Microbes and molecules

UNIT 1

European Initiative for Biotechnology Education

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ACTIVITIES

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However, users should be aware that errors and omissions can be made, and that different employers and educational authorities adopt different standards. Therefore, before doing any activity, users should *always* carry out their own risk assessment. In particular, any local rules issued by employers or educational authorities MUST be obeyed, whatever is suggested in the EIBE Unit.

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- practical work is carried out in a properly equipped and maintained science laboratory;
- any mains-operated equipment is properly maintained;
- care is taken with normal laboratory operations such as heating substances;
- good laboratory practice is observed when chemicals or living organisms are used;
- eye protection is worn whenever there is any recognised risk to the eyes;
- pupils and/or students are taught safe techniques for activities such as handling chemicals and microorganisms.

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INTRODUCTION

About this Unit

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

This Unit comprises a collection of activities designed to be used independently or in series as part of a teaching programme. They have been devised by practising teachers and educationalists from several European countries, brought together with support and encouragement from DGXII of the European Commission, under the auspices of EIBE, the *European Initiative for Biotechnology Education*.

All of the activities have been extensively tested on practical workshops involving teachers from across Europe.

The activities in this Unit consist of:

1. Inexpensive models of a DNA molecule and various microorganisms. These are intended to show the essential features of these systems and to provide an opportunity for students to consider of the relative sizes of microbes.

2. Simple, safe and inexpensive methods of extracting DNA, to give students experience of the basic techniques involved and allow them to see the genetic material.

3. A range of investigations emphasizing a) the presence of microorganisms in the environment and the selection of useful strains and; b) some of the products that microbes can provide (enzymes and antibiotics). Two of these investigations are qualitative; one provides a quantitative assay of enzyme production.

4. Several investigations of the effects of microbial growth, ranging from simple work with bread dough, through work on fermentation and finally the direct measurement of metabolic activity of yeast. In each of these investigations there is ample opportunity for further, extended investigations. To motivate students, activities have been selected to reflect biotechnological applications rather than theoretical principles alone.

5. Two demonstration activities which show natural methods of gene transfer, intended as a practical introduction to the principles of genetic modification.

Because two of the practical activities involve the production and action of antibiotics and the transfer of antibiotic resistance, additional information regarding these topics is included.

The authors have tried to provide a combination of both simple and more advanced activities, in the hope that any biology teacher will find something of interest and value amongst them. Two appendices provide some basic information about the use of microbiological techniques in the school laboratory.

In the near future, supplementary guides will be available, providing details of curriculum links, safety regulations, the availability of materials and other relevant information in different parts of the European Union.

Comments on these materials are very welcome, especially from teachers, at whom they are principally aimed. Comments and queries about this Unit should be sent to:

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MODEL MAKING

Cut-out DNA model

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

Aim

- To show the essential features of the structure of DNA (deoxyribonucleic acid).

Organisation

It takes about 30 minutes to make this model. If the cut-outs (opposite) have also to be coloured-in, allow more time.

Equipment and materials

Required by each student or group of students

- Scissors
- Strong paper glue, adhesive tape or a small stapler. *Note: double-sided tape is satisfactory, although it tends to come unstuck after some time.*
- Thread (to hang label from model, and to hang model up when completed)

Note: to provide a sturdier model, two sets of the sugar-phosphate 'backbones' can be glued together, back-to-back.

Assembly details

1. Cut out the sugar/phosphate 'back-bones' A and B.
2. Cut out the base pair 'rungs'.
3. Fold down the tabs on the rungs as shown opposite.
4. Stick the tab on one end of each rung onto the numbered boxes along back-bone A. You can attach the rungs in any order (pointing either way).
5. Stick the tabs on the other end of the rungs to the corresponding numbers on backbone B.
6. Attach the label to the double helix model, using thread. The diagrams below could also be used to label the model, if desired.

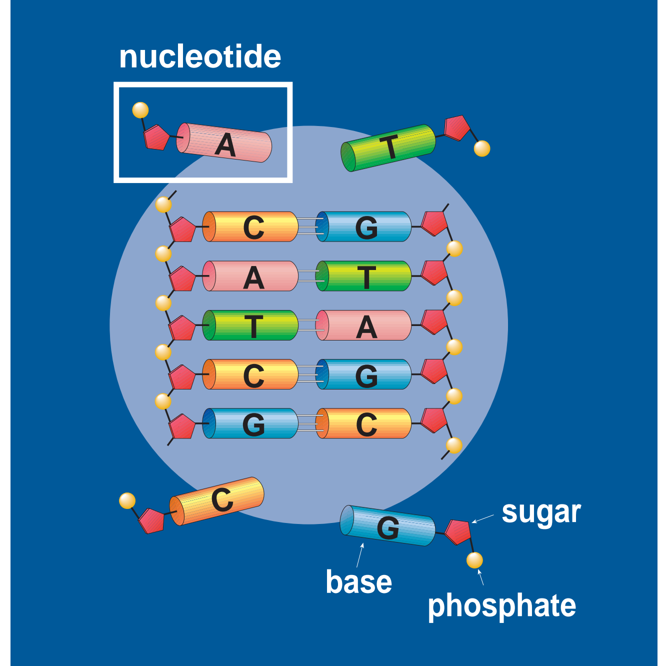
Acknowledgement

This model is adapted from one devised by the government research organisation CSIRO, in Australia, for their 'Double Helix' Science Club. EIBE is grateful to CSIRO for the original idea and for permission to adapt their model.

First position	Second position: T	C	A	G	Third position
T	PHE	SER	TYR	CYS	T
	PHE	SER	TYR	CYS	C
	LEU	SER	STOP	STOP	A
	LEU	SER	STOP	TRP	G
C	LEU	PRO	HIS	ARG	T
	LEU	PRO	HIS	ARG	C
	LEU	PRO	GLN	ARG	A
	LEU	PRO	GLN	ARG	G
A	ILE	THR	ASN	SER	T
	ILE	THR	ASN	SER	C
	ILE	THR	LYS	ARG	A
	MET	THR	LYS	ARG	G
G	VAL	ALA	ASP	GLY	T
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	GLY	A
	VAL	ALA	GLU	GLY	G

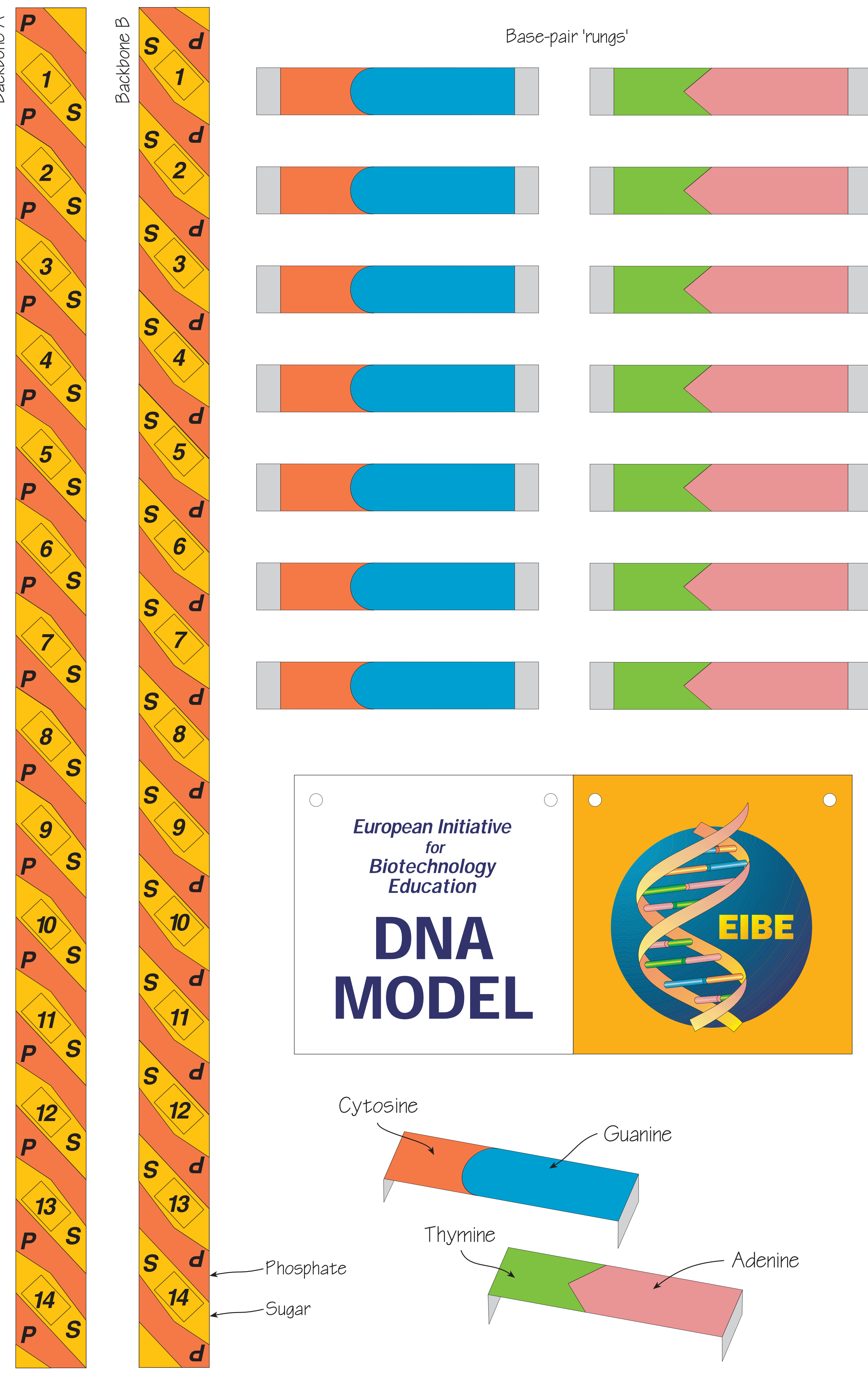
The genetic code

Each sequence of three bases on the DNA double helix encodes one of twenty amino acids represented in the body of the table by three-letter codes.



The structure of DNA

Chains of sugar and phosphate molecules form the two 'backbones' of DNA. Between these, four bases: thymine (T); cytosine (C); adenine (A) and guanine (G) are linked by hydrogen bonds.



Backbone A
Backbone B
Base-pair 'rungs'
European Initiative
for
Biotechnology
Education
DNA
MODEL
EIBE
Cytosine
Guanine
Thymine
Adenine
Phosphate
Sugar

MODEL MAKING

Model microbes

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

It is often difficult for students to appreciate the scale of microorganisms. In addition, the three-dimensional structure of microbes is difficult for students to understand from a two-dimensional view down a microscope.

By making models, this activity helps students to appreciate both the main features of and the relative sizes of microbes.

This activity is more successful if students are given freedom to devise their own models, rather than following a specified plan. Consequently, this task may be better completed at home (where a wider range of materials and more time may be available).

Note: for those misguided individuals who believe that model-making is beneath their dignity, it may be necessary to point out the long and distinguished history of model-making in molecular biology —although nowadays, physical models have been largely replaced by their computer counterparts.

Aim

- To show the essential features of the structure of a lambda bacteriophage and a variety of bacteria.
- To help students to appreciate the relative sizes of viruses and bacteria.

