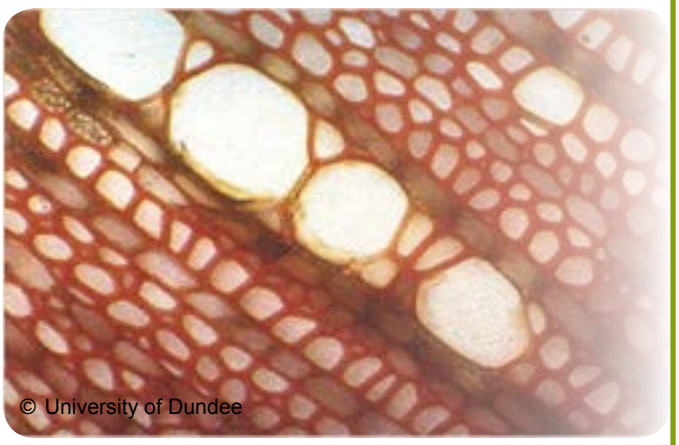
Advanced Biofuels - Lignocellulosic Bioethanol

Keywords

Bioenergy, biofuel, sustainable, renewable, biomass, yield, waste, bioethanol, lignocellulose, lignin, microbes, yeast, enzyme, fermentation, gribbles, varieties, pentose, hexose.



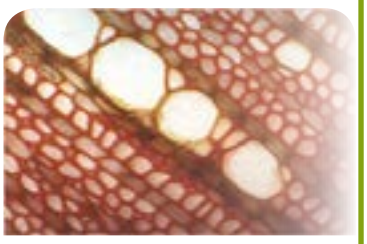
Plant cell wall lignins under the light microscope

Background

Bioethanol is produced by fermentation of sugars by yeast. Currently sugar beet and sugar cane are the main sources of sugar for bioethanol. In the future the biomass locked up in plant cell walls (lignocellulose) may be released for fermentation and production of bioethanol. This would enable bioethanol to be produced from wood, straw and waste materials.

Research to achieve this is going on in many areas including

- **Improving perennial biomass crops**
- **Manipulating lignin to optimise sugar release**
- **Improving release of sugars from plant cell walls**
- **Discovering new enzymes for sugar release**
- **Developing yeast strains to ferment sugars**
- **Bacterial fermentation of sugars to butanol**



Miscanthus planting measurements

We can use enzymes to break down plant biomass to release sugars for fermentation. In plants the sugars are locked into the cell walls as long chain polymers in ways we currently do not fully understand, preventing effective digestion by enzymes. If we can understand better how the plant sugars are arranged in the cell walls, we can select plants and match them with the most appropriate enzymes for more effective biofuel production.

Improving the properties of lignin in barley straw will make it easier to produce biofuel (or bioenergy) from this material without detrimental effects on the yield or quality of the crop. Lignin is a strengthening and waterproofing polymer that encrusts the sugar-based polymers in plant cell walls, making them hard to access for biofuel production. Lignin and its by-products are also toxic to microorganisms used in fermentation. Feedstocks rich in lignocellulose require treatment with acids, alkalis or steam explosion methods to hydrolyse hemicellulose and break down lignin, enabling access to the cellulose by enzymes. Steam explosion has significant potential as chemical methods have to be managed with recovery and waste water processes and can inhibit enzymes and fermentation, but all are energy intensive at present.

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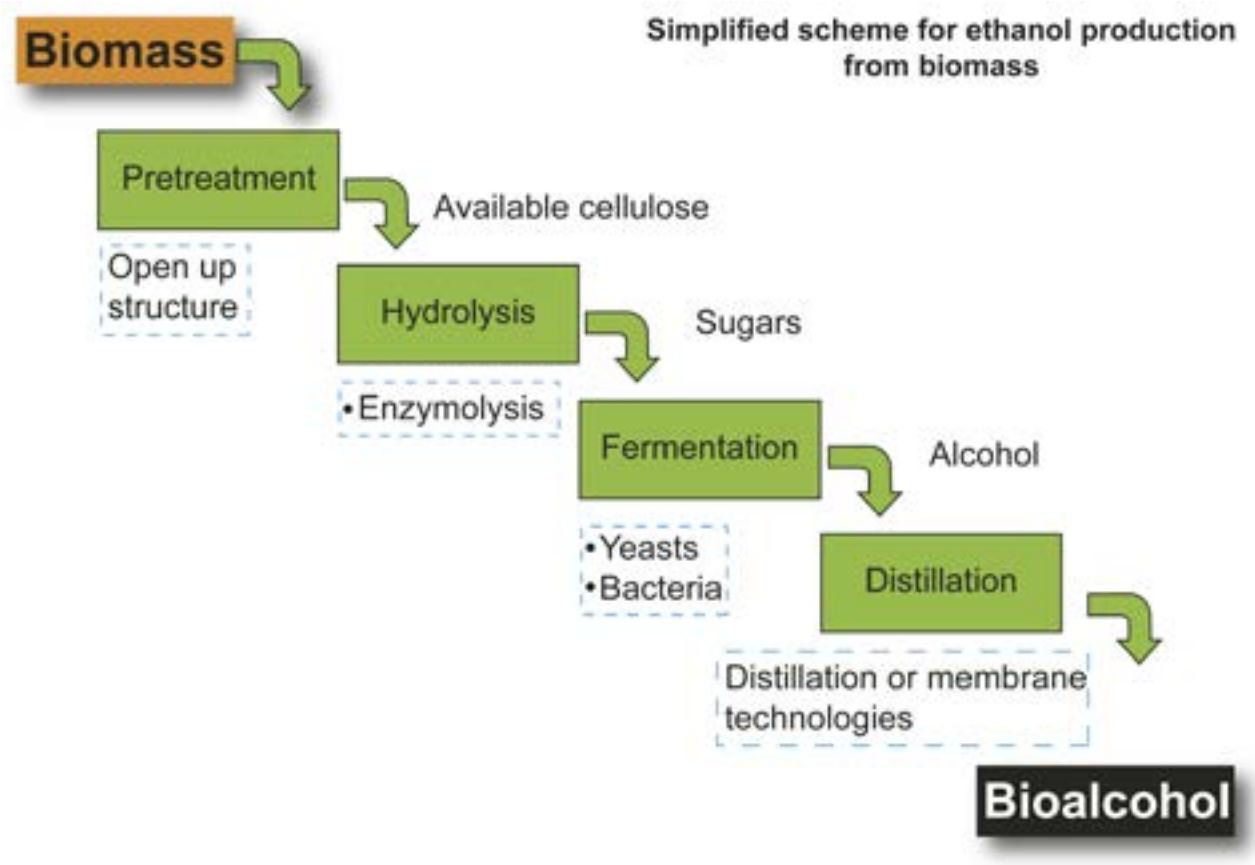
Discovering new enzymes in bacteria, fungi and marine wood borers (gribbles) will enable the conversion of non-food plant biomass into biofuels. Wood and straw contain polysaccharides (polymers of sugars) that can be converted into simple sugars suitable for fermentation to produce liquid biofuels. Currently we lack effective enzymes to digest these woody materials. However, marine wood borers are voracious consumers of lignocellulose and have all the enzymes needed for digestion of wood and straw. Scientists have already sequenced the genes that are expressed in the marine wood borer gut and which encode the digestive enzymes. Scientists will study the digestive process in borers as well as a range of microorganisms and investigate the industrial applications of their enzymes for biofuel production.

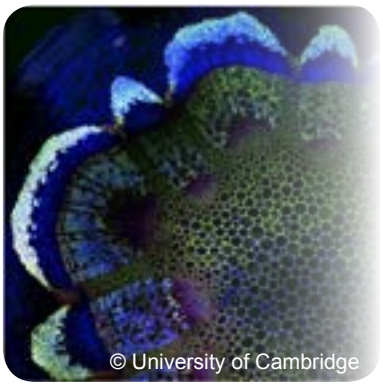
Marine wood borer (gribble)

To harness the potential of lignocellulosic (plant cell wall) materials for sustainable production of bioethanol, we need to optimise energy output without negative environmental, social or economic impacts. We need to optimise the release of sugars from plant cell walls in agricultural and wood-industry wastes to produce a fermentable feedstock that microorganisms can convert to fuels. Developing robust microbial strains that can use these feedstocks will enable sustainable production of bioethanol.



Advanced Biofuels - Lignocellulosic Bioethanol

Activity 2A - Plant material testing



Stained cross-section of plant stem

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

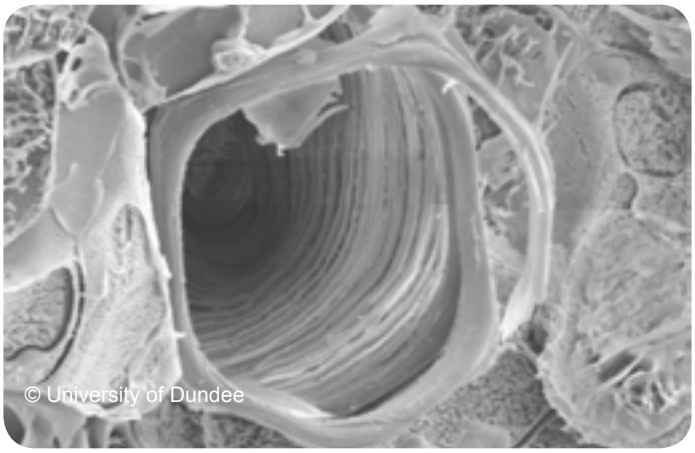
- Describe the main constituents of plant cells.
- Carry out staining for lignin and cellulose in the cell walls.
- Compare the constituents of different plant material and suggest the ideal components of biofuel crops.

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

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 require less fertiliser applications 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.



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).

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
- Forceps
- Scalpel
- Chopping board or dissection tray
- 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 possible use disposable scalpels or razors to cut the sections. 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. Used scalpels or razor blades should be disposed of in a stout sided container that is sealed and bagged before placing in the normal refuse.

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

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:

Refer to the suppliers safety guidelines when using stains. Some stains are more effective if prepared fresh but bear in mind they may be more hazardous as solids. Contact CLEAPSS for further information or Hazcards on the stains used.

Method

1. Prepare the stains to be used in advance (see below).
2. Carefully using a pair of forceps 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.

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.

Suppliers

Phloroglucinol, basic fuschin, 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.

Schulze's solution is available from 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

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.

Testing leaves for starch, Practical Biology. [Reference/webpage no longer available – July 2016]

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

What is wood? Science and Plants for Schools (SAPS)

Histochemical tests for fresh tissue slices, University of Illinois

Photosynthesis and starch production in Pelargonium leaf discs, Science and Plants for Schools (SAPS).

Growing the bioenergy field. BBSRC business spring, 2011

Rothamsted Research Willow Power

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

Sweet success for sustainable biofuel research

Biofuel from inedible plant material easier to produce following enzyme discovery

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2.

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

Plant variants point the way to improved biofuel production
www.bbsrc.ac.uk/news/industrial-biotechnology/2014/140922-pr-plant-variants-biofuel-production.aspx

Branching out: from model plants to coppiced trees
www.bbsrc.ac.uk/news/industrial-biotechnology/2014/140107-n-model-plants-to-coppiced-trees.aspx

Wind in the Willows [Reference/webpage no longer available – January 2017]

Miscanthus - A bioenergy crop for all seasons

Research groups

Prof. Paul Dupree, BSBEC Cell Wall Sugars Programme, Department of Biochemistry, University of Cambridge

Dr Angela Karp, BSBEC Perennial Bioenergy Crops Programme, Rothamsted Research

Professor Katherine Smart, BSBEC LACE Programme, School of Biosciences, University of Nottingham, Sutton Bonington Campus

Energy Crop Biology research group, Institute of Biological, Environmental and Rural Sciences, Aberystwyth University

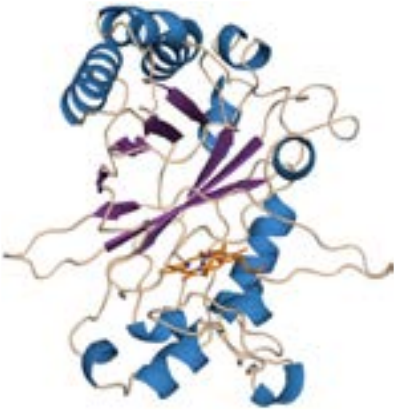
Advanced Biofuels - Lignocellulosic Bioethanol

Activity 2B - Hydrolysis of biofuel feedstocks

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

- Describe the enzymatic breakdown of cellulose.
- Analyse the effectiveness of enzymatic breakdown of plant material.
- Suggest effective enzymes and conditions for the production of fermentable sugars.

