/areas/introductory/energy/photosynthesis/testing-leaves-for-starch-the-technique.73.EXP.html

What sorts of carbohydrates do plants make? Science and Plants for Schools (SAPS) [Photosynthesis - A Survival Guide](http://www.saps.org.uk/secondary/teaching-resources/134-photosynthesis-a-survival-guide) <http://www.saps.org.uk/secondary/teaching-resources/134-photosynthesis-a-survival-guide>

What is wood? Science and Plants for Schools (SAPS) [Student Sheet 16 - What is Wood?](#)

Histochemical tests for fresh tissue slices, University of Illinois http://boneslab.bio.ntnu.no/old_root/histochemicaltests.htm

Photosynthesis and starch production in Pelargonium leaf discs, Science and Plants for Schools (SAPS). www.saps.org.uk/secondary/teaching-resources/145-photosynthesis-and-starch-production-in-pelargonium-leaf-discs-

Growing the bioenergy field. BBSRC business spring, 2011 www.bbsrc.ac.uk/news/industrial-biotechnology/2011/110314-f-growing-the-bioenergy-field.aspx

Rothamsted Research Willow Power www.rothamsted.bbsrc.ac.uk/Research/Centres/Content.php?Section=ForThePublic&Page=WillowPower

NNFCC Miscanthus crop fact sheet www.nnfcc.co.uk/publications/nnfcc-crop-factsheet-miscanthus

Sweet success for sustainable biofuel research www.bbsrc.ac.uk/news/industrial-biotechnology/2010/100125-pr-success-sustainable-biofuel-research.aspx

Biofuel from inedible plant material easier to produce following enzyme discovery www.bbsrc.ac.uk/news/industrial-biotechnology/2010/100913-pr-biofuel-from-inedible-plant-material.aspx

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2. <http://royalsociety.org/Sustainable-biofuels-prospects-and-challenges/>

Nuffield Council on Bioethics, April 2011, *Biofuels: ethical issues* www.nuffieldbioethics.org/biofuels-0

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Research groups

Prof. Paul Dupree, BSBE Cell Wall Sugars Programme, Department of Biochemistry, University of Cambridge www.bsbec.bbsrc.ac.uk/programmes/cell-wall-sugars.html www.bioenergy.cam.ac.uk

Dr Angela Karp, BSBE Perennial Bioenergy Crops Programme, Rothamsted Research www.bsbec.bbsrc.ac.uk/programmes/perennial-bioenergy-crops.html

Professor Katherine Smart, BSBE LACE Programme, School of Biosciences, University of Nottingham, Sutton Bonington Campus www.bsbec.bbsrc.ac.uk/programmes/index.html www.nottingham.ac.uk/bioenergy/index.aspx www.nottingham.ac.uk/bioenergy/lace/