Organisation

It takes about 30 minutes to make the bacteriophage model. If the net (opposite) has also to be coloured-in, assembly will take longer. Depending upon the care taken, the bacteria models may take an hour or more to constract. *Note: This activity may usefully be set as homework.*

Equipment and materials

Required by each student or group of students

- Text books, showing the structure of viruses and bacteria
- A selection of model-making materials e.g. card, discarded packaging, table-tennis balls, beads etc.
- Scissors
- Glue, adhesive tape or a small stapler
- Paints

Instructions to students

Virus

Use the cutout net provided to make a model of a lambda bacteriophage.

Bacteria

Use the materials provided to make models of bacteria (rods and cocci).

For each of your models:

1. Identify the basic features that are shown e.g. cell membranes, genetic material;
2. Work out the size of the organism your model represents;
3. Think of a simple way of explaining the relative sizes of the models to pupils younger than you e.g. 'if real bacteria were this size, you would (by comparison) be as tall as a house'.

Extension

Students could also be asked to make models of plant and/or animal cells. These could be used to reinforce the differences between prokaryotes and eukaryotes, and to emphasize the serial endosymbiosis hypothesis (explaining the supposed evolutionary origin of eukaryotes).

Additional information

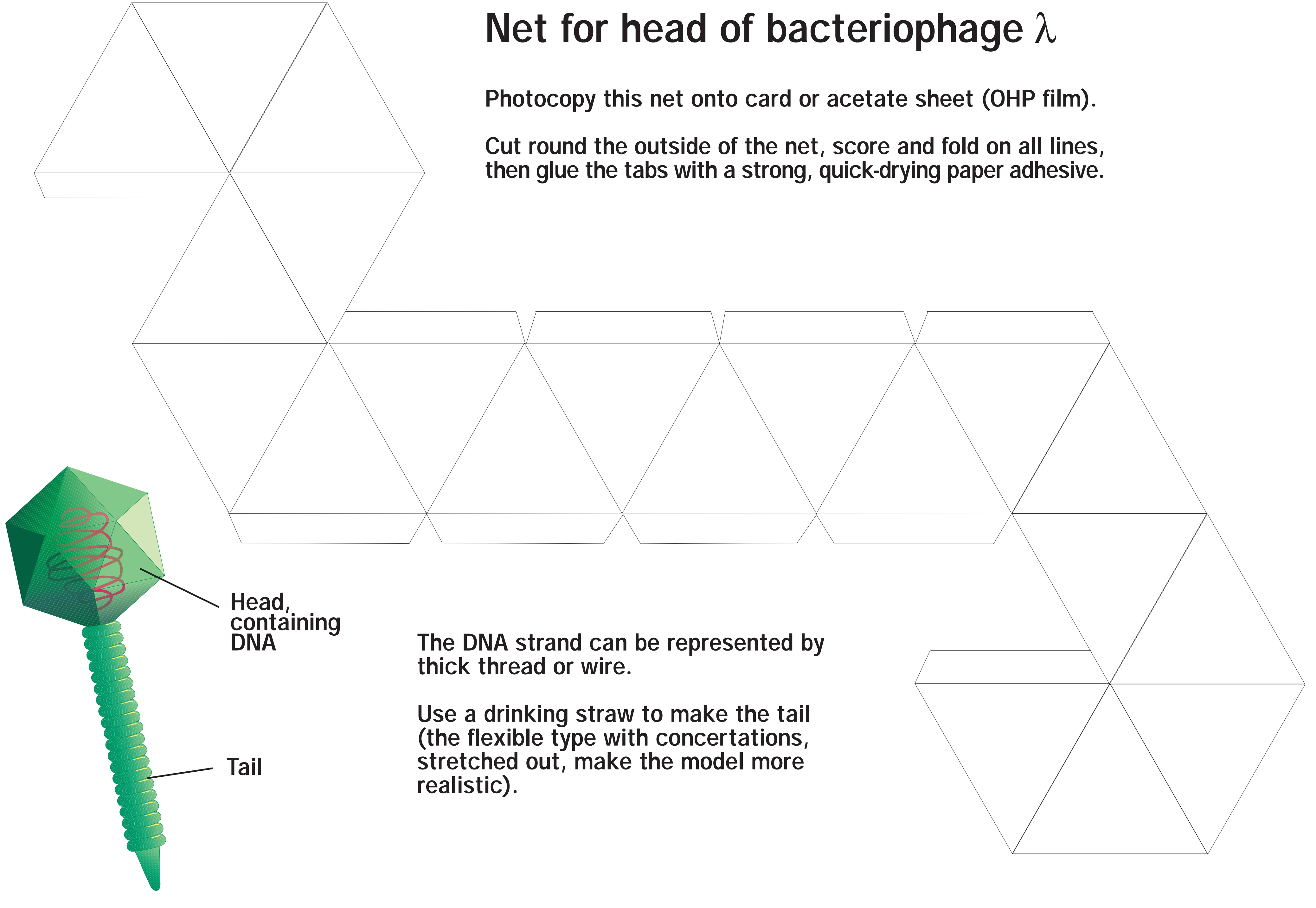
Model-making activities are also described in the following publication:

Nicholl, L. and Nicholl, D. (1987) Modelling the eukaryotic chromosome: a stepped approach. *Journal of Biological Education* 21 (**2**) 99–104.

Net for head of bacteriophage λ

Photocopy this net onto card or acetate sheet (OHP film).

Cut round the outside of the net, score and fold on all lines, then glue the tabs with a strong, quick-drying paper adhesive.



The DNA strand can be represented by thick thread or wire.

Use a drinking straw to make the tail (the flexible type with concertations, stretched out, make the model more realistic).

DNA EXTRACTION

Isolation of DNA from bacteria

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

This activity uses lysozyme (an enzyme) to degrade the cell walls of bacteria and household detergent to disrupt the inner cell membranes, releasing nucleic acids (DNA and RNA).

Aim

- To isolate nucleic acids from the bacterium *Escherichia coli* K-12.

Advance preparation

Cultures of bacteria, grown on nutrient broth, must be prepared *at least* 4 days in advance (only mature cultures yield significant quantities of nucleic acids).

Organisation

This activity takes about 50 minutes to complete, including a period of 30 minutes during which the bacteria are incubated with the enzyme.

Equipment and materials

Required by each student or group of students

- Mature culture of *Escherichia coli* K-12 (see *Advance preparation*, above)
- Lysozyme powder - only the smallest amount that can be held on the tip of a spatula is required
- Cold ethanol, 6 ml (IMS is satisfactory)
- Household detergent e.g. *Pril* (Henkel), *Woolite* (Reckitt & Colman), 0.5 ml
- Inoculation loop
- Distilled water, 3 ml
- Test tubes, 2
- Pipette or 1 ml plastic syringe, for dispensing water and lysozyme solution
- Water bath, set at 60 °C
- Incubator, set at 37 °C

Procedure

1. Prepare a culture of *Escherichia coli* K-12, at least 4 days before you wish to isolate the DNA.
2. Add a small quantity of lysozyme to 5 ml of bacterial suspension and mix well.
3. Incubate the mixture for 30 minutes at 37 °C.
4. Add 0.5 ml of household detergent to the bacterial suspension.
5. Incubate the mixture for 2 minutes at 60 °C in a beaker of water.
6. Allow the mixture to cool in cold water for a few minutes.
7. Slowly and very carefully pour a layer of cold ethanol onto the surface of the mixture.
8. The nucleic acids (DNA and RNA) will precipitate into the upper (ethanol) layer.

Extension

1. The DNA may be stained e.g. using aceto-orcein or methylene blue solution.

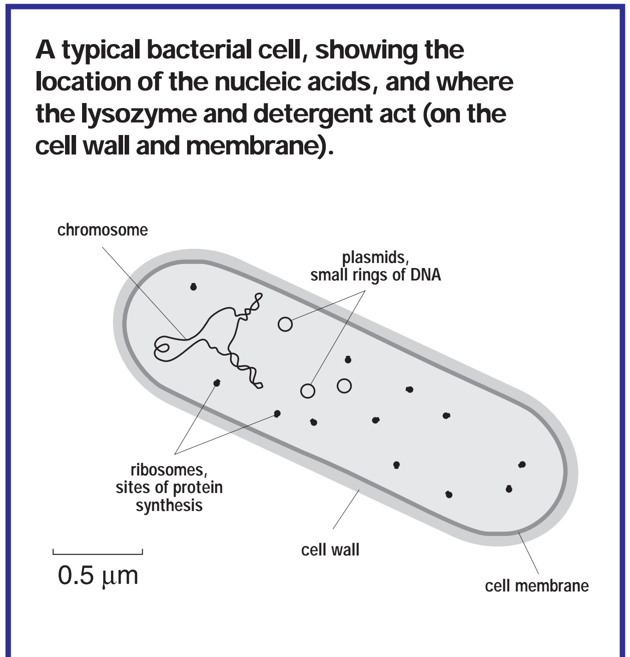
Safety precautions

Standard microbiological safety procedures should be followed when handling cultures. Care should be taken when using hot water (step 5).

Acknowledgement

This procedure is based on one devised by Hertel *et al.* and Süßmuth *et al.* (1987) who isolated DNA from *Bacillus subtilis*. We would like to thank Professor Joseph Lengeler of Osnabrück for the suggestion of using household detergent for this work.

A typical bacterial cell, showing the location of the nucleic acids, and where the lysozyme and detergent act (on the cell wall and membrane).



1. Prepare a culture of *E.coli* K-12 on nutrient broth.
2. Add a small quantity of lysozyme to 5 ml of the bacterial suspension, and mix well.
3. Incubate the mixture for 30 minutes at 37 °C.
4. Add 0.5 ml of household detergent to the bacterial suspension.
5. Incubate the mixture for 2 minutes at 60 °C in a beaker of water.
6. Allow the mixture to cool in cold water for a few minutes.
7. Slowly and very carefully pour a layer of cold ethanol onto the surface of the mixture.
8. The nucleic acids (fine white strands) will precipitate into the upper (ethanol) layer.

DNA EXTRACTION

Isolation of DNA from onions

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

This is a crude method of isolating DNA and RNA from plant tissue. First, the tissue is broken up mechanically. Household detergent is used to degrade both the cell membranes and those surrounding the nuclei. Cell fragments are separated by filtration; the nucleic acids and soluble proteins remain. An enzyme is used to degrade the proteins, then the nucleic acid is precipitated into ice cold ethanol.

Aim

- To isolate nucleic acids from onion tissue. *Note: nucleic acids prepared in this way will not be very pure. The essential purpose of the technique described is to demonstrate the major principles involved in the extraction of DNA from tissue.*

Advance preparation

The ethanol used must be ice cold. Place it in a plastic bottle in a freezer *at least* 24 hours before you attempt this activity.

Organisation

This activity takes about 35 minutes, including an incubation period of 15 minutes.