Keywords Bioenergy, biofuel, sustainable, renewable, biomass, yield, waste, bioethanol, sugar, lignocellulose, microbes, yeast, enzyme, fermentation, gribbles, varieties, hydrolysis.



The structure of the newly identified lignin-degrading enzyme from *Rhodococcus*

Background

Sustainable liquid biofuels can be produced from lignocellulosic biomass such as wood and straw. These materials contain polysaccharides that can be converted through enzymatic hydrolysis into simple sugars which can be fermented to produce liquid biofuels.

Bioethanol produced on a large scale in Brazil and the USA is made from sugar cane or maize respectively. Sugars from sugar cane can be fermented by *Saccharomyces cerevisiae* without prior treatment as they are already disaccharides, but starch polymers from maize or wheat need conversion to di- or monosaccharidic sugars, by a hydrolysis reaction known as saccharification, prior to fermentation. The enzyme mixtures used in saccharification of starch are amylases, enzymes also found in human saliva and secreted by the pancreas.

In plants the majority of sugars are locked into the cell walls in ways we do not fully understand, preventing effective digestion by enzymes. Currently we lack effective enzymes to digest these woody materials as amylases hydrolyse a different type of linkage between individual sugars to the linkages found in cell wall polysaccharides. One of the aims of current research is to discover enzymes that can release sugars from currently indigestible cell wall components. Lignocellulose and hemicelluloses are broken down by the actions of a range of enzymes including cellulases and hemicellulases.

In this activity students can compare the effectiveness of enzymes at hydrolysing a variety of feedstocks. Straw, maize and rapeseeds are recommended substrates. The popcorn mimics the process of steam explosion that can be used to open up plant cell walls to allow enzymes access to polysaccharides. In the table below the two enzymes that are compared for their ability to produce fermentable sugars from the feedstocks are cellulase and pectinase. Cellulase breaks down accessible cellulose molecules whereas pectinases break down the pectin in cell walls that holds the cellulose molecules in place. Pectin is predominantly found in non-woody parts of plants (as it is associated with the primary cell wall found around all plant cells) and holds cells together.

Advanced Biofuels - Lignocellulosic Bioethanol

Age Range: This experiment is suitable for secondary and post-16 students.

Duration: 50-60 minutes.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of enzymes, carbohydrates and sugars.

What you will need

- Cellulase, pectinase (Pectinex®)
- Variety of biofuel feedstocks: straw, popcorn, rapeseeds
- Boiling tubes or conical flasks
- Beaker
- Stirrers
- Water bath
- Timer
- Mortar and pestle
- Buffers across a pH range of 3-8 e.g. Tris buffer
- Glucose test strips

Optional

- Blood glucose monitor
- Amylase

Health and Safety

Care should be taken with enzymes particularly due to their allergenic nature and ability to act as sensitisers. CLEAPSS® Recipe book RB37 (Enzymes), Hazcard 33 (Enzymes), RB3 (Alginate beads), RB19 (Calcium chloride and nitrate(V) solutions), Guide 3.015 (Enzymes), Laboratory handbook page 1441-1443. Solutions equal to or stronger than 1% (w/v) should be labelled as irritant - CLEAPSS® Recipe book. The coloured part of glucose testing strips are hazadous and students should be instructed not to touch them or cut them up.

Method

The activity should be trialled in advance to ensure suitable conditions are chosen for use in class. Take care choosing appropriate buffers as some may be inhibitory to enzymes.

1. The biofuel feedstocks should be ground down in a mortar and pestle to enable the enzymes to access the cellulose.
2. Students should weigh out 1 g of each feedstock and add it to a boiling tube.
3. Add 10 ml of buffer.
4. Add 0.5 ml of enzyme.
5. Remove a small volume (approximately 0.5ml) at 5 minute intervals and test the glucose concentration using a glucose test strip, such as 'Diabur Test® 5000' (semi-quantitative) or 'Diastix' (qualitative) or blood glucose monitor. There may be a delay in getting a result and students should be instructed not to wait for this before timing the removal of the next test aliquot. Students should carefully follow the glucose testing instructions.

6. Present the results in a graph.

							Control	Control	Control
Feedstock	straw	popcorn	rapeseeds	straw	popcorn	rapeseeds	straw	popcorn	rapeseeds
Enzyme	Cellulase	Cellulase	Cellulase	Pectinase	Pectinase	Pectinase	×	×	×
pH	5	5	5	5	5	5	5	5	5
Temperature	35°C	35°C	35°C	35°C	35°C	35°C	35°C	35°C	35°C

									Control
Feedstock	popcorn	popcorn	popcorn	popcorn	popcorn	popcorn	popcorn	popcorn	popcorn
Enzyme	Cellulase	Cellulase	Cellulase	Cellulase	Pectinase	Pectinase	Pectinase	Pectinase	×
pH	3	4	5	6	3	4	5	6	5
Temperature	35°C	35°C	35°C	35°C	35°C	35°C	35°C	35°C	35°C

Extension activities

Repeating the experiment with amylases will allow comparison of the effectiveness of saccharification versus breakdown of the cellulose.

					Control
Feedstock	starch	starch	starch	starch	popcorn
Enzyme	Amylase	Amylase	Amylase	Amylase	×
pH	3	4	5	6	5
Temperature	35°C	35°C	35°C	35°C	35°C

Students can immobilise the enzyme in alginate beads and investigate the effect on the reaction rate. Recover the enzyme and repeat to investigate the viability of use in continuous flow processes. Placing the enzyme-alginate beads in a column or syringe will enable student to replicate a continuous flow process and test the effect of repeated passage through the column.

Preparing immobilised enzymes

1. Prepare a 2% sodium alginate solution with warm distilled or deionised water, mix thoroughly and leave overnight in a fridge. The initial mixture can be very lumpy but will become smooth overnight.

2. Add the stock enzyme solution to the sodium alginate solution to obtain the correct final concentration desired and mix thoroughly. If required add a small amount of distilled or deionised water but take care to ensure the final solution of immobilised enzyme-alginate is not too runny.

3. Prepare a 1.5% calcium chloride solution with calcium chloride dihydrate ($CaCl_2.2H_2O$). The calcium ions cause the sodium alginate to set and hence using distilled or deionised water for the alginate and enzyme solutions is important as is avoiding contact of the syringe with the calcium chloride solution.

4. Draw the enzyme-alginate solution up into a syringe.
5. Add the enzyme-alginate solution into a 1.5% $CaCl_2$ solution drop by drop. Carefully observe the shape of the drops. If the drops take on a 'comet' shaped appearance add a small amount of distilled or deionised water to the enzyme-alginate solution, mix and retry.
6. Allow the enzyme-alginate beads to set for at least 10 minutes.
7. Carefully strain the beads and rinse with distilled or deionised water.

The enzyme kinetics of cellobiase can be investigated with older students. This activity would be suitable for A-level students. Bio-Rad produce a kit that enables students to investigate cellobiase rates of reaction under different conditions by observing a colour change using a spectrophotometer.

Suppliers

A variety of enzymes including cellulase (*Celluclast®*) can be obtained from National Centre for Biotechnology Education (NCBE) www.ncbe.reading.ac.uk/menu.html University of Reading, 2 Earley Gate, Whiteknights Road, Reading, RG6 6AU tel: 0118 9873743 fax: 01189 750140.

Diastix and sodium alginate, can be obtained from Philip Harris Education, Hyde Buildings, Hyde, Cheshire, SK14 4SH tel: 0845120 4520 fax: 0800 138 8881 and Timstar Laboratory Suppliers Ltd, Timstar House, Marshfield Bank, Crewe, Cheshire, CW2 8UY tel: 01270 250459 fax:01270 250601 Daibur-Test® 5000 strips as well as a wide range of blood glucose monitors and detection strips can be obtained from local chemists.

Biofuel enzyme kit for investigating the activity of cellobiase can be obtained from Bio-Rad Laboratories www.bio-rad.com.

Further reading and links

King, A.J. *et al*, 2010 Molecular insight into lignocellulose digestion by a marine isopod in the absence of gut microbes. *PNAS*,107(12), 5345-5350.

Enzyme from wood-eating gribble could help turn waste into biofuel [Reference/webpage no longer available – January 2017]

Cows' stomachs could hold key to green fuels www.roslin.ed.ac.uk/news/2011/07/29/cows%27-stomachs-could-hold-key-to-green-fuels/

New insights into biomass breakdown www.bbsrc.ac.uk/news/industrial-biotechnology/2014/140609-pr-insights-into-biomass-breakdown.aspx

Significant step forward in biofuels quest [Reference/webpage no longer available – January 2017]

Immobilised yeast - Immobilisation of yeast in calcium alginate beads, Dean Madden, 2007.

Glucose detector. National Centre for Biotechnology Education (NCBE) Practical Biotechnology. [Reference/webpage no longer available – October 2016]

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2.

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

Research groups

Professor Simon McQueen-Mason, BSBEC Marine Wood Borer Enzyme, Discovery Programme, The University of York, Heslington, York, YO10 5DD

Professor Paul Dupree, BSBEC Cell Wall Sugars Programme, Department of Biochemistry, University of Cambridge

Professor David Archer, BSBEC LACE programme Strand 2, University of Nottingham

Professor Timothy Bugg, Department of Chemistry, University of Warwick www2.warwick.ac.uk/fac/sci/chemistry/research/bugg/bugggroup/research/

Professor Walton and Professor Davies, Critical Enzymes for Sustainable Biofuels from Cellulose (CESBIC), Department of Chemistry, University of York www.york.ac.uk/chemistry/staff/academic/t-z/pwalton/
www.york.ac.uk/chemistry/staff/academic/d-g/gdavies/

Advanced Biofuels - Lignocellulosic Bioethanol

Activity 2C – Fermentation of lignocelluloses

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

- Describe the process of ethanol production from lignocelluloses.
- Carry out pretreatment and yeast fermentation of a range of substrates.
- Assess the effectiveness of pretreatment and enzymatic hydrolysis of lignocelluloses substrates.

Keywords Bioenergy, biofuel, sustainable, renewable, biomass, yield, waste, residues, bioethanol, lignocellulose, microbes, yeast, enzyme, fermentation, gribbles, varieties, pentose, hexose, pretreatment.

Steam Explosion Unit

Background

Current bioethanol production uses food crops such as sugar cane and maize. Large amounts of sugar molecules are present in the lignocellulose of plant material and current research aims to 'unlock' the fermentable sugars in agricultural or forestry wastes and residues from cereal production such as straw, bran, brewers grain and wood as well as from *Brassicas* and food-chain waste.

