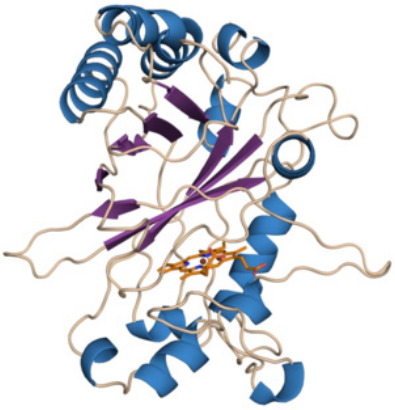
Activity 2B - Hydrolysis of biofuel feedstocks

Learning outcomes: 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 monosaccharide 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

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
- 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.

Method

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 1 ml aliquot 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.
6. Graph the results.

							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.

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First wood-digesting enzyme found in bacteria could boost biofuel production www.bbsrc.ac.uk/news/industrial-biotechnology/2011/110609-pr-wood-digesting-enzyme.aspx

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/

Immobilised yeast - Immobilisation of yeast in calcium alginate beads, Dean Madden, 2007. www.eurovolvox.org/Protocols/immobilisedyeast.html

Glucose detector. National Centre for Biotechnology Education (NCBE) Practical Biotechnology. www.ncbe.reading.ac.uk/ncbe/protocols/PRACBIOTECH/PDF/glucdet.pdf

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

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

Advanced Biofuels

Research groups

Professor Simon McQueen-Mason, BSBEC Marine Wood Borer Enzyme, Discovery Programme, The University of York, Heslington, York, YO10 5DD www.bsbec.bbsrc.ac.uk/programmes/marine-wood-borer-enzyme-discovery.html

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

Professor David Archer, BSBEC LACE programme Strand 2, University of Nottingham, Regulation of cellulase enzyme expression in Trichoderma www.nottingham.ac.uk/bioenergy www.nottingham.ac.uk/bioenergy/lace

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