Equipment and materials

Required by each student or group of students

- Domestic food blender
- Sharp vegetable knife and chopping board
- Large plastic funnel
- Water bath, set at 60 °C
- Ice, in a jug
- 250 ml beakers, 2
- Coffee filter paper (do not use laboratory filter paper)
- Onion, about the size of a cricket ball
- Washing-up liquid, 10 ml (do not use the thicker, concentrated type)
- Table salt, 3 g
- Distilled water, 100 ml
- 10 ml plastic syringe, for measuring out liquids
- Boiling tube
- Glass stirring rod
- Protease enzyme, e.g. *Neutrase* (Novo Nordisk), 2–3 drops
- Ice cold ethanol, about 6 ml (IMS is suitable).

Procedure

1. Add the table salt to the washing-up liquid. Make up to 100 ml with distilled water.
2. Chop the onion into small pieces, but no smaller than 10 mm x 10 mm.
3. Add the chopped onion to a beaker with the salty washing-up liquid solution.
4. Put the beaker in a water bath at 60 °C for *exactly* 15 minutes.
5. Cool the mixture by standing the beaker in an ice water bath for 5 minutes, stirring frequently.
6. Pour the mixture into a blender and blend it for *no more than 5 seconds.*
7. Filter the mixture into a second beaker. Ensure that the foam on top of the liquid does not contaminate the filtrate.
8. Add 2–3 drops of protease to about 6 ml of the onion tissue extract in a boiling tube and mix well.
9. Pour ice cold ethanol carefully down the side of the boiling tube, to form a layer on top of the onion extract. Leave the tube for a few minutes without disturbing it.
10. Nucleic acids will precipitate into the upper (ethanol) layer.

Extension

1. The DNA may be stained e.g. using aceto-orcein or methylene blue solution.
2. DNA may also be extracted from certain animal tissues (e.g. cod roe, liver, calf thymus i.e. sweetbread). Here, a mortar and pestle (with silver sand) instead of a blender provides a gentler method of breaking up the tissue, ensuring that the DNA is not sheared.

Safety precautions

This procedure presents no particular safety hazards, although care should be exercised when chopping the onion or using the blender.

Acknowledgement

This method is adapted from *A Sourcebook of Biotechnology Activities* by Alison Rasmussen and Robert Matheson (1990) National Association of Biology Teachers / North Carolina Biotechnology Center. ISBN: 0 941212 09 2.

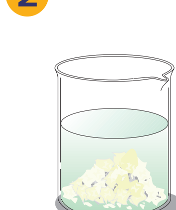
The full publication is available from the NABT, 11250 Roger Bacon Drive #19, Reston, Virginia 22090, USA.

1

10 ml washing-up liquid
3 g table salt
100 ml water
1 medium-sized onion

Add the table salt to the washing-up liquid. Make up to 100 ml with water. Stir well to dissolve the salt.

2



Add the chopped onion to the salty washing-up liquid solution.

The washing-up liquid breaks down the cell membranes — releasing DNA from the nucleus inside each cell.

3

Stand the beaker in hot water at 60 °C for 15 minutes.

Hot conditions speed up the process ...

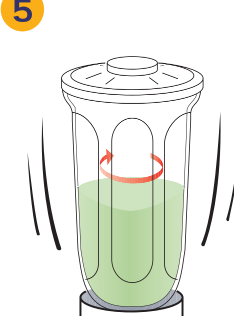
... and denature DNases that might degrade the DNA.

4

Cool the mixture by standing the beaker in a jug of ice for a few minutes.

... but leave it too long and the DNA gets broken up too ... so an ice bath is necessary.

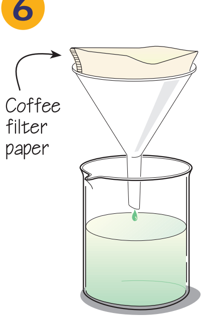
5



Blend for no more than 5 seconds.

The blender helps to break open the onion cells — but do not blend for too long or you will shear the DNA.

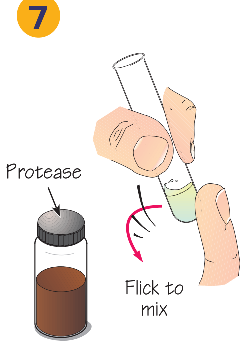
6



Filter the blended mixture.

This separates the cell wall material from the DNA and proteins, which are now in solution.

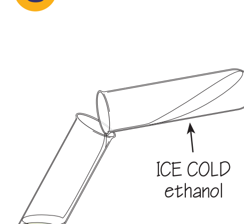
7



Add 2–3 drops of protease enzyme to about 10 ml of the onion extract.

The protease breaks down the protein in the solution. Only a little is needed.

8



Very carefully pour an equal volume of ice cold ethanol onto the surface of the onion extract.

The ethanol* MUST be ice cold — keep it in the freezer overnight beforehand.

* *you can use industrial methylated spirits (IMS)*

Isolation of DNA from onions

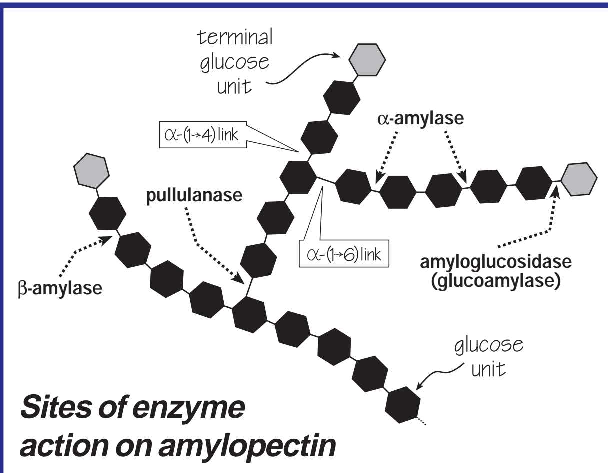
DNA forms in the upper (ethanol) layer.

DNA does not dissolve in ethanol — so it comes out of solution into the upper layer.

Amylase production by soil microbes

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

Starch is made from glucose units linked to form either a linear polymer called amylose or a branched polymer called amylopectin. These polymers are broken down by extra-cellular amylases that are produced by many kinds of organisms, including bacteria and fungi. Large numbers of microbes are screened to find those suitable for commercial enzyme production. In this investigation, soil microbes are screened for extra-cellular enzyme production.



Sites of enzyme action on amylopectin

Glucose units in amylose and amylopectin are linked by α–(1→4) bonds; in amylopectin, side branches are linked to these chains by α–(1→6) bonds. The major amylases that are available commercially are:

***α-amylase** – hydrolyses α–(1→4) bonds in glucose polymers, but only within chains, yielding shorter chains (dextrins). Obtained commercially from bacteria (e.g. Bacillus spp.).*

***β-amylase** – hydrolyses α–(1→4) bonds in glucose polymers, breaking off successive maltose units from the ends of the chains. Cannot bypass α–(1→6) bonds. Obtained commercially from barley and malt.*

***amyloglucosidase** – breaks α–(1→4) bonds, cleaving glucose units progressively from the ends of the chains. Also hydrolyses α–(1→6) bonds, but only slowly. Obtained commercially from the fungi Aspergillus spp. and Rhizopus oryzae.*

***pullulanase** – hydrolyses α–(1→6) bonds. Obtained commercially from the bacteria Bacillus acidopullulyticus and Klebsiella pneumoniae.*

Aim

- To screen naturally-occurring soil microorganisms for amylase production.

Prerequisite knowledge

Students will need to understand standard microbiological procedures, including aseptic techniques. Knowledge of the starch-iodine reaction would be helpful.

Advance preparation

Iodine solution should be prepared at least a day ahead. The iodine crystals take a while to dissolve completely.

Starch/nutrient agar and bottles of **sterile water** should be prepared before the lesson. Ideally, **soil samples** should be air-dried before use.

Sterile cotton wool swabs. The plastic shaft of shop-bought cotton wool swabs will melt when they are autoclaved. Some commercial cotton swabs may also be treated with an anti-microbial agent. Make your own swabs for this investigation by twirling a small amount of cotton wool round the tip of a cocktail stick. Autoclave in a McCartney bottle or loosely-wrapped in aluminium foil at 121 °C for 15 minutes.

Organisation

Preparation and inoculation of Petri dishes: 45 minutes

Observation of results, 2–3 days later: 15 minutes

Equipment and materials

Required by each student or group of students (It is assumed that normal laboratory equipment is also available)

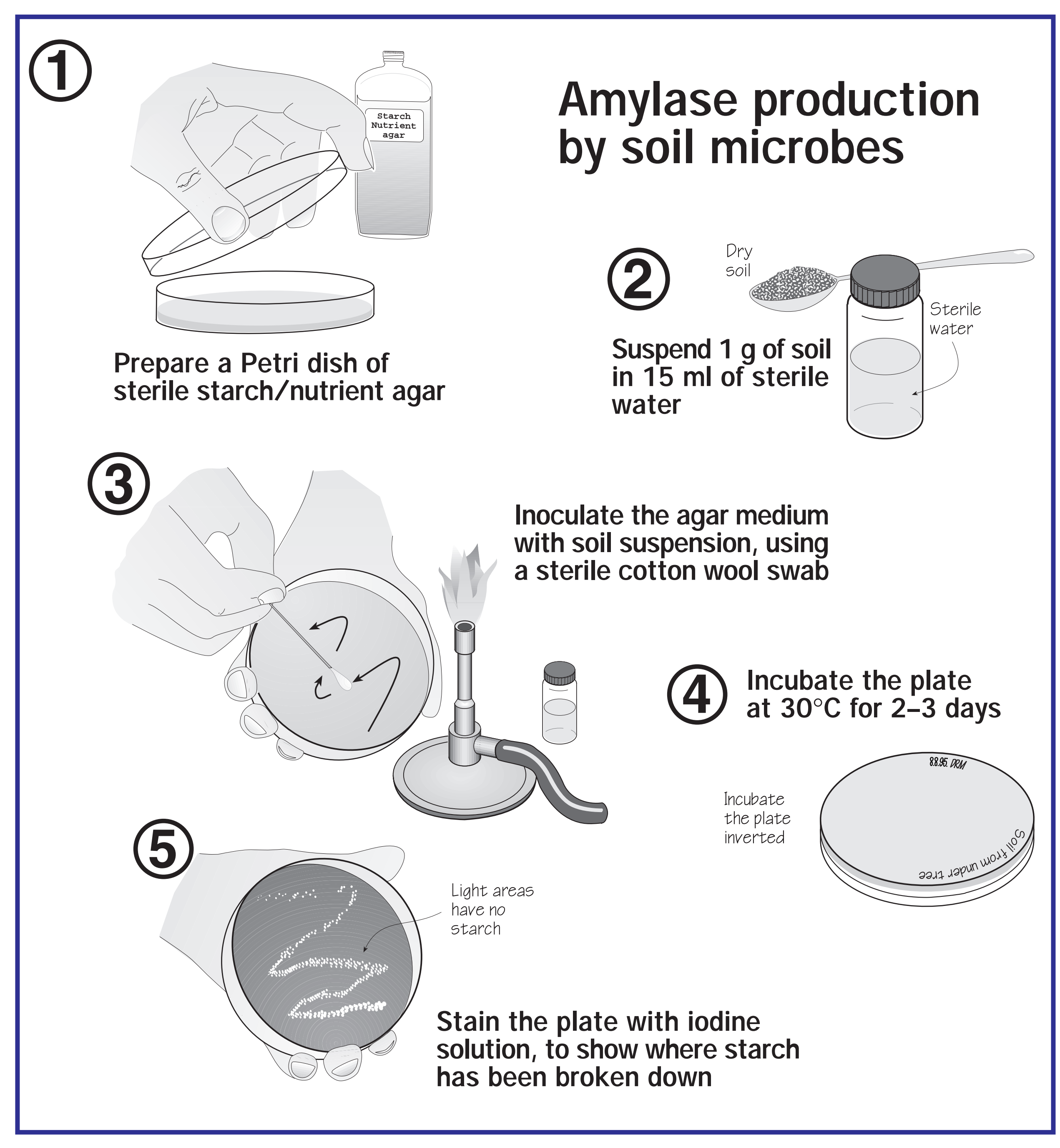
- Sterile Petri dish, containing 15–20 ml of sterile starch/nutrient agar, prepared from commercial nutrient agar with 0.2% soluble starch added
- 1 g of dry soil, taken from the surface 10 cm
- A solution of iodine, made by dissolving 1 g of iodine crystals and 2 g of potassium iodide in 300 ml of distilled water.
- 15 ml of sterile distilled water, in a McCartney bottle
- Sterile, home-made cotton wool swab (see *Advance preparation*, above).
- Marker pen (to label Petri dish)