Fermentation by yeast currently uses sugar beet and sugar cane as the main sources of sugar for bioethanol. Starches from maize or grain feedstocks are hydrolysed with amylase enzymes (saccharification) to produce sugar that can be fermented. Yeast have been used for centuries in brewing alcoholic drinks. The yeast *Saccharomyces cerevisiae* produces ethanol by fermentation of sucrose or glucose (hexose, C6 sugars) but is unable to ferment pentose (C5) sugars. *Saccharomyces diastaticus* is able to utilise starch for fermentation. The National Collection of Yeast Cultures recommends certain strains for the production of bioethanol, such as *Pachysolen tannophilus*, *Candida succiphilia*, *Candida tenuis* and *Pichia stipitis*, due to their ability to degrade cellulose or ferment xylose. To make use of a greater proportion of lignocelluloses it may be possible to genetically engineer yeast to ferment pentose (five carbon) and hexose (six carbon) sugars with equal efficiency. Most yeast varieties use hexose sugars as a substrate for fermentation. It may also be possible that scientists out in the field can discover new varieties of yeast that are able to preferentially ferment pentose sugars. To enable yeast to carry out fermentation the sugars trapped in plant cell wall lignocellulose, must be 'released' by pretreatment with steam or chemicals followed by hydrolytic breakdown of the released polysaccharides with enzyme cocktails. Currently chemical treatment involves either strong acid or mild alkali but due to the requirement for specialised equipment to carry out the procedure as well as the treatment of waste chemicals before disposal, research is focusing on steam treatments at present. Pretreatments change the structure of cell walls and polymers by disrupting intermolecular forces holding them together, allowing greater access by enzymes and water. Enzymatic digestion of exposed polysaccharides can produce mono-, di- and tetrasaccharides. Enzymes are expensive and need to be recovered from industrial processes. Immobilising enzymes enables easier recovery and development of more efficient continuous processing. However, it restricts the ability of the enzymes to carry out cleavage of the insoluble polysaccharides.

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In this activity students can compare the effectiveness of a variety of pretreatments on fermentation rates of yeast. The recovery and reuse of resources is important in making biofuel production economic and environmentally friendly and students could investigate the rate of fermentation with and without immobilising enzymes or yeast in sodium alginate balls.

1. Fermentation of polysaccharides with a range of pretreatments (steam explosion or alkali treatment).
2. Fermentation of polysaccharides with or without enzyme digestion (Popcorn, starch and oatbran, with or without amylase or cellulase).
3. Fermentation of substrates with immobilised yeast or co-immobilised yeast and cellulase.
4. Fermentation rates for monosaccharide versus polysaccharide substrates using *Saccharomyces cerevisiae*, *Saccharomyces diastaticus* or more specialised yeast strains.

It is suggested students are split into groups to assess the effects of different variables and report their results back to the rest of the class.

This activity is based on ones described in the NCBE booklet Practical Fermentation – A guide for Schools and Colleges: Yeast cells and enzyme together they can do it, bioreactor practical (replacing lactose/ lactase with cellulose/cellulase) [Reference/webpage no longer available – October 2016], a resource developed by Dr Jen Bromley in conjunction with Science and Plants for Schools (SAPS) www.sapa.org.uk and the work of the Society of General Microbiology www.microbiologyonline.org.uk.

Age Range: This experiment is suitable for secondary and post-16 students.

Duration: Set up 60 minutes, analysis of results and reporting back 60 minutes. Allow up to a week between sessions to enable sufficient fermentation for measurable levels of carbon dioxide to be produced. The experiment can be set up and run in one day for a science fair or exhibition with adjustment of the fermentation conditions.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of microbes, fermentation, enzymes, alcohols, fuels and the properties of gases.

What you will need

- Conical flask (100 ml) or boiling tubes
- 8% glucose solution
- 0.1M phosphate(V) buffer pH 7
- Brewer's or baker's yeast (*Saccharomyces cerevisiae*)
- Deionised or distilled water
- Stirrers
- Balloons or bubble counters
- Measuring cylinder (50 ml)
- Pressure cooker or autoclave
- Popcorn maker
- Alkali (1M NaOH) and acid (1M HCl)
- Cellulase (*Celluclast*)
- Amylase
- Oatbran or popcorn
- Cornflour or potato starch
- Thermometer
- Timers
- Beaker of disinfectant
- Eye protection

Optional

- Water bath
- Sodium alginate
- Syringe
- 1.5% Calcium chloride (CaCl) solution
- Buffer solutions at varying pH
- Strainer
- Fermentation locks
- Universal indicator solution
- Cotton wool
- Magnetic stirrer and fleas
- Alternative yeast strains

Health and Safety

The following factors should be considered when planning to carry out any investigations involving microorganisms; nature of the organism used, source of the organism, temperature of incubation, culture medium used, type of investigation and the facilities available, chance of contamination, expertise of people involved. If necessary change the conditions or limit the involvement of students perhaps by carrying out the experiment as a demonstration. CLEAPSS® handbook - "perfectly safe if the organisms studied are known to be non-pathogenic, such as brewer's and baker's yeast, the bacteria in yoghurt or edible mushrooms"

If fermentation occurs for longer than 1 day fermenters and their contents should be sterilised at 121°C for 15 minutes prior to disposal.

CLEAPSS® laboratory handbook – Section 14.9 Fermenters (Safety, Practical considerations) page 1443-1451, section 15.2 Microbiology (COSHH, good practice and safety precautions, levels of practical work, using microorganisms in practical work, equipment and materials, sterilisation and disinfection) page 1505

CLEAPSS® Recipe book RB3 (Alginate beads), RB19 (Calcium chloride and nitrate(V) solutions), RB99 (Testing for gases).

CLEAPSS® Hazcards 19A (Calcium salts), 20 (Carbon dioxide), 40A (Ethanol), 40C (Carbohydrates).

CLEAPSS® Guidance PS 04 (COSHH: risk assessments in situations where microorganisms might be involved), PS 15 (Ventilation and levels of Carbon dioxide and other gases in the laboratory & prep room),PS 89 (Measurement of anaerobic respiration in yeast)

CLEAPSS® guides L214 (examining autoclaves, pressure cookers) R101 (Steam sterilisation: Autoclaves & pressure cookers)

CLEAPSS® Model risk assessment 3.026 (Microorganisms used in food production).

Further advice can also be sought from the Society for General Microbiology and the Microbiology in Schools Advisory Committee

1% VirKon is a suitable disinfectant for general surface cleaning and sterilisation as well as for discard pots (follow manufacturer's instructions).

Care should be taken with enzymes particularly due to their allergenic nature and ability to act as sensitisers. CLEAPSS® Recipe book RB37 (Enzymes), Hazcard 33 (Enzymes), Guide 3.015 (Enzymes), Laboratory handbook page 1441Q-1443. Solutions equal to or stronger than 1% (w/v) should be labelled as irritant.

Diluted enzymes do not work well when immobilised in alginate beads, so concentrates may be necessary and the appropriate safety measures should be taken.

Method

You may want to set up one simple experiment to show the rate of fermentation with and without pretreatment, alternatively you may want to divide a class up into groups to investigate different variables and report back after they have conducted their experiments. The activity should be trialled in advance to ensure suitable conditions are chosen for use in class. Take care choosing appropriate buffers as some may be inhibitory to enzymes.

The tables below show a number of possible experiments that can be conducted. *Celluclast* catalyses the breakdown of the glucose polymers that comprise cellulose to glucose, cellobiose (i.e.pairs of glucose units) and longer chains of glucose units. The optimum conditions for activity of this enzyme preparation are in the range pH 4.5-6.0, and about 50-60 °C.

1. The biofuel feedstocks should be pretreated with either steam (using a pressure cooker, autoclave or popcorn maker) or alkali (1M sodium hydroxide [NaOH] for 1 hour at room temperature) prior to the activity. Ensure the alkali-treated solution is neutralised before fermentation with 1M Hydrochloric acid (HCl). This may require repeated additions of acetic acid with agitation and time for the feedstock to equilibrate. Use a caslibrated pH meter to check the neutralisation and adjust with NaOH if required.

2. Prepare the fermentation stock solutions in phosphate (V) buffer. Ready made phosphate buffers can be obtained at the desired pH and are usually supplied as tablets or stock solutions.

3. Students should label the conical flasks then add the yeast, feedstocks and buffers.

4. If immobilising yeast in sodium alginate ideally the solution of yeast and sodium alginate is prepared the day before.

5. Resuspend the yeast in a small amount of distilled or deionised water in order to make a final 3% solution in the fermentation reaction. To ensure active cultures incubate in malt extract broth for 48 hours at room temperature before inoculating. To ensure pure cultures streak out the yeast on malt agar plates and inoculate from single colonies. These steps should not be carried out by students and they should not open incubated petri dishes.

6. Add the following to the conical flasks or bioreactor

- 100 ml of phosphate buffer
- Feedstock equivalent to 10 g of original dry weight
- 5 ml of enzyme (refer to the suppliers data sheet to choose suitable concentrations)

7. Inoculate with yeast

8. Stopper the flasks with bungs holding fermentation locks and attached bubble counters or add the balloons.

Yeast	✔	✔	✔	✔	✔	✔
Feedstock	Oatbran	Oatbran	Oatbran	Popcorn	Popcorn	Popcorn
Pretreatment	✕	Alkali	Steam	✕	Alkali	Steam
Enzyme	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase

Yeast	✔	✔	✔	✔	✔	✔	✔	✔	✔
Feedstock	Oatbran	Oatbran	Oatbran	Popcorn	Popcorn	Popcorn	Starch	Starch	Glucose
Pretreatment	✕	Alkali	Alkali	✕	Steam	Steam	✕	✕	Alkali
Enzyme	Cellulase	Cellulase	✕	Cellulase	Cellulase	✕	Amylase	✕	Cellulase

In order to calculate the rate of fermentation the amount of carbon dioxide produced can be measured over time. This can be done in a number of ways including the use of bubble counters available from National Centre for Biotechnology Education (NCBE), collection of carbon dioxide (CO_2) in inverted water-filled measuring cylinders, with balloons attached to the neck of the conical flask or boiling tubes or with gas syringes. Choose the method used according to the equipment and time available for the experiment. The volume of carbon dioxide produced can be calculated by multiplying the number of bubbles recorded by a bubble counter and the volume of one bubble. If using balloons the volume can be measured by carefully tying off the balloon used to collect the gas produced, immersing it in a large measuring cylinder and measuring the displaced volume. Note: the gas in the balloon is not likely to be predominantly carbon dioxide and is instead displaced air, with the denser carbon dioxide produced remaining at the bottom of the flask. This experiment is suitable for public demonstrations and science fairs providing appropriate risk assessment is carried out.

Extension activities

Enzymes and microbes can prove to be expensive components in an industrial bioreactor operation. Being able to recover and reuse them is enhanced if they are immobilised. Students can immobilise yeast and enzymes in sodium alginate and assess the impact on the ability of the reactions to carry out fermentation.

Yeast	✔	✔	✔	✔	✔	✔	✔	✔	✔
Feedstock	Oatbran	Oatbran	Oatbran	Oatbran	Popcorn	Popcorn	Popcorn	Popcorn	Glucose
Pretreatment	Alkali	Alkali	Alkali	Alkali	Steam	Steam	Steam	Steam	Alkali
Enzyme	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase
Immobilisation	Yeast	Yeast and enzyme	Enzyme	✕	Yeast	Yeast and enzyme	Enzyme	✕	✕

Preparing immobilised yeast

1. Prepare a 2% sodium alginate solution with warm distilled or deionised water, mix thoroughly and leave overnight in a fridge. The initial mixture can be very lumpy but will become smooth overnight.
2. Resuspend the yeast in a small amount of distilled or deionised water so that the final solution of immobilised yeast-alginate is not too runny.
3. Add the resuspended yeast solution to the sodium alginate solution and mix thoroughly.
4. Prepare a 1.5% calcium chloride solution with calcium chloride dihydrate ($CaCl_2.2H_2O$). The calcium ions cause the sodium alginate to set and hence using distilled or deionised water for the alginate and yeast solutions is important as is avoiding contact of the syringe with the calcium chloride solution.
5. Draw the yeast-alginate solution up into a syringe.
6. Add the yeast-alginate solution into a 1.5% $CaCl_2$ solution drop by drop. Carefully observe the shape of the drops. If the drops take on a ‘comet’ shaped appearance add a small amount of distilled or deionised water to the yeast-alginate solution, mix and retry.
7. Allow the yeast-alginate beads to set for at least 10 minutes.
8. Carefully strain the beads and rinse with distilled or deionised water.