Procedure

1. Place 1 g of dry soil in 15 ml of sterile distilled water. Agitate well to disperse the soil.
2. Inoculate the starch/nutrient agar plate, using a sterile cotton wool swab to streak soil suspension over the surface of the agar.
3. Label the Petri dish with your initials, the date and the source of the inoculum.
4. Incubate the inoculated plate, inverted, for 2–3 days at 30 °C.
5. Carefully pour iodine solution into the incubated plates until the entire surface of the agar is covered to a depth of about 1 mm. Spots where starch is still present are tinted blue-black (iodine molecules enter the central space of the helical chains of glucose molecules in starch). Light brown zones (the colour of the iodine solution) develop along the edges of the colonies if the microorganisms have degraded starch.

Safety precautions

IMPORTANT: In some countries, it may be forbidden to carry out this investigation, as plates, inoculated with unspecified organisms, are opened after incubation. If necessary, a culture of *Bacillus subtilis* could be used instead of soil organisms. Standard microbiology safety procedures should be followed when carrying out this work and when disposing of cultures, although aseptic techniques are not strictly necessary up to the inoculation of the plates. It is not advisable to subculture (unidentified) organisms from the inoculated plates. Iodine is toxic, and must be handled with care.



PRODUCTIVE MICROBES

Cellulase production

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

There is great interest in utilising cellulose wastes as feedstocks for fermentation processes, thereby converting low cost starting materials into products of greater value. Most commercial cellulases are produced by submerged fermentation of the fungus *Trichoderma reesei*. However, the bacterium *Cellulomonas* grows more rapidly on a Petri dish and the production of extra-cellular cellulases by it is easy to measure.

Aim

- To provide a quantitative assay of cellulase production by *Cellulomonas*.

Advance preparation

Cultures of *Cellulomonas* in nutrient broth (e.g. in McCartney bottles) should be prepared for classroom use. This should be done two or three days before it is intended to carry out this practical work. The cultures should be incubated at 25–30 °C.

CMC medium contains the following: 0.5 g carboxymethylcellulose (a soluble form of cellulose); 0.1 g $NaNO_3$; 0.1 g K_2HPO_4; 0.1 g KCl; 0.05 g $MgSO_4$; 0.05 g yeast extract; and 0.1 g glucose in 100 ml of water. The medium should be solidified using 1.7 % w/v agar.

Organisation

Preparation and inoculation of Petri dishes: 45 minutes

Observation of results, 2–3 days later: 15 minutes

Equipment and materials

Required by each student or group of students (It is assumed that normal laboratory equipment is also available)

- Slope culture of *Cellulomonas* sp.
- Sterile Petri dish, containing 15 ml of sterile CMC medium (see *Advance preparation*, above)
- Sterile water (dispensed into a McCartney bottle)
- Sterile 1 ml syringes (without needles), 2 or a pipette filler and 2 sterile, plugged pipettes
- Discard beaker containing 5% solution of Chlorate I solution e.g. *Domestos* (Lever)
- Congo red solution (made with 1 mg per ml of water)
- 1M sodium chloride solution
- Ethanol (for flaming cork borer)
- Cork borer, 5 mm diameter
- Incubator set at 25–30 °C
- Pen for marking Petri dishes

Procedure

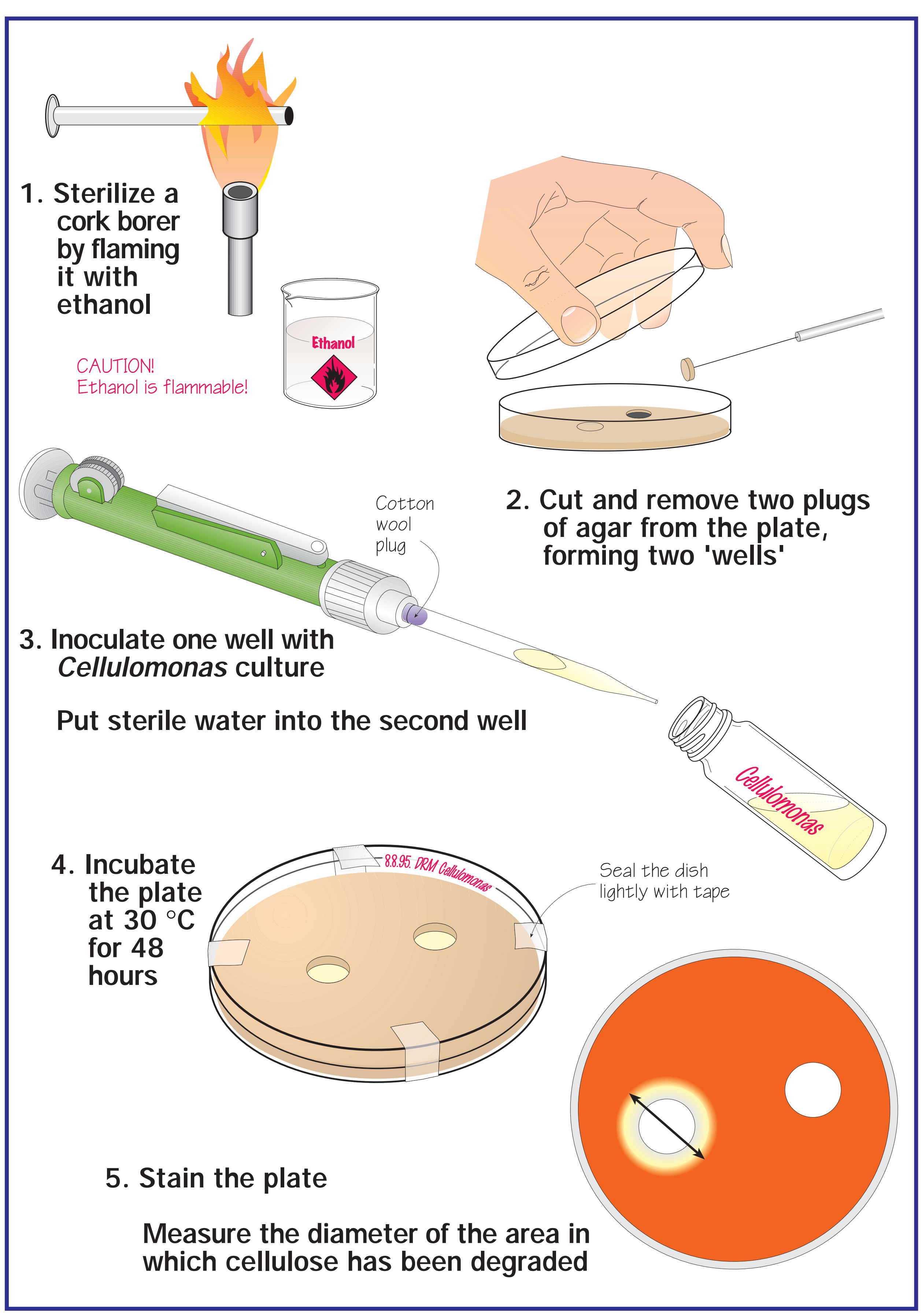
1. Dip a 5 mm diameter cork borer into alcohol. Set fire to the alcohol and let it burn away. CAUTION! Take care to hold the borer horizontally while doing this so that flames do not travel up the centre of the borer and burn your hand.
2. Hold the lid of a CMC medium plate slightly to one side and use the borer to cut a well in the agar. Remove the agar plug from the borer if necessary using a mounted needle.
3. Repeat steps 1 and 2 so that you have two wells in the agar.
4. Label each well on the base of the Petri dish. A suitable code would be: **C** (*Cellulomonas*); **W** (sterile water, a 'control').
5. Into the appropriate well place 0.2 ml of either microbial culture or sterile water, using a separate sterile syringe or pipette for each. Place the syringes, as they are used, in a beaker of disinfectant.
6. Incubate the plates for up to a week at 25–30 °C. *Cellulomonas* will produce clear zones up to 16 mm in diameter after 48 hours at 30 °C.

After incubation...

7. Flood the plates with Congo red solution for 15 minutes, then de-stain with the salt solution for 10–15 minutes. Unstained areas indicate where the CMC has been broken down to β-(1→4) glucans that contain seven or fewer glucose residues. The diameter of the clear zone can be measured to provide a quantitative comparison of cellulolytic activity.

Extension

1. Soil suspensions can be pipetted into wells on the plates to screen their microbial flora for cellulase activity.
2. The same technique can be used to assay the activity of commercial cellulase preparations.
3. The course of cellulase breakdown can be followed over several days by using duplicate plates.



Safety precautions
Standard microbiological safety procedures, including aseptic techniques, must be observed when carrying out this work.

IMPORTANT: If soil samples are screened for cellulase-producers (*Extension*, Activity 1), local regulations may forbid the reopening of inoculated plates.

PRODUCTIVE MICROBES

Antibiotic production

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

Many microorganisms produce antibiotics — substances which inhibit the growth of or kill their bacterial competitors. Since the development of penicillin in the 1940s (produced by the fungus *Penicillium*), these substances have been highly successful in the fight against disease. Today, the most important antibiotics are produced by the bacterium *Streptomyces*. More information about the action of antibiotics, and the phenomenon of bacterial resistance is provided in the accompanying pages.

Aim

- To demonstrate the production of the antibiotic streptomycin by *Streptomyces griseus*, and to show its effect on the growth of a variety of microorganisms.

Prerequisite knowledge

Origin and effects of antibiotics; the development and spread of resistance to antibiotics. (See accompanying text).

Below: a three-day-old culture of *Streptomyces griseus* (left). The test organisms are (from the top): *Candida utilis, Micrococcus luteus, Pseudomonas fluorescens, Bacillus mycoides, Escherichia coli.*

Advance preparation

Active cultures of *Streptomyces griseus* are required. These must be grown in for 2–3 days on plates, each containing 15–20 ml of basal agar medium.