Students could investigate the ability of yeast to ferment different sugar substrates, see activity 1G - Yeast fermentation.

The fermentation reaction may also be set up as a bioreactor with recording of pH and temperature with data logging software.

Suppliers

Bioreactors, bubble counters and a variety of enzymes including cellulose (*Celluclast®)* can be obtained from National Centre for Biotechnology Education (NCBE) www.ncbe.reading.ac.uk/menu.html University of Reading, 2 Earley Gate, Whiteknights Road, Reading, RG6 6AU, tel: 0118 9873743, fax: 01189 750140

Popcorn and oatbran can be obtained from local supermarkets.

Brewer's or baker's yeast can be obtained from local supermarkets, brewery stores, or bakeries.

Dried yeast can also be obtained from Blades Biological Limited www.blades-bio.co.uk Cowden, Edenbridge, Kent, TN8 7DX, tel:01342 850 242, fax: 01342 850 924.

Sodium alginate and universal indicator can be obtained from Philip Harris Education, Hyde Buildings, Hyde, Cheshire, SK14 4SH, tel: 0845120 4520 fax: 0800 138 8881.

Further reading and links

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Yeast fermentation and distillation instructions from Practical Chemistry

Practical Fermentation – A guide for Schools and Colleges. 1999. National Centre for Biotechnology Education (NCBE) and Society of General Microbiology (SGM). [Reference/webpage no longer available – October 2016]

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Cows' stomachs could hold key to green fuels www.roslin.ed.ac.uk/news/2011/07/29/cows%27-stomachs-could-hold-key-to-green-fuels/

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2.

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

Research groups

Lignocellulosic Conversion To Bioethanol

Professor Katherine Smart, BSBEC LACE Programme, School of Biosciences, University of Nottingham, Sutton Bonington Campus

Professor Simon McQueen-Mason, BSBEC Marine Wood Borer Enzyme, Discovery Programme, The University of York, Heslington, York, YO10 5DD

Prof. Paul Dupree, BSBEC Cell Wall Sugars Programme, Department of Biochemistry, University of Cambridge

National Collection of Yeast Cultures, Institute of Food Research, Norwich Research Park, Norwich, NR4 7UA www.ncyc.co.uk/

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Keywords

Bioenergy, biofuel, sustainable, renewable, biomass, yield, waste, bioethanol, biobutanol, lignocellulose, microbes, bacteria, enzyme, fermentation, varieties, soil, synthetic biology, nutrient broth.

Bacterial cultures examined in a containment cabinet

Background

Genetically modified strains of *Escherichia coli* are able to break down cellulose and produce bioethanol, and some strains of bacteria, such as *Clostridium acetobutylicum*, are able to produce biobutanol. Many naturally occurring strains of bacteria and fungi, such as those found in soil, produce cellulases that can convert lignocellulose to fermentable sugars. Research to identify cellulases in bacteria and fungi as well as optimising the lignocellulosic conversion process will hopefully lead to more efficient production of biofuels.

Biobutanol is widely recognised as a superior biofuel to ethanol, in terms of energy content, ease of distribution, versatility and applications. However, the strains of bacteria currently used to produce biobutanol generate unwanted by-products and are inefficient. *Clostridium acetobutylicum* is able to convert starch to butanol but yields are three times less efficient than for ethanol production. Currently Clostridia form spores when the butanol levels get too high and fermentations need to be carried out in the absence of oxygen, making the development of an easy to manage industrial process harder.

Bioethanol is produced by fermentation of sugars by yeast or *Escherichia coli*. The bacterium *Zymomonas mobilis* is a promising alternative to yeast due to its greater sugar uptake, yields and resistance to ethanol concentrations. Neither yeast nor bacteria are capable of efficiently fermenting pentose sugars (monosaccharides with five carbon atoms) in comparison to their ability to ferment hexose sugars (monosaccharides with six carbon atoms) such as glucose. Research to modify their metabolism to provide this ability may enable the fermentation of lignocellulosic derived sugars in the future.

Experimental industrial scale bioreactors

Moreover, they are unable to utilise lignocellulose directly as a feedstock. Research aims to generate bacterial strains, using synthetic biology, which can efficiently utilise lignocellulose to produce butanol and ethanol. Once developed, these strains must be tested for their ability to work on an industrial scale in an environmentally friendly and sustainable process using non-food biomass.

Researchers have identified the gene for breaking down lignin in a soil-living bacterium called *Rhodococcus jostii.* Although such enzymes have been found in fungi, bacteria can be modified more easily to produce large amounts of the required enzyme. In addition, bacteria are quick and easy to grow, making it easier to produce enzymes which can break down lignin on an industrial scale. There is hope that similar enzymes can be found in bacteria which live in very hot environments such as near volcanic vents. Enzymes in these bacteria have evolved to work best at high temperatures meaning they are ideally suited to be used in industrial processes.

Activity 2D - Bacterial cellulase

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

- Describe the use of cellulose in paper and sources of naturally produced cellulases.
- Carry out an experiment to investigate the presence of cellulase producing bacteria in soil.
- Assess the pros and cons of the method for identifying cellulase producing bacteria.

Keywords Bioenergy, biofuel, sustainable, renewable, biomass, yield, waste, cellulose, cellulase, lignocellulose, microbes, yeast, enzyme, fermentation, varieties, bioprospecting.

Background

The production of cellulases by bacteria can be investigated by sourcing bacteria such as *Cellulomonas sp*. which produces extracellular cellulase or *Pseudomonas flourescens*, or testing samples of soil, and incubating them in nutrient broth with paper as a source of cellulose. Bioprospecting involves searching in suitable environments for organisms that have beneficial features for producing biofuels. Researchers focus on agricultural and forestry microecosystems where fermentation is taking place such as manure, leaf litter, rotting wood and straw rich soil. Once researchers isolate yeast, bacteria or filamentous fungi, they can create a profile of the phenotype by testing their ability to ferment different biofuel feedstocks and resist pretreatment conditions.

In this activity students can carry out their own bioprospecting to see if they can discover cellulase producing bacteria and test their ability to breakdown the cellulose in paper. This activity is based on one from the Society of General Microbiology (SGM) publication Practical Microbiology for Secondary Schools.

Age Range: This experiment is suitable for primary and secondary students.

Duration: set up 30 minutes, incubation 1-2 weeks.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of microbes, soil constituents, enzymes, the carbon cycle, decomposition and ecosystems.

What you will need

- Conical Flasks
- Test tubes
- Test tube rack
- Pipettes
- Nutrient broth
- Paper samples cut into strips
- Cotton wool
- Soil
- Nitrile gloves
- Autoclave
- Eye protection

Optional

- *Cellulomonas sp.*

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Health and Safety

Students should use aseptic technique if culturing the amples themsleves. Use disposable pipettes or autoclave the pipettes after the activity. Immeditaley following use students should put them in 1% Virkon. Ensure hands are washed following this activity. Be aware of the risk of inadvertently culturing pathogenic microorganisms and treat this activity as if potentially harmful microorganisms could be cultured from the soil samples. Do not allow students to open the test tubes once they have been incubated. Paper samples should only be observed inside the test tubes while recording results. The samples and equipment used should be sterilised at 121°C for 15 minutes prior to disposal.

The following factors should be considered when planning to carry out any investigations involving microorganisms: nature of the organism used, source of the organism, temperature of incubation, culture medium used, type of investigation and the facilities available, chance of contamination, expertise of people involved. If necessary change the conditions or limit the involvement of students perhaps by carrying out the experiment as a demonstration. CLEAPSS® handbook - "perfectly safe if the organisms studied are known to be non-pathogenic, such as brewer's and baker's yeast, the bacteria in yoghurt or edible mushrooms".

CLEAPSS® laboratory handbook – section 15.2 Microbiology (COSHH, good practice and safety precautions, levels of practical work, using microorganisms in practical work, equipment and materials, sterilisation and disinfection) page 1505.

CLEAPSS® Guidance PS 04 (COSHH: risk assessments in situations where microorganisms might be involved).

CLEAPSS® guides R101 (Steam sterilisation: Autoclaves & pressure cookers).

CLEAPSS® Model risk assessment 3.026 (Microorganisms used in food production).

Further advice can also be sought from the Society for General Microbiology and the Microbiology in Schools Advisory Committee.

1% VirKon is a suitable disinfectant for general surface cleaning and sterilisation as well as for discard pots (follow manufacturer's instructions).

For Gram staining and preparation of slides see CLEAPSS® Guidance leaflet 95, Recipe sheet 90 and Hazcards 32, 36A, 40A and 85.

Method

1. Make up nutrient broth in a conical flask and autoclave.
2. Collect soil samples or obtain a sample of *Cellulomonas.*
3. Set up test tubes as below and label with contents, name and date.

					Control
Nutrient	✔	✔	✔	✔	✔
Soil or Cellulomonas	✔	✔	✔	✔	✘
Paper	Newspaper	Filter paper	Rice paper	Glossy paper	Newspaper

4. Add 5 ml of nutrient broth to the control tube using a pipette, and seal.
5. Add the soil sample to 30 ml of nutrient broth in a conical flask. Swirl the flask to form an evenly distributed soil suspension and then allow the particulate debris to settle for 1-2 minutes.
6. Add 5 ml of the soil suspension to each test tube using a pipette.
7. Add the paper samples to the tubes and seal the tubes.
8. Incubate the test tubes for about 1 week at room temperature. Ensure the test tubes are securely stored so that students cannot access and open them while they are being incubated
9. Autoclave the test tubes at 121°C for at least 15 minutes before allowing students to examine them. Discard the test tubes shortly after examination. If the tubes are opened and left for more than a few hours they will require sterilisation again.

Extension activities

If you can source cellulase producing bacteria such as *Cellulomonas sp.* for the students, or students identify a particularly effective source of cellulase-producing microorganisms, their ability to resist lignocellulosic bioethanol pretreatment conditions can be investigated. Bacteria could be exposed to varying temperatures or alkali treatments prior to repeating the test with different types of paper. If exposing bacteria to varying temperatures, ensure that they are exposed for a short duration and that temperatures above 30°C are not used to avoid growing any potentially pathogenic microorganisms.

To demonstrate the presence of bacteria and microorganisms in the soil samples students could examine samples under the microscope. Students can create a soil suspension with water and then visualise the bacteria, by using a technique such as Gram staining. Alternatively students could add cultures of *Cellulomonas* bacteria to paper laid on to the surface of a sterile nutrient agar plate. This should be done using aseptic technique. The students can then observe the paper a few days later for sugns of hydrolysis using a microscope with a 10x objective without opening the dishes.

Suppliers

A variety of bacteria including cellulase producing *Cellulomonas* on agar slopes can be obtained from National Centre for Biotechnology Education (NCBE) www.ncbe.reading.ac.uk/menu.html University of Reading, 2 Earley Gate, Whiteknights Road, Reading RG6 6AU tel: 0118 9873743, fax: 01189 750140 or *Pseudomonas flourescens* on agar slopes can be obtained from Blades Biological Limited www.blades-bio.co.uk Cowden, Edenbridge, Kent TN8 7DX tel:01342 850 242, fax: 01342 850 924

Further reading and links

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Engineering Synthetic Microbial Communities for Biomethane Production - VIDEO www.bbsrc.ac.uk/news/industrial-biotechnology/2014/140717-n-synthetic-microbial-communities-biomethane.aspx

Bugs produce diesel on demand [Reference/webpage no longer available – January 2017]

First wood-digesting enzyme found in bacteria could boost biofuel production

Cows' stomachs could hold key to green fuels www.roslin.ed.ac.uk/news/2011/07/29/cows%27-stomachs-could-hold-key-to-green-fuels/

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

Research groups

Professor Nigel Peter Minton, BSBEC Second Generation, Sustainable, Bacterial Biofuels Programme, School of Molecular Medical Sciences, The University of Nottingham, Nottingham, NG7 2RD

Professor Katherine Smart, BSBEC LACE Programme, School of Biosciences, University of Nottingham, Sutton Bonington Campus

Biofuel Research Centre, Edinburgh Napier University www.napier.ac.uk/bfrc

Professor Timothy Bugg, Department of Chemistry, University of Warwick www2.warwick.ac.uk/fac/sci/chemistry/research/bugg/bugggroup/research/

Professor Frank Sargent, Molecular Microbiology, College of Life Sciences, University of Dundee www.lifesci.dundee.ac.uk/groups/frank_sargent/

Professor David Archer, BSBEC LACE programme Strand 2, University of Nottingham

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Professor Soyer, University of Warwick http://osslab.lifesci.warwick.ac.uk/adLola.html

Professor John Love, Biosciences, University of Exeter http://biosciences.exeter.ac.uk/staff/index.php?web_id=john_love&tab=profile

Activity 2E - Cellulase enzyme activity

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

- Describe the breakdown of cellulose by cellulases and cellulase producing microbes.
- Carry out quantitative assays of enzyme activity.
- Assess the relative merits of immobilised cellulases and microbe produced cellulases.