A culture with which to inoculate these plates must also be prepared in advance. To do this, resuspend a loop of *Streptomyces* from a slope culture in 1 ml of sterile basal broth, then pipette this into a test tube into which 5 ml sterile basal broth has been placed. Incubate the culture for 24 hours at 30 °C.

Inoculate the Petri dishes with the overnight culture of *Streptomyces griseus* by streaking the plate with a single vertical line as far over to the left as possible so that the right part of the culture medium remains sterile.

Incubate the plates for 3–4 days at 30 °C.

In addition, prepare overnight cultures of test organisms (chosen from *Bacillus mycoides, Candida utilis, Escherichia coli* K-12, *Micrococcus luteus, Pseudomonas fluorescens*).

Organisation

Preparation of the media and inoculua: 60 minutes +

Initial incubation of the *Streptomyces* culture: 24 hours, then an additional 72–96 hours

Inoculation of plates: 20 minutes

Incubation: 24–72 hours

Equipment and materials

Required by each student or group of students (It is assumed that normal laboratory equipment is also available)

- Access to an incubator, set at 30 °C
- Inoculating loop
- Sterile Petri dish, containing 15–20 ml of basal agar medium, onto which *Streptomyces griseus* has been streaked and allowed to grow for 48–72 hours (see *Advance preparation*, above)
- A selection of the following organisms, on agar slopes, as test organisms*:
  - *Candida utilis* (a yeast)
  - *Micrococcus luteus* (a bacterium)
  - *Pseudomonas fluorescens* (a bacterium)
  - *Bacillus mycoides* (a bacterium)
  - *Escherichia coli* K-12 (a bacterium)

**Refer to local regulations for information regarding the use of these organisms in schools (the use of some may be prohibited in certain countries).*

Procedure

1. Inoculate each *prepared Streptomyces* plate with the test organisms as follows:
   a) Sterilise an inoculation loop by passing it through a Bunsen burner flame and allowing it to cool briefly.
   b) Load the sterile loop with culture of the test organisms, then make a single horizontal streak for each organism on the clear part of the medium (to the right of the *Streptomyces*). The streaks must always be at right angles to the *Streptomyces* culture and be drawn close up to the edge of it. **IMPORTANT! Do not touch the *Streptomyces* culture with the loop.**
   c) Re-sterilise the inoculation loop by passing it through a flame once more.

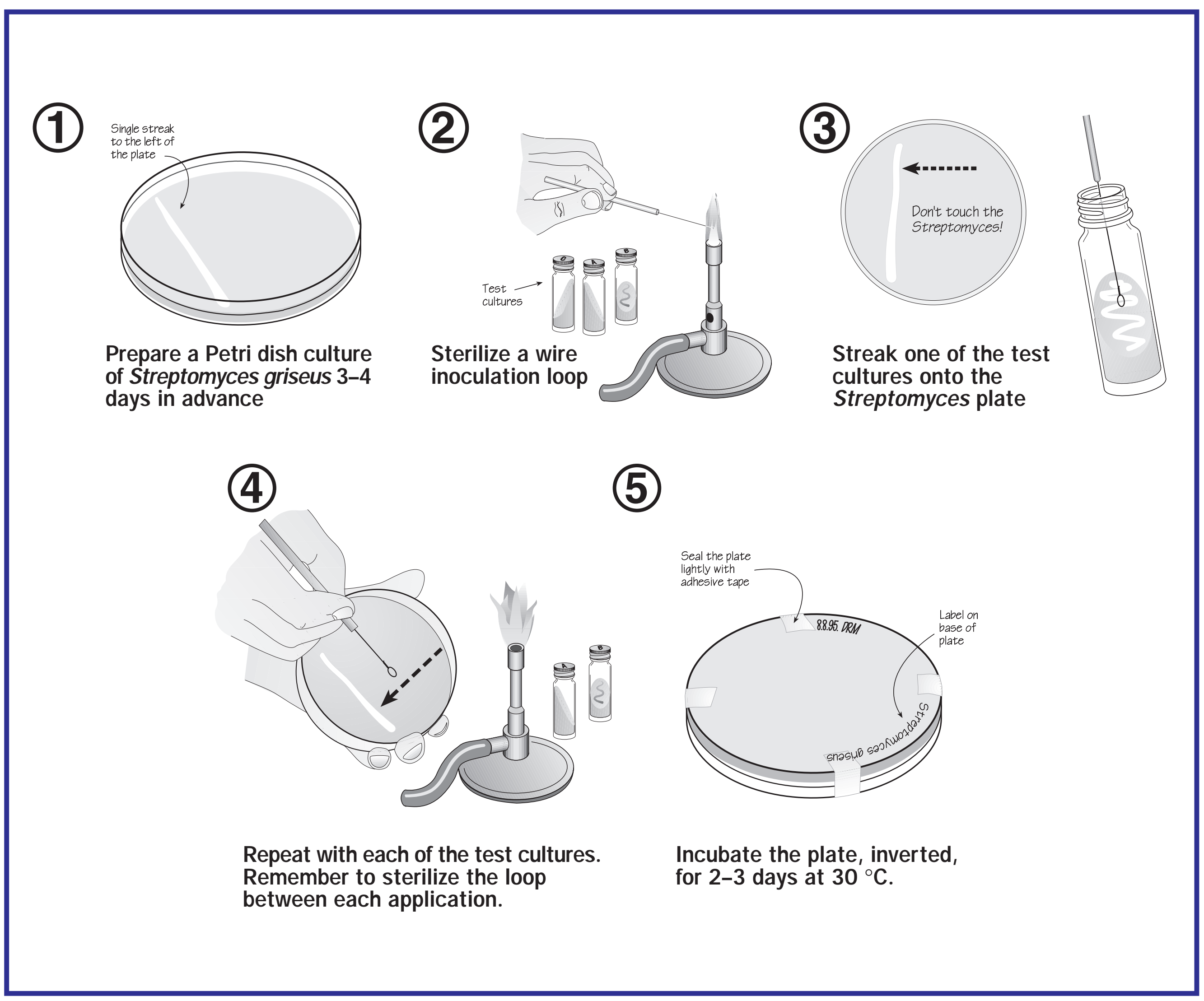
   Repeat (a)–(c) for each of the test cultures.
2. Incubate the plates for 2 days at 30 °C.
3. Examine the plates.

Safety precautions

This work must be carried out in a laboratory. Standard microbiology safety procedures should be followed when carrying out this work and when disposing of cultures. The amount of antibiotic produced during this investigation does not constitute a safety hazard.

Note

Streptomyces griseus produces the antibiotic streptomycin which diffuses into the culture medium. Streptomycin and allied antibiotics interfere with protein synthesis by binding to the 30S component of bacterial ribosomes. Some organisms (*Bacillus mycoides, Escherichia coli, Micrococcus luteus*) are sensitive; others (*Pseudomonas fluorescens*) are resistant. Yeasts (*Candida utilis*) are not affected by streptomycin as they are eucaryotes with a different ribosomal structure. For more information, refer to the accompanying text, which describes antibiotic production and action in detail.



MICROBIAL ACTIVITY

Making bread dough

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

Bread-making is one of the oldest examples of biotechnology, with accounts of leavened bread dating from ancient Egypt (4,000 BC). In the United Kingdom, bread is traditionally made from a dough of wheat flour, water, salt and possibly fat, depending upon the recipe. This forms a matrix in which yeast is trapped. Amylases in the moistened flour convert starch to glucose, which nourishes the immobilised yeast cells.

In addition, the yeast requires a source of nitrogen. Peptones and amino acids are provided by partial hydrolysis of flour protein (collectively termed gluten). The yeast's anaerobic respiration generates carbon dioxide and alcohol.

Gluten contributes to the elasticity and plasticity of the dough, ensuring that the carbon dioxide remains trapped as it enlarges the air bubbles within the dough, causing it to rise.

Aims

- This outline protocol may be used to investigate the effect of various recipe components which modify either flour proteins or enzyme activity.

Organisation

This activity can be completed in 50 minutes, depending upon the conditions (temperature, etc.).

Equipment and materials

For each batch of dough

- Dried yeast, 1 g
- Strong flour, 75 g
- Water, 50 ml
- Additional ingredients as desired e.g. ascorbic acid, α-amylase, potassium bromate (see *Extension*, below)
- Small beakers for mixing dough in
- Stout glass rods for mixing the dough
- 100 ml measuring cylinders, 2
- Stop clock

Procedure

1. Resuscitate the dried yeast in the water. Add the flour and mix well.
2. Roll the dough into a sausage shape, and place it in one of the measuring cylinders.
3. Record the height of the dough in the cylinders every 10 minutes over a one hour period.