Keywords Bioenergy, biofuel, sustainable, renewable, biomass, yield, waste, bioethanol, lignocellulose, cellulase, microbes, yeast, bacteria, gribbles, enzyme, varieties.

Background

In this practical the activity of cellulase enzyme is assessed using viscosity reduction. It is recommended that four sources of cellulase are compared: fruit extracts, commercially sourced cellulase, yeast and bacteria (immobilised in sodium alginate or on sterile paper discs). If yeast and bacteria are to be tested it is essential that strains producing cellulase are used. If enzymes and microbes cannot be obtained the experiment can easily be carried out comparing fruit sources of cellulase. During the ripening of some fruits cellulases are produced that break down the cellulose in cell walls causing softening.

This activity is based on the Science and Plants for Schools activity - Cellulase assays. A similar activity investigating starch and bacteria rather than cellulose called *Breakdown of starch by microbes* published in *Practical Microbiology for Secondary Schools* , Society for General Microbiology, may also be carried out.

Health and Safety

Care should be taken with enzymes particularly due to their allergenic nature and ability to act as sensitisers. CLEAPSS® Recipe book RB37 (Enzymes), Hazcard 33 (Enzymes), Guide 3.015 (Enzymes), Laboratory handbook page 1441-1443. Solutions equal to or stronger than 1% (w/v) should be labelled as irritant.

The following factors should be considered when planning to carry out any investigations involving microorganisms: nature of the organism used, source of the organism, temperature of incubation, culture medium used, type of investigation and the facilities available, chance of contamination, expertise of people involved. If necessary change the conditions or limit the involvement of students perhaps by carrying out the experiment as a demonstration.

CLEAPSS® laboratory handbook – section 15.2 Microbiology (COSHH, good practice and safety precautions, levels of practical work, using microorganisms in practical work, equipment and materials, sterilisation and disinfection) page 1505.

CLEAPSS® Guidance PS 04 (COSHH: risk assessments in situations where microorganisms might be involved)

CLEAPSS® Model risk assessment 3.026 (Microorganisms used in food production).

Further advice can also be sought from the Society for General Microbiology and the Microbiology in Schools Advisory Committee.

1% VirKon is a suitable disinfectant for general surface cleaning and sterilisation as well as for discard pots (follow manufacturer's instructions).

Ensure you use a wallpaper paste that does not have fungicides.

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Viscosity reduction method

Cellulase enzymes degrade cellulose fibres by cleaving glucose molecules predominantly from the ends of the polysaccharide chains. Wallpaper paste provides a source of cellulose fibres that can be degraded by cellulases resulting in a change in viscosity. This property can be exploited to set up a quantitative assay of cellulase activity. By loading samples of wallpaper paste mixed with a source of cellulase into a syringe and measuring the time taken for it to run out of the syringe a quantitative measure of cellulase activity can be made.

Age Range: This experiment is suitable for secondary and post-16 students.

Duration: 60 minutes.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of microbes, enzymes, carbohydrates and the properties of solids and liquids.

What you will need

- Boiling tubes
- Wallpaper paste (without fungicide)
- Syringe
- Retort stand
- Beaker
- Stirrers
- Water bath
- Timer
- Fruit extract

Method

1. Make up a 2% (w/v) wallpaper paste solution, sufficient to provide 25 ml for each sample to be tested.
2. Place 25 ml of the paste in a boiling tube and add 2 to 5 ml of fruit extract. Mix thoroughly.
3. Then draw up the mixture into the barrel of a syringe and clamp in a retort stand, pointing downwards into a small beaker. Note the time taken for all the mixture to drain through the syringe nozzle into the beaker.
4. Incubate the fruit or enzyme-wallpaper paste mixture at different temperatures, such as in a water-bath at 30°C, allow to return to room temperature and repeat the investigation, checking the change in viscosity.
5. To speed up the investigation for students, provide samples that have been incubated prior to the lesson or assign students to groups to investigate different variables.

The more active the enzyme the greater the reduction in viscosity, and so the shorter the drainage times. If investigating cellulase production by microbes you may need to provide nutrients and carry out an overnight incubation in the wallpaper paste prior to assessing the viscosity.

Extension activities

Effects on the zone of destruction can be tested under different conditions such as temperature and pH. Natural sources of enzyme have some advantages and if using commercial sources to investigate the effects of pH or temperature, check that they are not heat stable or have unusual pH profiles. Samples can be taken from the wallpaper paste prior to and following degradation to quantify the levels of sugar produced and evaluate the efficiency of the enzymes for producing fermentable sugars.

Advanced Biofuels - Bacterial Biofuels

Suppliers

A variety of bacteria, including cellulase producing *Cellulomonas,* on agar slopes can be obtained from National Centre for Biotechnology Education (NCBE) www.ncbe.reading.ac.uk/menu.html University of Reading, 2 Earley Gate, Whiteknights Road, Reading RG6 6AU tel: 0118 9873743 fax: 01189 750140 or *Pseudomonas flourescens* on agar slopes can be obtained from Blades Biological Limited www.blades-bio.co.uk Cowden, Edenbridge, Kent TN8 7DX tel:01342 850 242, fax: 01342 850 924

Whatman paper discs can be obtained from Sigma-Aldrich.

Further reading and links

King, A.J. *et al*, 2010 Molecular insight into lignocellulose digestion by a marine isopod in the absence of gut microbes. *PNAS*,**107**(12, 5345-5350.

Cellulase assays, Science and Plants for Schools (SAPS) www.saps.org.uk/students/projects/179-student-project-enzymes-and-their-activity-in-fruits-and-vegetables

Breakdown of Starch by microbes, *Practical Microbiology for Secondary Schools*, Society of General Microbiology [Reference/webpage no longer available – July 2016]

Seafarers' scourge provides hope for biofuel future

First wood-digesting enzyme found in bacteria could boost biofuel production

Cows' stomachs could hold key to green fuels www.roslin.ed.ac.uk/news/2011/07/29/cows%27-stomachs-could-hold-key-to-green-fuels/

Breakdown of starch by microbes published in *Practical Microbiology for Secondary Schools,* Society for General Microbiology

Glucoamylase production by yeast, 1993, National Centre for Biotechnology Education (NCBE). [Reference/webpage no longer available – October 2016]

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2.

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

Research groups

Professor Simon McQueen-Mason, BSBEC Marine Wood Borer Enzyme, Discovery Programme, The University of York, Heslington, York YO10 5DD

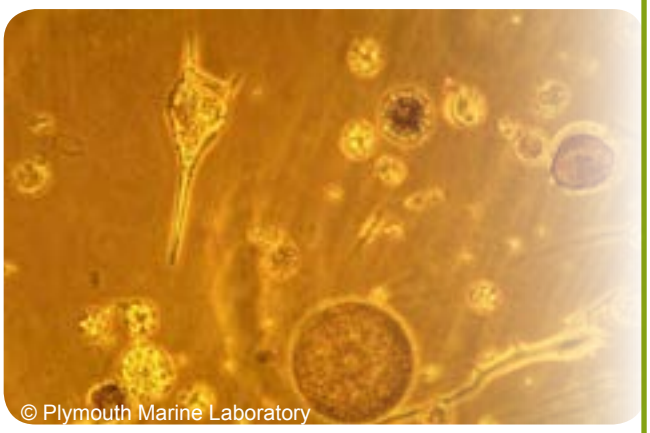
Professor Paul Dupree, BSBEC Cell Wall Sugars Programme, Department of Biochemistry, University of Cambridge

Professor Timothy Bugg, Department of Chemistry, University of Warwick www2.warwick.ac.uk/fac/sci/chemistry/research/bugg/bugggroup/research/

Advanced Biofuels - Algal Biofuels

Keywords

Bioenergy, biofuel, biodiesel, biogas, sustainable, renewable, biomass, yield, waste, microbes, enzyme, photosynthesis, algae, varieties, unicellular, multicellular, eukaryotic, oil, carbon dioxide, biodiesel, hydrocarbons, carbohydrates, carbon partitioning, bioprospecting, somatic fusion, hybrid, heterokaryon, directed evolution, synthetic biology, genetic modification.



Algae

Background

Algae are a diverse group of eukaryotic photosynthetic organisms that constitute over 40,000 species. They can be single-celled (unicellular) or multicellular such as seaweed. Microalgae have been described as nature's very own power cells and could provide alternatives to petroleum-based fuels without competing with crops.

Algae can harvest the power of the sun through photosynthesis and convert this into biomass including oil. Many species are fast growing and efficient at absorbing carbon dioxide (CO_2), being more productive than land plants per unit area. This makes them an important part of the carbon cycle and they are able to produce complex molecules, such as hydrocarbons and carbohydrates, including cellulose, proteins, fats and oils, from the carbon dioxide they absorb. How algae and plants convert carbon dioxide into different molecules, known as carbon partitioning, is of great interest to researchers. Research is being undertaken to uncover novel microalgal compounds that could provide alternatives to those from petrochemical sources. Algae produce more oils when they are starved of nitrogen but we don't yet understand why this should be. Uncovering the metabolic mechanisms behind such behaviour will be important if we want to harness the full potential of algae.

There are a wide range of bioenergy products that can be obtained from culturing algae including biomass for combustion to produce heat and electricity, fermentation to produce bioethanol, biobutanol or biogas, oil for conversion to biodiesel or even possibly algal synthesised biodiesel.

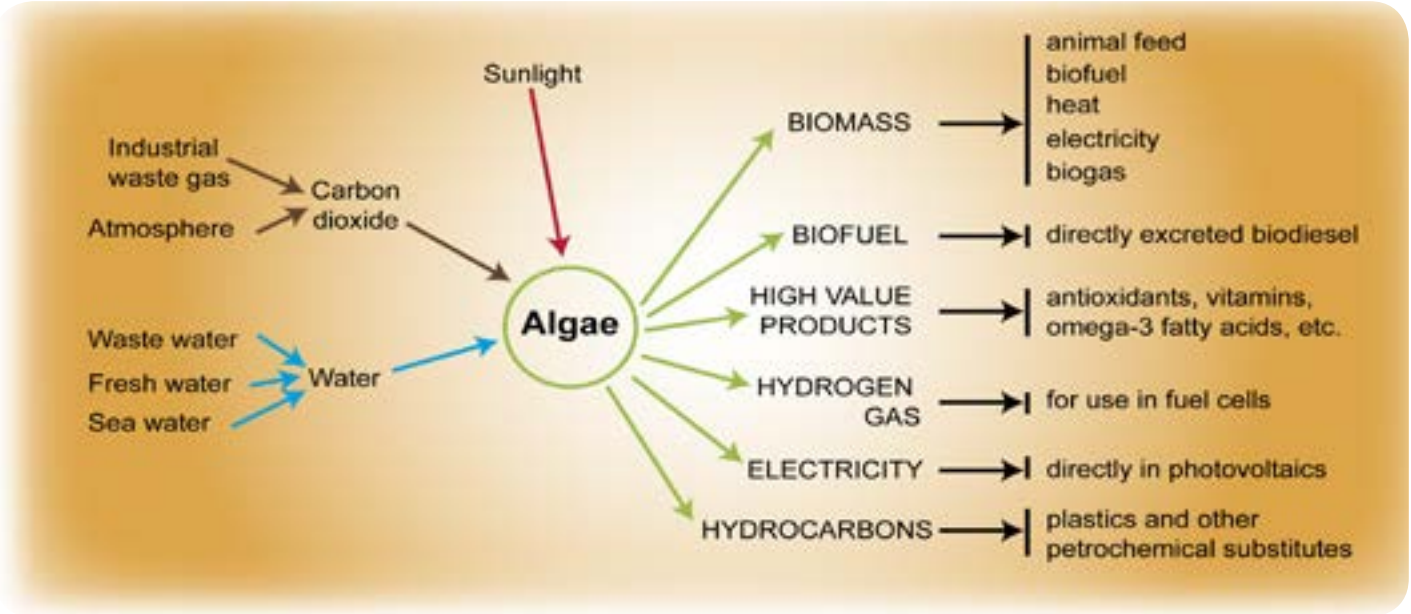
As well as producing hydrocarbons that can be converted into fuels or plastics some microalgae have unique abilities such as being able to produce hydrogen gas which can be used in fuel cells to produce electricity. Others, such as cyanobacteria, might one day be used in solar panels to generate electricity directly. Algae can grow in very nutrient rich environments that are toxic to other plants so they could be used for treating 'waste waters', from a range of industrial sources.

SEM image of algae

Unlike land plants microalgae produce only one cell type and don't divert resources into multicellular structures such as flowers, roots or vasculature, and so they grow much more quickly than land plants. Microalgae can be grown in large bioreactors and continually harvested unlike crops or macroalgae. They could be grown using the waste CO_2 from industrial processes, power stations or waste treatment plants. The oil they produce can then be converted into liquid fuel such as biodiesel. The ability of microalgae to capture industrial CO_2 emissions as their source of carbon for growth and be cultivated on non-agricultural land or in the sea reducing their competition with food crops for land, makes them an attractive proposition both economically and sustainably. Unfortunately, the culture of algae on a large commercial scale (mainly for biomass for aquaculture or specialised products such as natural food colourants, omega-3 oils and antioxidants) has so far been restricted to sunny climates, and mainly to those species that are tolerant to extreme environments such as high light or saline conditions.

Advanced Biofuels - Algal Biofuels

In order to develop biofuels from algae, research is being conducted to find suitable strains that produce high levels of oils, can tolerate heat and high concentrations of carbon dioxide, and are easy to harvest. Some of these strains may well be grown using bubble columns and photobioreactors in conjunction with CO_2 from flue gas emissions.



The strains of algae eventually used for processes outside of the research lab will depend on many factors: economics, engineering practicalities, overcoming scientific barriers, adoption of industrial standards, local planning applications, government incentives, social acceptance and more. Depending on the outcome of research and experimental pilot plants it is feasible that many of the individual algal applications can be combined in one facility such as waste water treatment, energy generation, animal feed production and the removal of carbon dioxide. The algae may be cultured in self-contained bioreactors, in open-air ponds or harvested from the environment. The process of harvesting algae is currently a significant challenge to obtaining economical yields. The problems associated with culturing and harvesting differ in bioreactors, marine environments or large ponds. The UK has a relatively cold climate, slowing growth of algae and reducing productivity, however, waste heat from industrial activity could be used to warm ponds and thereby increase growth rates.

There may well be naturally occurring algae that can perform many of the tasks that we might want and researchers carrying out bioprospecting hope to identify suitable strains by selective screening. When growing algae in open systems it is inevitable that the ponds will get contaminated with algae from the environment, so many developers of algal technologies are hoping to harness these environmental algae as the main source of the algal biomass in their ponds. Strain selection will be key to successfully developing algal derived biofuels.

There are a number of methods for developing suitable strains of algae, including breeding, somatic fusion, genetic modification, synthetic biology and directed evolution. Breeding algae is theoretically possible but currently faces significant scientific challenges, such as identifying why algal species possess the genes for sexual reproduction but are only observed to reproduce asexually in the lab. Therefore, alternative techniques such as somatic fusion and synthetic biology are being investigated. Somatic or protoplast

fusion involves combining the cells of different strains of algae. The technique has been used on plants and yeast and researchers are now investigating if stable algal hybrids can be created using this technique. This somatic breeding approach is particularly attractive since it allows the creation of novel strains by crossing species boundaries and exploiting the diversity found amongst the microalgae, but without using GM technology, which is currently rather limited for algae. Nevertheless, research is being conducted to develop methods for genetic modification to introduce desirable traits into algae, and synthetic biology approaches to re-engineer algal cells. One further option would be to use a technique called directed evolution. Here many algae are subjected to conditions that cause their DNA to change very slightly, the change in each individual alga's DNA will be subtlety different from the changes in any other alga's DNA. The algae are then selected based on some condition, perhaps how much oil they produce, and these selected 'best' algae then go through the process again. So in a gradual stepwise fashion algae that are 'better' at producing oils are selected – in a process analogous to evolution.

Another key issue faced in the development of algal biofuels is harvesting. The oil can be collected from algae in a variety of ways but may involve growing algae in batches rather than continuously. Separating the algae from culture, concentrating the algae, drying algae, extracting oils mechanically or chemically and recycling the nutrients and water to reduce waste, all present difficulties and potential energy costs. Some strains of algae, such as *Scenedesmus*, form thick sediments whereas others are extremely small or capable of moving. These characteristics influence the efficiency and methods used for harvesting algae.

Advanced Biofuels - Algal Biofuels

Activity 3A - Culturing algae

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

- Describe the requirements for algal growth.
- Culture algae in flasks or on agar.
- Compare the effects of growing conditions on algae and the growth of different species.
- Discuss the difficulties of growing algae in large quantities for biofuel production.

Keywords Bioenergy, biofuel, biodiesel, sustainable, renewable, biomass, yield, culture, photosynthesis, algae, varieties, photobioreactor.

Background

There are a wide range of bioenergy products that can be obtained from culturing algae including biomass for combustion to produce heat and electricity, fermentation to produce bioethanol, biobutanol or biogas, oil for conversion to biodiesel or even possibly algal synthesised biodiesel. The algae may be cultured in self-contained bioreactors, in open-air ponds or harvested from the environment. Microalgae can be grown in large bioreactors and continually harvested unlike crops or macroalgae. They could be grown using the waste carbon dioxide (CO_2) from industrial processes, power stations or waste treatment plants. Algae can grow in very nutrient-rich environments that are toxic to other plants so they could be used for treating 'waste waters', from a range of industrial sources. The ability of microalgae to capture industrial CO_2 emissions as their source of carbon for growth and be cultivated on non-agricultural land or in the sea reducing their competition with food crops for land, makes them an attractive proposition both economically and sustainably.

Photobioreactor

Unfortunately, the culture of algae on a large commercial scale has so far been restricted to sunny climates and produces either biomass for aquaculture or specialised products such as natural food colourants, omega-3 oils and antioxidants. The UK has a relatively cold climate, slowing growth of algae and reducing productivity, however, waste heat from industrial activity could be used to warm ponds and thereby increase growth rates.

The problems associated with culturing and harvesting algae differ in bioreactors, marine environments, greenhouses or large ponds. In order to develop biofuels from algae, research is being conducted to find suitable strains that produce high levels of oils, can tolerate heat and high concentrations of carbon dioxide, and are easy to harvest. Some of these strains may well be grown using bubble columns and photobioreactors in conjunction with CO_2 from flue gas emissions. When growing algae in open systems it is inevitable that the ponds will get contaminated with algae from the environment. Therefore scientists are trying to identify natural strains that can compete in the environment whilst also considering the potential environmental impacts of growing large numbers of algae.

Algae can be cultured in solutions or on solid media such as agar. Algae require specific nutrients, just like plants and other organisms, to grow well. In this activity students can set up and grow algae on solid media or in culture. This activity may take up to 6 weeks and would be best set up at the start of a topic or term, alternatively cultures can be prepared in advance for students to compare. Depending on the facilities available, and the variables students investigate, a variety of approaches to culturing the algae may be appropriate. If investigating the nutrients and concentrations that are optimal for algal growth a recipe for

micronutrient solution is provided and can be adjusted by changing the amounts or omitting minerals. For simpler experiments a prepared nutrient mix containing the minerals required by algae is available from Sciento. Alternatively liquid plant food or fish fertiliser will do, though these will be lacking the trace elements needed by algae. The algae can be cultured in anything from sterile conical flasks to used drinks bottles, or even in a photobioreactor.

Suitable algae and microorganisms to culture include:

Scenedesmus quadricauda. These algae form colonies typical of four cells and have no means of propulsion. They are hardy and ideal for investigating photosynthesis.

Chlorella vulgaris. Single-celled spherical algae with no means of propulsion through the water. These tiny cells are some of the world's smallest plants at 2-12 µm in diameter. Often found in aged tap water.

Euglena gracilis. Single-celled microorganism capable of photosynthesis and featuring flagella to propel itself towards the light. Up to 60 µm in length and often found in ponds or farmyard puddles.

Pinnularia nobilis. Very small single celled alga about 3 µm in diameter. Part of the group of algae known as diatoms. They are nutrient rich and contain up to 11% oil that enters the food chain and is concentrated in fish livers (cod-liver oil).

The cultured algae can be displayed at science fairs and are most easily transported on agar plates. A large range of conditions can be demonstrated and colonies of algal growth can be observed.

Age Range: These experiments are suitable for primary and secondary students.

Duration: 60 minutes to set up, 2-5 weeks to culture.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of microbes, photosynthesis and plants. An understanding of the nutrients and conditions required for plant growth and photosynthesis would help students plan investigations.

Algae on agar plates

What you will need

- Varieties of unicellular algae
- Petri dishes
- Agar
- BG11 solution (recipe below)
- A5 trace metal solution (recipe below)
- Distilled water
- Pipettes
- Measuring cylinder
- Conical flasks
- Bunsen burner
- Heatproof mat
- Streaking loops
- Light source or north facing window sill

Optional

- Prepared culture media
- Liquid plant food

Health and Safety

If carrying out culture of algae with primary pupils it is recommended that the following precautions are taken. Prepare culture media solutions or liquid plant food and dilute to the working concentration (according to the manufacturer's instructions) in advance. Use sterile disposable Pasteur pipettes to add 1 ml of algal suspension to the plates and swirl the plate to distribute the algae evenly. Take care with powdered nutrient algae medium (HARMFUL, IRRATATING, OXIDISING, avoiding contact with skin and eyes. If using artificial lighting ensure flourescent lighting is stable and cooland warn those with epileps about flickering lights.

The following factors should be considered when planning to carry out any investigations involving microorganisms: nature of the organism used, source of the organism, temperature of incubation, culture medium used, type of investigation and the facilities available, chance of contamination, expertise of people involved. If necessary change the conditions or limit the involvement of students perhaps by carrying out the experiment as a demonstration.

CLEAPSS® laboratory handbook – section 14.9 Fermenters (Safety, Practical considerations page 1443-1451, section 15.2 Microbiology (COSHH, good practice and safety precautions, levels of practical work, using microorganisms in practical work, equipment and materials, sterilisation and disinfection) page 1505, section 15.5 Plants and seeds (choosing suitable plant material, growing and cultivating plants, sources and suppliers of plants pages 1540-1567

CLEAPSS® Recipe book RB93 (Stains for plant material).

CLEAPSS® Guidance G5p (Using chemicals safely, PS 04 (COSHH: risk assessments in situations where microorganisms might be involved).