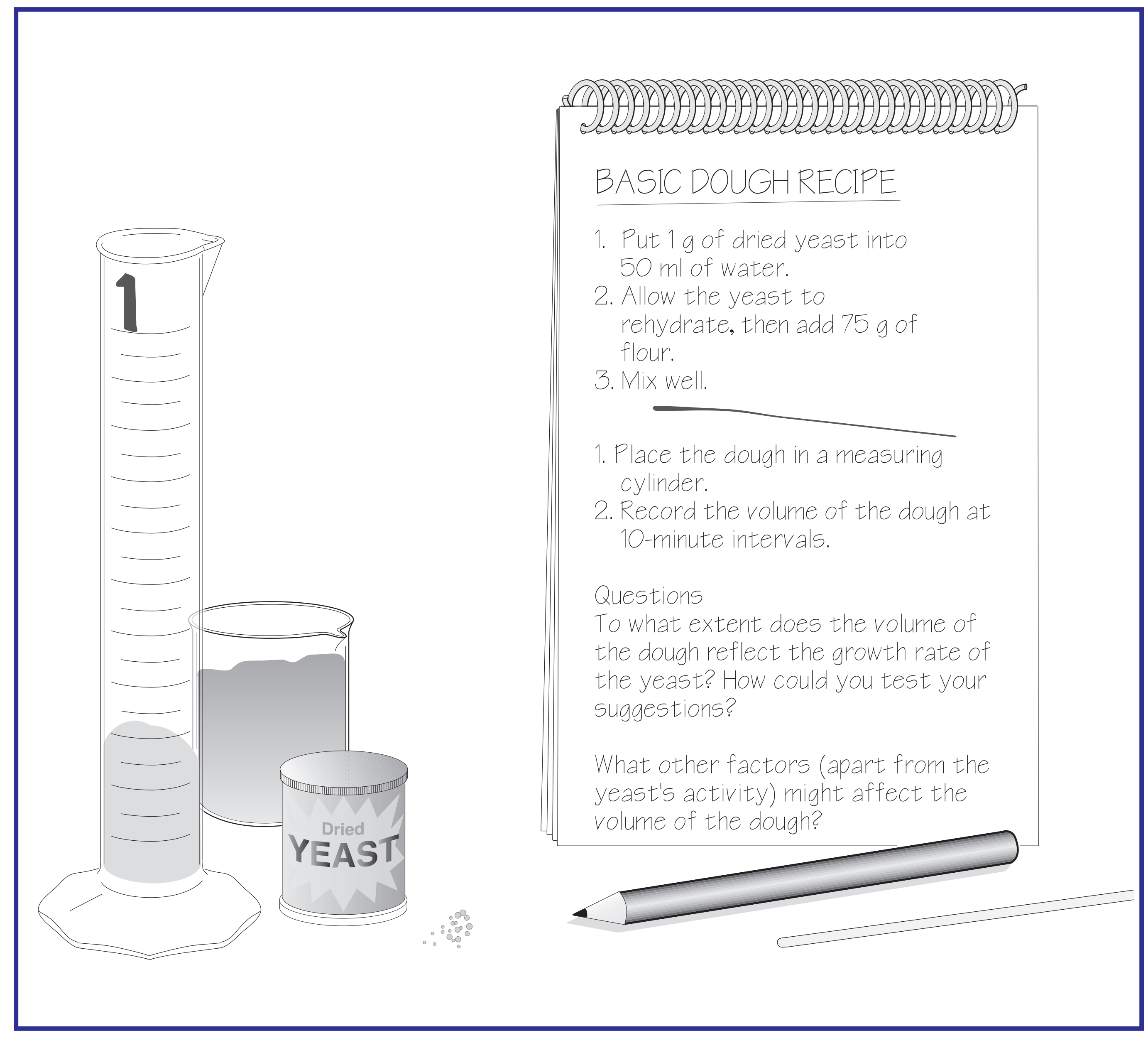
Extension

1. What effect do different types of dried yeast have on the rate at which dough rises? (e.g. normal dried yeast *vs.* the newer 'mix-in' type of yeast.)
2. What differences are there between the rates at which doughs made with different types of flour rise? (e.g. strong white, white and wholemeal flours. 'Strong' flours contain plenty of gluten but very little α-amylase. Wholemeal flour is rich in α-amylase.)
3. What effect does the flour improver ascorbic acid (vitamin C) have on the rate at which the dough rises? Ascorbic acid interacts with enzymes in the dough to limit the extent to which sulphydryl bonds are formed between gluten proteins. (Add 1 g to the recipe given for dough above.)
4. What effect does the addition of α-amylase have on the rate at which dough rises? How can any observed differences be explained?
5. Flour improver potassium bromate facilitates the formation of sulphydryl bonds between adjacent gluten proteins so that more elastic, expandable doughs are made. Of course, too much expansion would weaken the dough. Compare doughs made using flour with and without this additive.
6. Salt inhibits the activity of proteases, and so prevents gluten from being weakened into a sticky mass that can not retain carbon dioxide gas. Excess salt forms strong ionic bonds with side chains of protein molecules, making them less stretchy and leading to a tough bread. Excess salt also inhibits yeast growth. Try to determine the optimum salt content of dough.

Additional information

On food and cooking. The science and lore of the kitchen by Harold McGee (1991) Harper Collins Publishers. ISBN: 0 00 412657 2.

Pritchard, P.E. (1992) 'Studies on the bread-improving mechanism of fungal alpha-amylase'
Journal of Biological Education 26, (1) 12–18.



MICROBIAL ACTIVITY

Immobilised yeast cells

Ordinary bakers' yeast, *Saccharomyces cerevisiae*, is unable to ferment the sugar lactose. The enzyme β-galactosidase breaks down lactose to glucose and galactose. Yeast that is co-immobilised with this enzyme is able to grow in a medium that contains lactose. Of the two sugars formed by enzyme action, glucose is used preferentially. Once supplies of this sugar have been exhausted, the yeast adjusts its metabolism and the other breakdown product of lactose, galactose, is utilised. The activity of the yeast is readily-monitored simply by measuring the volume of gas (carbon dioxide) evolved during the fermentation.

Aim

- To provide an introduction to the quantitative study of fermentation

Advance preparation

Sodium alginate is not readily soluble in water. Both hot water and stirring are necessary to dissolve it. Sodium alginate solution must therefore be made up in advance. If you wish to store sodium alginate solution, it is advisable to autoclave it first. *Important: use distilled or dionised water for all solutions, as calcium ions in tap water will cause the alginate to 'set'.*

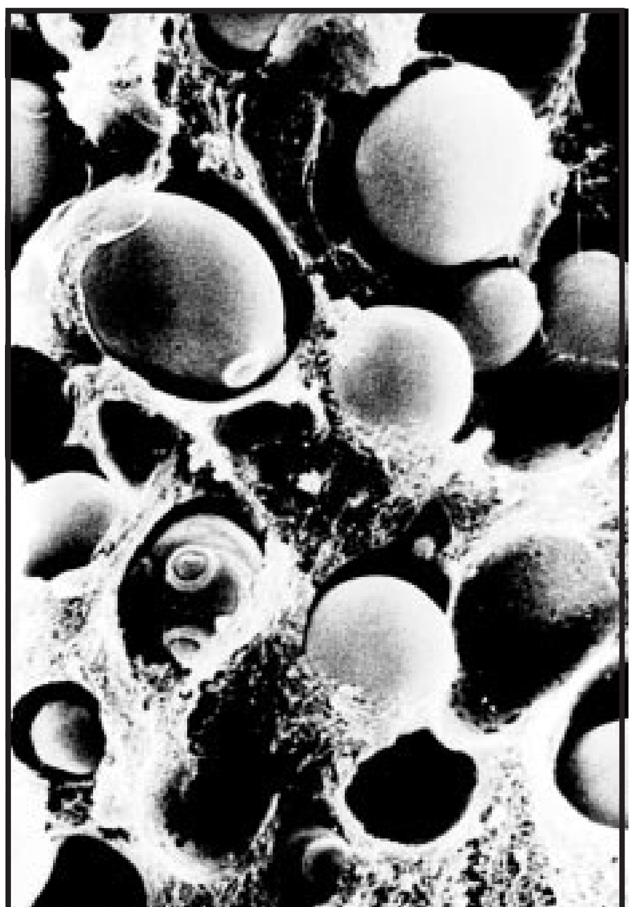
Organisation

Immobilised yeast cells can be prepared in 10–15 minutes. Fermentation can take up to a week.

Equipment and materials

Required by each student or group of students

- 4% sodium alginate solution, 10–15 ml
- 1.5% calcium chloride solution, 100 ml
- 10 ml syringe, without needle
- Tea strainer
- 200 ml beakers, 2
- 250 ml conical flasks, 2
- Bungs, to fit fermentation flasks, bored and fitted with delivery tubes, 2
- Large e.g. 500 ml beakers, 2
- 100 ml measuring cylinders, 2
- Chemical sterilizing solution e.g. sodium metabisulphite solution or a proprietary product such as *Milton* (Sodium hypochlorite solution, Procter & Gamble).



Photograph courtesy Dr Duncan Casson

Yeast cells immobilised in a matrix of calcium alginate.

- 13% sodium chloride solution, about 1 litre (ordinary table salt is adequate for this work)
- 100 ml of medium, containing 2 g glucose, 1 g yeast extract and 1 g of peptone
- 100 ml of medium, containing 2 g lactose, 1 g yeast extract and 1 g of peptone
- β-galactosidase enzyme, e.g. *Lactozym* (Novo Nordisk), 2 ml
- Bakers' yeast, fresh or dried

Procedure

Prepare the immobilised enzyme and yeast pellets as follows:

1. Mix the dried yeast with 25 ml distilled water in a small beaker. Cover and leave to rehydrate for 10 minutes at room temperature.
2. Mix the sodium alginate solution and β-galactosidase together in the other small beaker. Stir in 10 ml of the yeast suspension.
3. Draw up some of the yeast / enzyme / alginate mixture into the syringe. Add it, a

drop at a time, to the calcium chloride solution in the second large beaker.

4. Leave the immobilised enzyme / yeast cell pellets to harden in the calcium chloride solution for 10 minutes.
5. Separate the pellets from the solution using the tea strainer. Rinse them well with tap water.

The pellets may be stored in sterile water in a refrigerator for up to 3 days before they are used.

Set up the fermentation vessels as follows:

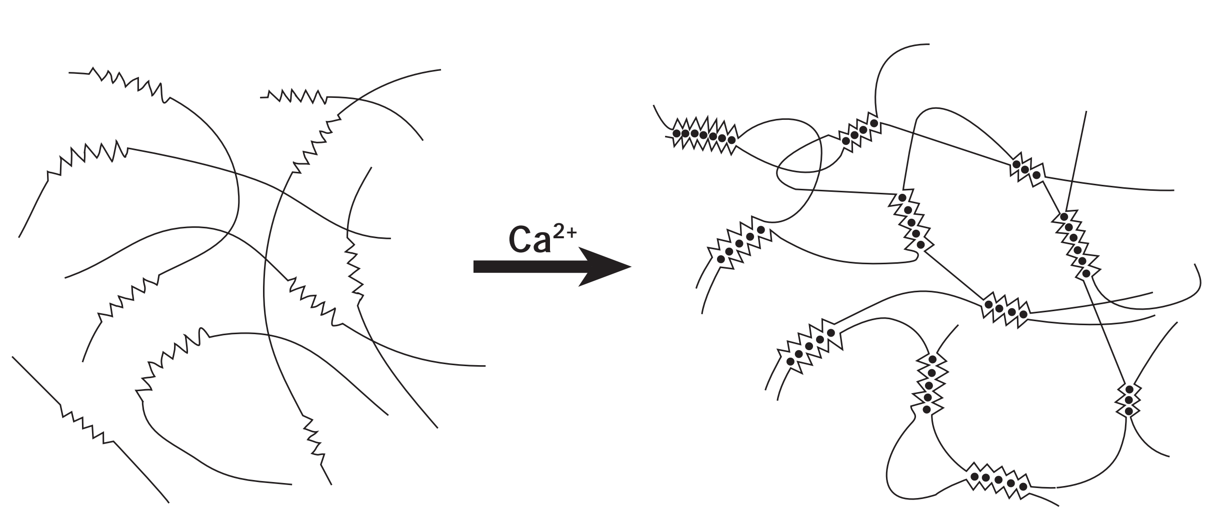
1. Sterilize the flasks using sodium metabisulphate solution or a similar chemical. Rinse the flasks with water after sterilization is complete.
2. Add the sterile growth media to the flasks. Use glucose medium for one (as a control), and lactose medium in the other.
3. Add an equal number or mass of immobilised yeast pellets to each flask.
4. Stopper the flasks with bungs that have been fitted with delivery tubes.
5. Maintain the flasks at 21–25 °C. Collect the gas that is produced over a 13% solution of sodium chloride (carbon dioxide will not dissolve in this solution). Record the volume of gas that has been collected at convenient intervals.
6. Plot the results on a graph, showing the volume of gas evolved against time.

Extension

1. Different types of yeast e.g. wine-makers' or bakers' yeast may be used.
2. The concentration of β-galactosidase, incubation temperature and other variables may be altered.