Practical Fermentation – A guide for Schools and Colleges. 1999. National Centre for Biotechnology Education (NCBE) and Society of General Microbiology (SGM). [Reference/webpage no longer available – October 2016]

Burdass, D., Grainger, J.M. and Hurst, J. (editors) 2006, Basic Practical Microbiology – A Manual and Grainger, J.M. and Hurst, J. (editors) 2007, Practical Microbiology for Secondary schools available free from the Society for General Microbiology (SGM)

Further advice can also be sought from the Society for General Microbiology and the Microbiology in Schools Advisory Committee.

Method

This activity can be carried out with ordinary agar. Some algae are sensitive to impurities present in agar and researchers over come this by a process of washing agar.

1. Add the BG11 2x base (it is 2x, therefore dilute this 1:2) to the agar in conical flasks, stopper with foam bungs or non-absorbent cotton wool, foil and autoclave.
2. After autoclaving, while still warm enough that the agar can be poured, add A5 (1:1000).
3. Pour plates.
4. Using aseptic technique streak plates with algal suspension, seal the plates and place under a light source or on a north facing window sill. With primary pupils use sterile disposable Pasteur pipettes and add 1 ml of suspended algae.
5. Algae will grow best at room temperature (18-22°C) under constant fluorescent illumination.
6. If adjusting the conditions, try different light sources and temperatures ensuring only one variable is changed at a time, e.g. consider that if growing algae in the fridge an equivalent light source will be needed to the algae grown outside the fridge.

For liquid cultures just omit the agar.

BG11 – 2x base:	
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
$NaCO_3$	0.02 g

Make up to 500 ml with distilled H_2O and autoclave. The pH should be 7.1 after sterilisation.

Trace metal A5_1000x mix:	
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L

Autoclave the final solution.

Algae in solution

What you will need

- Varieties of unicellular algae
- BG11 solution (recipe above)
- A5 trace metal solution (recipe above)
- Distilled water
- Pipettes
- Measuring cylinder
- Conical flasks
- Bunsen burner
- Light source or north-facing window sill

Optional

- Prepared culture media
- Liquid plant food
- Shaker
- Light bank
- Aquarium air pump
- Graticule
- Densitometer
- Cafetiére
- Coffee filter and filter papers

Method

1. Make up the nutrient solution in conical flasks If using the BG11 2x base dilute this 1:2, stopper with foam bungs or non-absorbent cotton wool, foil and autoclave.
2. After autoclaving, while still warm enough that the agar can be poured, add A5 (1:1000).
3. Using sterile technique add equal inoculations of algae to each culture, 5-10 ml into 250 ml culture media should be sufficient. Swirling or inverting the algae prior to inoculation will distribute the algae equally.
4. Seal the flasks and place under a light source or on a north-facing window sill.
5. Algae will grow best at room temperature (18-22°C) under constant fluorescent illumination with agitation and added carbon dioxide. If a light bank and shaker are available these can provide the light and mixing required. Aeration can be easily achieved with an aquarium pump and is significantly safer and cheaper than a CO_2 canister.
6. Under constant illumination the density of algae in the culture should reach its maximum in 3-5 weeks.
7. If adjusting the conditions, try different light sources and temperatures ensuring only one variable is changed at a time, e.g. consider that if growing algae in the fridge an equivalent light source will be needed to the algae grown outside the fridge.

Extension activities

Post-16 students could monitor the growth of the algae by measuring algal density through cell counts in addition to recording the colour of the culture. Use microscopes, slides and cover slips or a counting chamber to do cell counts of the cultures over time. Ensure students record the data and create graphs of cell density over time.

Compare methods of cell number quantification using microscopes or densitometers.

Compare culture media using different concentrations of nutrients or omitting certain nutrients. Date can be recorded as cell density versus nutrient concentration for each sample time. Graphs can then be produced for each nutrient treatment.

As well as adjusting the light and temperatures the algae are cultured in, try replicating extreme environments and assessing the effect of salinity on algal growth. Create graphs of final cell density versus temperature or salinity.

The algal culture may also be set up as a bioreactor with recording of pH and temperature with data logging software. If setting the algal culture up this way try to ensure that the light source does not transfer too much heat to the culture. It may be worthwhile setting up a control culture without algae, if relying on sunlight, to monitor any difference in temperature.

Try different techniques of harvesting the biomass, dry it and measure the mass to assess the growth rates and yield. Cafetiéres and coffee filters can be easily obtained and used to separate the algae from the culture medium.

Visualise algae under the microscope, staining for cellulose, lignin or lipid content see activity 2A - plant material testing-for more detail.

Suppliers

Algae and culture media can be obtained from Sciento, www.sciento.co.uk/ 61 Bury Old Road, Whitefield, Manchester, M45 6TB tel: 0161 773 6338 fax: 0161 773 6338

Algae can also be obtained from Blades Biological Limited www.blades-bio.co.uk Cowden, Edenbridge, Kent, TN8 7DX tel:01342 850 242 fax: 01342 850 924.

Bioreactors can be obtained from National Centre for Biotechnology Education (NCBE) www.ncbe.reading.ac.uk/menu.html University of Reading, 2 Earley Gate, Whiteknights Road, Reading RG6 6AU tel: 0118 9873743 fax: 01189 750140

Further reading and links

Pittman, J., Dean, A. and Osundeko, O, 2011. The potential of sustainable algal biofuel production using wastewater resources. *Bioresour Technol*, **102**(1, 17-25.

Science and Plants for Schools (SAPS) www.saps.org.uk/ Cambridge University Botanic Garden 1 Brookside, Cambridge CB2 1JE tel: 01223 748455, saps@hermes.cam.ac.uk

Microbial Discovery Activity Effect of Nitrate and Phosphate levels on the Growth of Algae American Society for Microbiology, Education Department, 1752 N Street, NW, Washington, DC 20036 EducationResources@asmusa.org

Making Algae (Phytoplankton Grow, The Feeding Frenzy: Seasonal Upwelling Teaching Box, Digital Library for Earth System Education (DLESE)

Synthetic biology, green algae and seaweed provide promise for sustainable fuels of the future [Reference/webpage no longer available – January 2017]

Towards the quintessential green technology, BBSRC business, Autumn 2010.

Scientists aim to improve photosynthesis to increase food and fuel production

www.biomara.org/
www.walesonline.co.uk/news/uk-news/2010/03/08/seaweed-the-green-fuel-of-the-future-for-our-cars-91466-25983211/

How to make an algae test photobioreactor www.instructables.com/id/How-To-Make-an-Algae-Photo-BioreactorPart-One/

Algal Research in the UK: A Scoping study for BBSRC, July 2011.

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2.

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

Advanced Biofuels - Algal Biofuels

Research groups

Professor Alison Smith, Department of Plant Sciences, University of Cambridge

The Algal Bioenergy Consortium

Professor Johnathan Napier, Rothamsted Research www.rothamsted.ac.uk

Dr Saul Purton, University College London www.ucl.ac.uk/biology/academic-staff/purton/purton.htm

Dr Sohail Ali, Plymouth Marine Laboratory www.pml.ac.uk/about_us/pml_people/sohail_ali.aspx

Carole Llewellyn, Plymouth Marine Laboratory

Dr Jon Pittman, University of Manchester, Faculty of Life Sciences, Michael Smith Building, Oxford Road, Manchester, M13 9PT. Utilisation of microalgae for sustainable biotechnology.

Dr D Jim Gilmour, Microbial Physiology of Extremophiles, University of Sheffield

BioMara project at The Scottish Association for Marine Science, Dunstaffnage marine laboratory, Oban www.sams.ac.uk

Culture Collection of Algae and Protozoa (CCAP) national algae collection www.ccap.ac.uk

Activity 3B - Algal photosynthesis

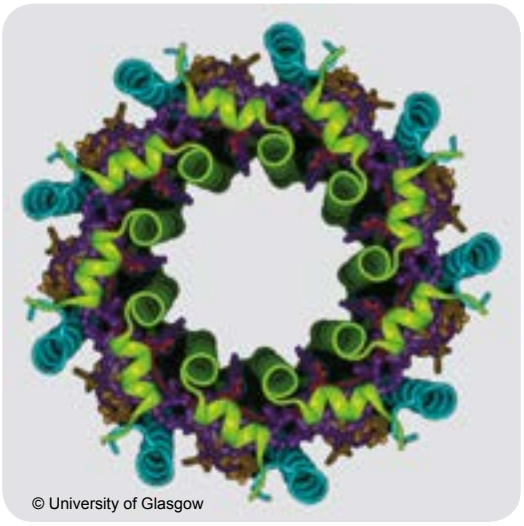
Learning objectives: By the end of the session students should be able to:

- Describe the requirements of photosynthesis.
- Take measurements to assess the rate of photosynthesis under varying conditions.
- Evaluate the benefits of producing biofuels from algae and the conditions required.

Keywords Bioenergy, biofuel, biodiesel, sustainable, renewable, biomass, yield, waste, photosynthesis, carbon dioxide, algae, varieties, photobioreactor.

Background

Algae are a diverse group of eukaryotic photosynthetic organisms that constitute over 40,000 species. They can be single-celled (unicellular) or multicellular such as seaweed. Microalgae have been described as nature's very own power cells and could provide alternatives to petroleum-based fuels without competing with crops.



Light Harvesting complex 2

Algae can harvest the power of the sun through photosynthesis and convert this into biomass including oil. Many species are fast growing and more productive than land plants. This makes them an important part of the carbon cycle and they are able to produce complex molecules, such as hydrocarbons and carbohydrates, including cellulose, proteins, fats and oils, from the carbon dioxide they absorb. How algae and plants convert carbon dioxide into different molecules, known as carbon partitioning, is of great interest to researchers who aim to develop algae that can produce the substances we require. Photosynthesis is only 6% efficient and it may be possible to improve this to produce faster growing algae, higher yielding plants or develop novel ways of capturing solar energy.

For efficient and economic production of algal biofuels a number of conditions are required and suitable algae varieties need to be identified and developed. The UK has a relatively cold climate that is not particularly sunny, slowing growth of algae and reducing productivity. The possible solutions to this are culturing algae in self-contained photobioreactors, rather than open air ponds, or using surplus heat from industrial activity to warm ponds and thereby increase growth rates. In this activity students can investigate the most suitable conditions for algal photosynthesis and compare the photosynthetic ability of varieties of algae.

This activity will enable students to observe the photosynthesis reaction in algae using an indicator method for carbon dioxide uptake.

A range of photosynthesis experiments have been developed by Science and Plants for Schools (SAPS) including photosynthesis using algae wrapped in jelly balls:

www.saps.org.uk/secondary/teaching-resources/235-student-sheet-23-photosynthesis-using-algae-wrapped-in-jelly-balls

Advanced Biofuels - Algal Biofuels

Age Range: This experiment is suitable for secondary and post-16 students.

Duration: 60 minutes.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of microbes, photosynthesis, plants, the properties of gases, indicators and chemical reactions. Knowledge of photosynthesis and the properties of light will help students interpret the results of their investigations.

What you will need

- Varieties of unicellular algae
- Hydrogen carbonate indicator solution
- Test tubes and test tube rack or Universals
- Light source
- Pipettes

Optional

- Sodium alginate
- Syringe
- Beaker
- Strainer
- $CaCl_2$ solution
- Colorimeter

Health and Safety

The following factors should be considered when planning to carry out any investigations involving microorganisms: nature of the organism used, source of the organism, temperature of incubation, culture medium used, type of investigation and the facilities available, chance of contamination, expertise of people involved. If necessary change the conditions or limit the involvement of students perhaps by carrying out the experiment as a demonstration.

CLEAPSS® laboratory handbook – section 15.2 Microbiology (COSHH, good practice and safety precautions, levels of practical work, using microorganisms in practical work, equipment and materials, sterilisation and disinfection) page 1505.

CLEAPSS® Recipe book RB3 (Alginate beads), RB48 (Indicators-carbon dioxide)

CLEAPSS® Guidance PS 04 (COSHH: risk assessments in situations where microorganisms might be involved).

Burdass, D., Grainger, J.M. and Hurst, J. (editors) 2006, Basic Practical Microbiology – A Manual and Grainger, J.M. and Hurst, J. (editors) 2007, Practical Microbiology for Secondary schools available free from the Society for General Microbiology (SGM)

Further advice can also be sought from the Society for General Microbiology and the Microbiology in Schools Advisory Committee.

Ensure suitable light sources are used for this activity and that water is kept away from electrical connections.they are protected from water

Method

1. Different species of algae should be cultured prior to the activity (you may want to carry out activity 3A - culturing algae first).
2. Prepare solutions of equal algal density, a dark green coloured solution will provide sufficient algae to enable measurement of photosynthesis over a short period. Some cultures will readily settle if placed in the dark overnight, other cultures will require filtering or centrifugation. The concentration of algae in culture can be assessed by cell counts, relative colour or using a densitometer. Larger concentrated vials of *Scenedesmus* algae sufficient to produce a class set of algal balls are available from Sciento if there is insufficient time to culture algae.
3. Add 2 ml of algal solution to a test tube or universal and add 2 ml of hydrogen carbonate indicator.
4. Alternatively the algae can be immobilised in sodium alginate balls prior to adding to the indicator.
5. The algae are exposed to equivalent levels of light using a light box.
6. Photosynthesis can be measured as the indicator turns from yellow to purple as carbon dioxide is removed from the solution by the algae. Hydrogen carbonate indicator is very sensitive to the levels of carbon dioxide (CO_2) present.
7. The relative levels of dissolved carbon dioxide can be compared to a set of prepared standard solutions *or* if the algae have been immobilised in alginate balls a colourimeter can be used.
8. The concentration of carbon dioxide in the indicator can be measured using a colourimeter to test the absorbance of light at 550nm.

   The amount of light that will pass through the indicator decreases as it turns purple and the % of carbon dioxide is calculated by the colourimeter.

Extension activities

If the time and sufficient equipment is available students can immobilise the algae in the sodium alginate prior to the activity. Prepared algal balls will keep for some time in distilled water in a fridge. Allow them time to warm up and acclimatise to the light before checking that they are still active.

Preparing immobilised algae

1. Prepare a 2-3% sodium alginate solution with warm distilled or deionised water, mix thoroughly and leave overnight in a fridge. The initial mixture can be very lumpy but will become smooth overnight. Sodium alginate from different suppliers can vary in viscosity. A magnetic stirrer can be used to stir the mixture overnight but do not use a heated stirrer as this will reduce the efficacy of the gel matrix.

2. Concentrate the algae suspension by gentle centrifugation or allowing to settle overnight in a dark room. Resuspend in a small amount of distilled or deionised water so that the final solution of immobilised algae-alginate is not too runny.

3. Add the resuspended algae to the sodium alginate solution and mix thoroughly. Ideally a dark green solution will be produced in order to have enough algae to be able to carry out a sufficient rate of photosynthesis to be easily measured in relatively short timescales.

4. Prepare a 1.5% calcium chloride solution with $CaCl_2.2H_2O$. The calcium ions cause the sodium alginate to set and hence using distilled or deionised water for the alginate solution and resuspending the algae is important as is avoiding contact of the syringe with the calcium chloride solution.

5. Draw the algae-alginate solution up into a syringe.

6. Add the algae-alginate solution into a 1.5% $CaCl_2$ solution drop by drop. Carefully observe the shape of the drops. If the drops take on a 'comet' shaped appearance add a small amount of distilled or de-ionised water to the algae-alginate solution, mix and retry.

7. Allow the algae-alginate beads to set for at least 10 minutes.

8. Carefully strain the beads and rinse with distilled or deionised water.

A number of variables can be investigated and the results graphed by students. The ability of algae to carry out photosynthesis with different types of lights could be assessed. Filters can be used to block specific wavelengths of light to investigate the chromophores present in the algae.

Suppliers

Algae and culture media can be obtained from Sciento, www.sciento.co.uk/ 61 Bury Old Road, Whitefield, Manchester M45 6TB, tel: 0161 773 6338 fax: 0161 773 6338

Photosynthesis kits and hydrogen carbonate indicator can be obtained from National Centre for Biotechnology Education (NCBE) www.ncbe.reading.ac.uk/menu.html University of Reading, 2 Earley Gate, Whiteknights Road, Reading RG6 6AU tel: 0118 9873743 fax: 01189 750140

Colorimeters can be obtained from Philip Harris Education, Hyde Buildings, Hyde, Cheshire SK14 4SH, tel: 0845120 4520 fax: 0800 138 8881

Further reading and links

Eldridge, D. (2004) A novel approach to photosynthesis practicals. *School Science Review*, **85** (312, 37-45.

Photosynthesis using algae wrapped in jelly balls, SAPS www.saps.org.uk/secondary/teaching-resources/235

Investigating photosynthesis using immobilised algae, Practical Biology [Reference/webpage no longer available – July 2016]

Immobilised algae - Immobilised algae for studying photosynthesis, Debbie Eldridge

Investigating Photosynthesis – students guide, National Centre for Biotechnology Education (NCBE), [Reference/webpage no longer available – October 2016]

Investigating factors affecting the rate of synthesis, Practical Biology [Reference/webpage no longer available – July 2016]

Investigating the light dependent reaction in photosynthesis [Reference/webpage no longer available – July 2016]

Identifying the conditions needed in photosynthesis [Reference/webpage no longer available – July 2016]

Photosynthesis and starch production in Pelargonium leaf discs, Science and Plants for Schools (SAPS).

CLEAPSS® Recipe book Hydrogen carbonate indicator, page 48.

CLEAPSS® Guide R57 (2000) Colorimeters

Colorimetry and Higher Still, SSERC 1999, Biology Notes, Bulletin 197, 16.

Algal Research in the UK: A Scoping study for BBSRC, July 2011.

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2.

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

Research groups

Professor Alison Smith, Department of Plant Sciences, University of Cambridge

The Algal Bioenergy Consortium

Professor Johnathan Napier, Rothamsted Research www.rothamsted.ac.uk

Dr Saul Purton, University College London www.ucl.ac.uk/biology/academic-staff/purton/purton.htm

Dr Sohail Ali, Plymouth Marine Laboratory www.pml.ac.uk/about_us/pml_people/sohail_ali.aspx

Carole Llewellyn, Plymouth Marine Laboratory

BioMara project at The Scottish Association for Marine Science, Dunstaffnage marine laboratory, Oban www.sams.ac.uk

Culture Collection of Algae and Protozoa (CCAP) national algae collection www.ccap.ac.uk

Activity 3C - Algae chromatography

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

- Extract pigment from algae.
- Separate and compare the pigments in red and green algae.
- Analyse the distance of pigment migration.

Keywords Bioenergy, biofuel, biodiesel, sustainable, renewable, biomass, yield, waste, photosynthesis, algae, varieties, chromatography.

The green algae contain chlorophyll a and chlorophyll b (green pigments). The red algae contain chlorophyll a and phycobilin (a red pigment). Although chlorophyll is the major pigment for photosynthesis, the other pigments help algae harvest light of different wavelengths, which is useful when they are deeper in water. You should point out to the students that algae are simple (lower) plants. It is also worth mentioning that all land plants originated from one group of green algae.

The aim of this activity is to show the diversity of algae, and to demonstrate that red algae also contain green pigments (the green chlorophyll) and photosynthesise. By extracting the pigments, and then analysing them using chromatography, it is possible to demonstrate that the red algae also contain green pigment, which is usually masked by their red pigment.

This activity was developed by the Department of Plant Sciences at Cambridge University for the Royal Society Summer Science Exhibition at the Royal Festival Hall in London, entitled 'Meet the Algae: diversity, biology and energy '.

Age Range: This experiment is suitable for secondary students.

Duration: Activity takes about 30 minutes. Preparation will need to be done in advance to culture the algae and collect a concentrated pellet.

Suggested prior knowledge: It is recommended that you elicit the existing student knowledge of microbes, photosynthesis, plants, variation in organisms and techniques for separation including chromatography and solvents. Knowledge of photosynthesis and the properties of light will help students interpret the results of their investigations.

What you will need:

- Green algae culture (*Chlorella, Euglena*)
- Red algae culture (*Porphyridium*)
- Small mortar and pestle
- 1 ml plastic pipettes
- Centrifuge or test tubes
- Strips of blotting paper (Whatman 3MM)
- Ethanol
- Screw topped jars
- Fine paint brushes

Optional

- Centrifuge

Health and Safety

Wear eye protection. Ethanol is highly flammable, therefore there must be no naked flames and you must wash your hands afterwards. If using a centrifuge ensure that the centrifuge tubes are balanced and that the tubes used for centrifugation are sealed.

The following factors should be considered when planning to carry out any investigations involving microorganisms; nature of the organism used, source of the organism, temperature of incubation, culture medium used, type of investigation and the facilities available, chance of contamination, expertise of people involved. If necessary change the conditions or limit the involvement of students perhaps by carrying out the experiment as a demonstration.

CLEAPSS® laboratory handbook – section 15.2 Microbiology (COSHH, good practice and safety precautions, levels of practical work, using microorganisms in practical work, equipment and materials, sterilisation and disinfection) page 1505.

CLEAPSS® Recipe book RB26 (Chromatography solvents and locating agents)

CLEAPSS® Hazcards 40A (Ethanol)

CLEAPSS® Guidance PS 04 (COSHH: risk assessments in situations where microorganisms might be involved), PS 67-14 (Chromatography)

Burdass, D., Grainger, J.M. and Hurst, J. (editors) 2006, Basic Practical Microbiology – A Manual and Grainger, J.M. and Hurst, J. (editors) 2007, Practical Microbiology for Secondary schools available free from the Society for General Microbiology (SGM)

Further advice can also be sought from the Society for General Microbiology and the Microbiology in Schools Advisory Committee.

Method

1. Prepare two stocks of algae: red algae (*Porphyridium*) and green algae (*Chlorella*). *Euglena* is another green-coloured alga you can use.

2. Centrifuge 10 ml of culture in a centrifuge tube to form an algae pellet. If you do not have a centrifuge, put the algae mixture in a small test tube in the dark and allow to settle. This should take around one hour.

3. Carefully pour off the liquid, trying to avoid disturbing the pellet of algae cells at the bottom. Then place the tube containing the algae pellet in a freezer overnight.

4. Grind the pellet in a small mortar and pestle and resuspend the ground pellet in a small amount of water using a 1 ml plastic pipette. Start off with 1-2 drops and add more if necessary, but try to use the minimum so that the solution is as concentrated as possible.

5. Draw a line in pencil about 2 cm from the bottom of the paper . Use a fine paintbrush to place small dots of samples of green *Chlorella* and red *Porphyridium* next to each other. Add the samples a little at a time leaving them to dry in between.

6. Place the paper in 5 ml of ethanol (**Note**: make sure the solvent level is not above the level of the pencil line) in a screw topped jar and close the lid. Leave for around 10 minutes to allow the solvent to move up the paper. **What do you observe?**

The green spot moves up the paper, displaying no further colours. The red spot becomes bright red and green pigments move up the paper.

Algae chromatography

Suppliers

Red algae *Porphyridium purpureum*, other red algae, and the green *Chlorella* **and** *Euglena* can be obtained from Sciento, www.sciento.co.uk/ 61 Bury Old Road, Whitefield, Manchester M45 6TB tel: 0161 773 6338 fax: 0161 773 6338

A microcentrifuge suitable for school use can be obtained from National Centre for Biotechnology Education (NCBE) www.ncbe.reading.ac.uk/menu.html University of Reading, 2 Earley Gate, Whiteknights Road, Reading RG6 6AU, tel: 0118 9873743 fax: 01189 750140

Further reading

Meet the algae: diversity, biology and energy

Algal Research in the UK: A Scoping study for BBSRC, July 2011.

The Royal Society, January 2008. *Sustainable biofuels: prospects and challenges*, ISBN 978 0 85403 662 2.

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

Research groups

Professor Alison Smith, Department of Plant Sciences, University of Cambridge