Safety precautions

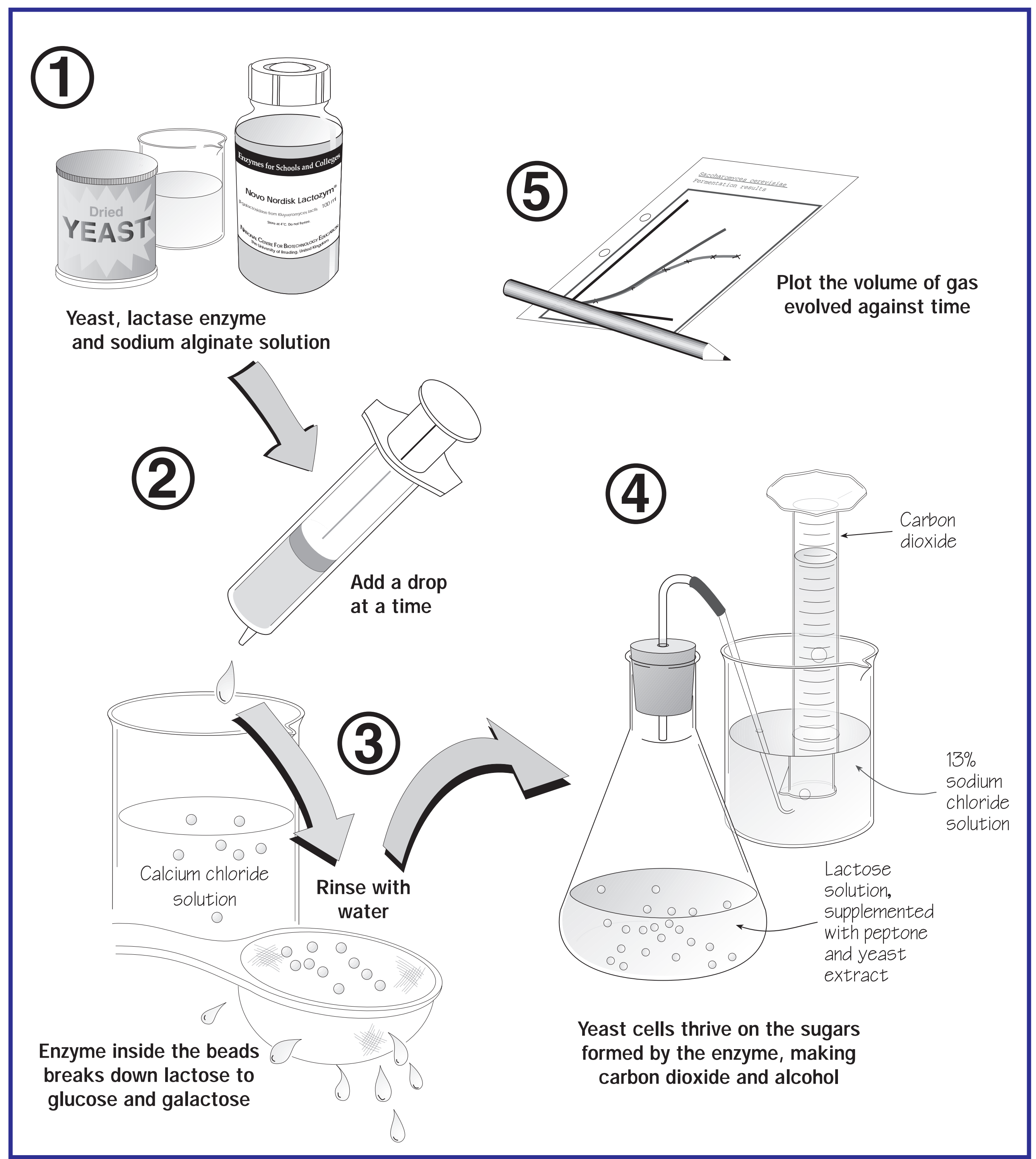
Build-up of gas within glass vessels could be dangerous. Ensure that the fermenter vessels are adequately vented. Care should be taken when using chemical sterilizing agents, and any guidelines for their use given by manufacturers must be followed.



Entrapment within calcium alginate is the most widely used technique for immobilising cells. It is especially suited to living cells as it requires only very mild conditions. Applications of this versatile method include immobilisation of living or dead cells in bioreactors, entrapment of plant protoplasts and plant embryos ('artificial seeds') for micropropagation, immobilisation of hybridoma cells for the production of monoclonal antibodies, and the entrapment of enzymes and drugs (see table, overleaf).

The cells or enzymes to be entrapped are first mixed with a solution of sodium alginate. This is then dripped into a solution containing multivalent cations (usually Ca^{2+}). The droplets form spheres automatically as they fall, entrapping the cells in a three-dimensional lattice of ionically cross-linked alginate (see diagram, above).

For more information, see Smidsrød, O. and Skjåk-Bræk, G. (1990) Alginate as an immobilization matrix for cells. *Trends in Biotechnology* 8 (**3**) 71–78.



Examples of uses of alginate-immobilised cells (after Smidsrød and Skjåk-Bræk, 1990)

Cells	Product/purpose
Bacteria	
Erwinia rhapontici	Isomaltulose
Pseudomonas denitrificans	Cleaning of drinking water
Zymomonas mobilis	Ethanol
Cyanobacteria	
Anabena sp.	Ammonia
Fungi	
Kluyveromyces bulgaricus	Hydrolysis of lactose
Saccharomyces cerevisiae	Ethanol
Saccharomyces bajanus	Champagne production

Cells	Product/purpose
Algae	
Botryococcus braunii	Hydrocarbons
Plant cells	
Chatharanthus roseus	Alkaloids for cancer therapy
Various plants	Artificial seeds
Plant protoplasts	Cell handling, microscopy
Mammalian cells	
Hybridomas	Monoclonal antibodies
Islets of Langerhans	Insulin/implantation
Fibroblasts or lymphoma cells	Interferons (α or β)

MICROBIAL ACTIVITY

The microbial fuel cell

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

For decades, microbes that produce electricity were a biological curiosity. Now, researchers foresee a use for them in watches and cameras, as power sources for the Third World and for bioreactors to turn industrial waste into electricity. The microbial fuel cell described here generates a small electrical current by diverting electrons from the electron transport chain of yeast. It may be used to study respiration in a novel and stimulating manner. Further information is given in *BIO/technology Education*, Volume 1, Number 4, pages 163–168.

Aims

- To provide a stimulating introduction to the study of respiration
- To permit the study of some of the factors which influence microbial respiration
- To show how waste organic material may be used to generate electricity

Advance preparation

Solutions of reagents should be prepared in advance. Pre-soak the cation exchange membrane in distilled water for 24 hours before use. The dried yeast can be resuscitated as the fuel cell is assembled.

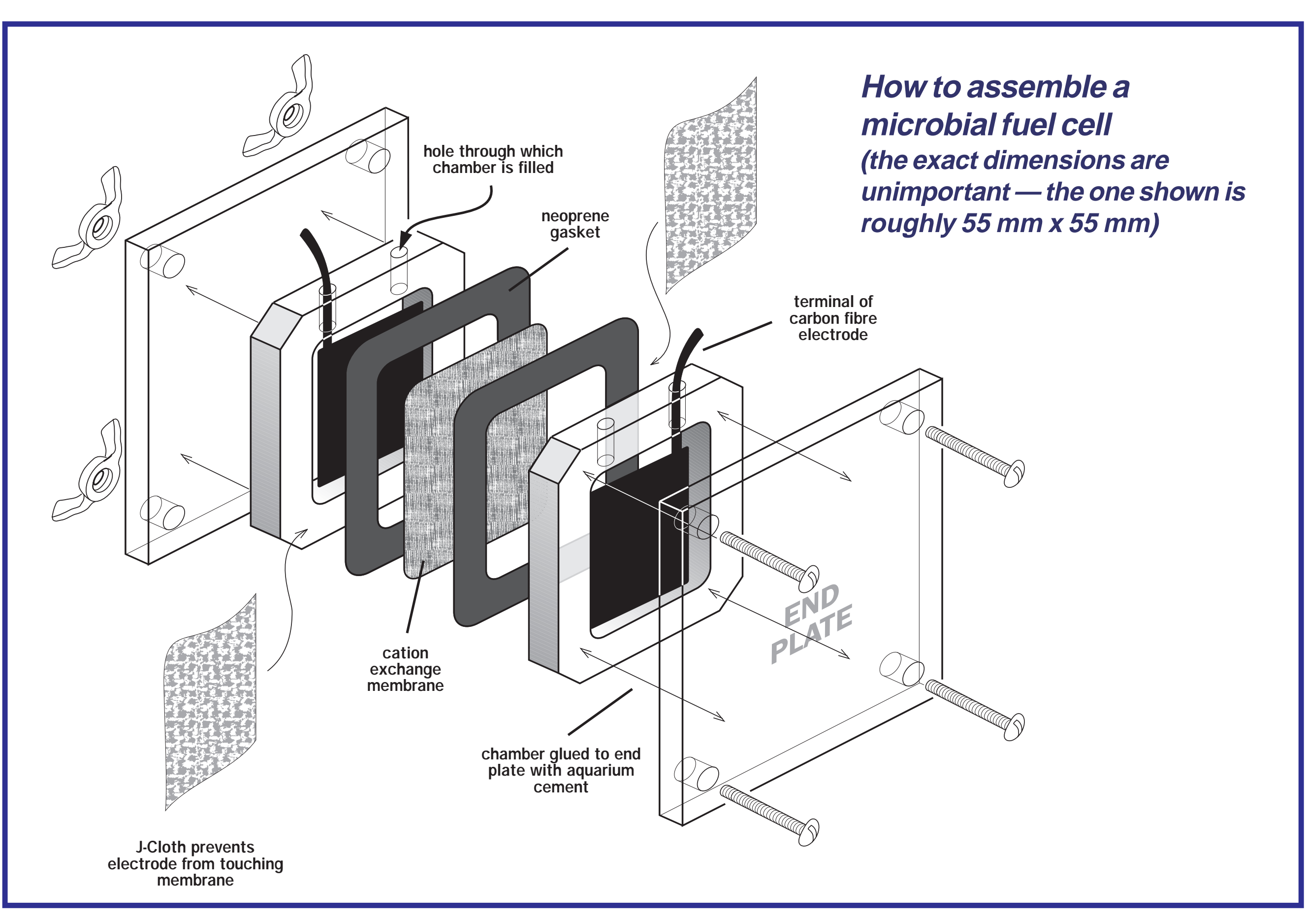
Organisation

Assembly (through to electricity generation) takes about 30 minutes.

Equipment and materials

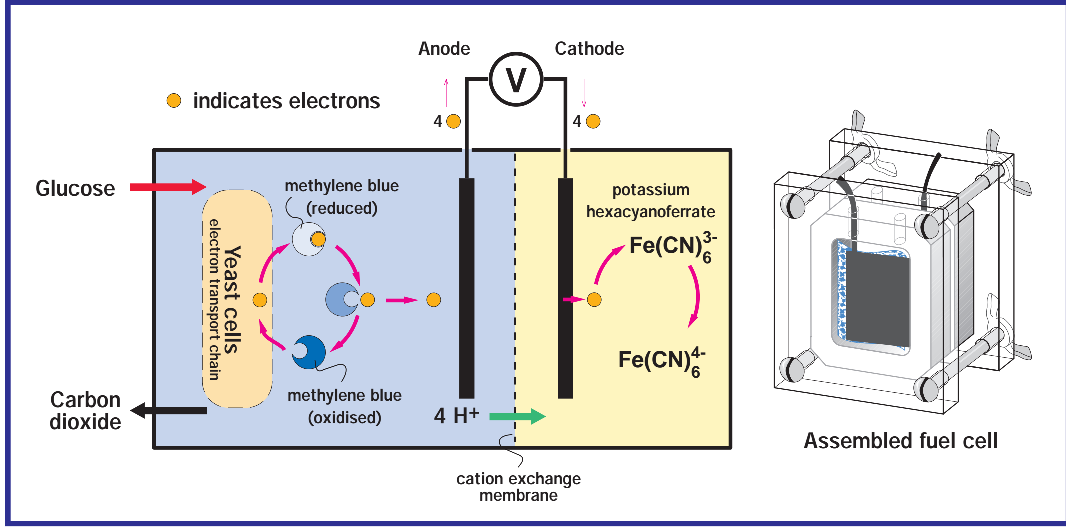
Required by each student or group of students

- *Perspex* (ICI) fuel cell, cut from 4 mm thick sheet
- Neoprene gaskets, 2
- Cation exchange membrane, cut to fit between chambers of the fuel cell. *The membrane may be re-used indefinitely, but will melt if it is autoclaved.*
- Carbon fibre tissue electrodes, cut to fit, 2
- *J-Cloth* (Johnson & Johnson), cut to fit inside cell, 2 pieces
- 10 ml plastic syringes, 2, for dispensing liquids
- Petri dish base or lid on which to stand fuel cell
- Electrical leads with crocodile clips, 2



How to assemble a microbial fuel cell
(the exact dimensions are unimportant — the one shown is roughly 55 mm x 55 mm)

How the fuel cell works



- 0–5 V voltmeter or multimeter and / or low current motor
- Scissors

All the solutions listed below should be made up in 0.1 M phosphate buffer, pH 7.0, instead of water

- Dried yeast, made into a thick slurry in 0.1M phosphate buffer (do *not* add glucose solution without first resuscitating the yeast in buffer)
- 10 mM methylene blue solution, 5 ml
- 1 M glucose solution, 5 ml
- 0.02 M potassium hexacyanoferrate (III) solution, 10 ml (also called potassium ferricyanide)

To make 0.1 M phosphate buffer, pH 7.0

Dissolve 4.08 g Na_2HPO_4 and 3.29 g NaH_2PO_4 in 500 ml distilled water.

Procedure

1. Cut out two carbon fibre electrodes as shown on the accompanying page.
2. Cut out two pieces of *J-Cloth* to fit inside the fuel cell.
3. Assemble the fuel cell as shown on the accompanying page.
4. Stand the assembled fuel cell on a Petri dish base or lid to catch any liquid which may leak.
5. Combine the yeast slurry, glucose and methylene blue solutions. Syringe the mixture into one chamber of the fuel cell.
6. Syringe potassium hexacyanoferrate (III) solution into the other side of the cell.
7. Connect a voltmeter or multimeter (via the crocodile clips) to the electrode terminals. Fuel cells of this type typically generate between 0.4–0.6 V. *A current should be produced immediately — if the meter registers zero, check the connections and ensure that the carbon fibre electrodes are not touching the cation exchange membrane.*

Extension

1. Several fuel cells may be joined together to give a greater voltage. Increasing the size of the cell (or the electrode area) will increase the current generated (but not the voltage).
2. Different mediators and / or types of yeast e.g. wine-makers' or bakers' yeast may be used. *Note: For safety reasons, the use of this fuel cell with other microorganisms is not recommended.*
3. Investigate the effect of temperature on the action of the fuel cell (remember to consider what 'controls' are necessary when making comparisons of this type).

Safety precautions

Potassium hexacyanoferrate (III) is poisonous. Eye protection should be worn when handling this material. If the solution comes into contact with the eyes, flood them with water and seek medical attention. If swallowed, give plenty of water to drink and seek medical attention. Local regulations should be observed when disposing of used solution.

Acknowledgement

The microbial fuel cell was developed by Dr Peter Bennetto at King's College, London.

Natural gene transfer by bacterial conjugation

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

There are various natural methods by which genes may transferred between bacteria. These mechanisms have probably evolved to allow organisms to adapt to rapidly-changing environmental conditions. As a rule, genes with the greatest mobility are located on plasmids.

Plasmids are rings of DNA which replicate independently of the bacterial chromosome. They carry a small number of genes conferring on their holders such things as an ability to break down harmful substances in the environment, like heavy metals or antibiotics.

Three types of natural gene transfer are recognised:

Transformation is the uptake of free DNA from the cell's surroundings;

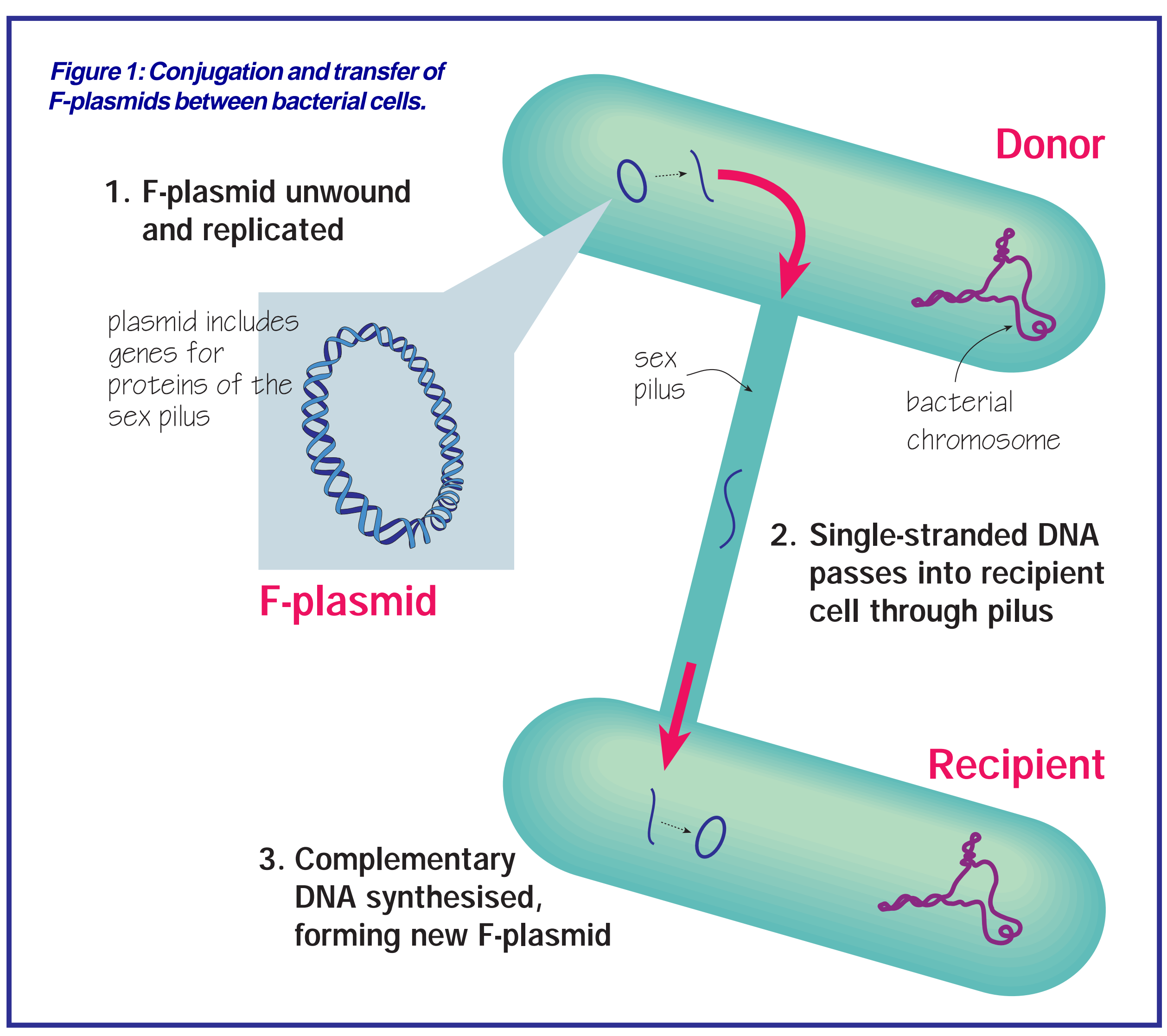
Transduction is the movement of DNA from one cell to another through the intervention of a bacteriophage;

Conjugation is the transfer of specialised 'F-plasmids' through a narrow tube (sex pilus) which links two bacterial cells (see Figure 1).

All of these mechanisms (transformation especially) are used by genetic engineers to ferry selected genes into bacteria.

In the following investigation, conjugation between two naturally-occurring bacterial strains is examined. *Note: This work uses naturally-occurring bacteria, plasmids and processes and is not, therefore, 'genetic engineering'.*

Figure 1: Conjugation and transfer of F-plasmids between bacterial cells.



F-plasmids

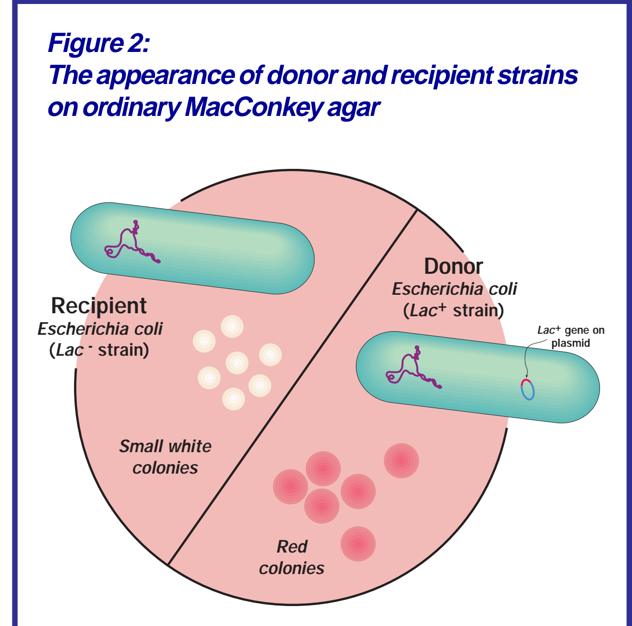
The so-called 'F-plasmids' (F stands for 'fertility') contain genes which enable the transfer of a copy of the plasmid between donor and recipient cells (in other words, bacterial conjugation).

F-plasmids may carry other genes as well. For example, in the investigation described here, the donor strain carries a plasmid called F *Lac*. It is so named because it carries genes which allow its bacterial host to metabolise lactose (the bacterium is therefore called a *Lac*$^+$ strain). In contrast, the recipient strain, with no plasmid, is unable to metabolise lactose (it is a *Lac*$^-$ strain).

On plates of MacConkey agar these different strains can be distinguished by their appearance: the donor strain forms red colonies, while the recipient strain forms white colonies (see Figure 2).

When donor and recipient strains are mixed, F *Lac* plasmids are transferred from the donor to the recipient. In this way the recipient acquires the ability to metabolise lactose.

Figure 2:
The appearance of donor and recipient strains on ordinary MacConkey agar



A genetic 'trick' enables the donor, recipient and transconjugant strains to be distinguished. A recipient strain is chosen with a chromosomal gene that renders it insensitive to the antibiotic streptomycin. The donor strain has no such gene and is inhibited by streptomycin. Hence the recipient strain and transconjugants can be identified by their ability to grow on a medium that contains streptomycin (Figure 3).

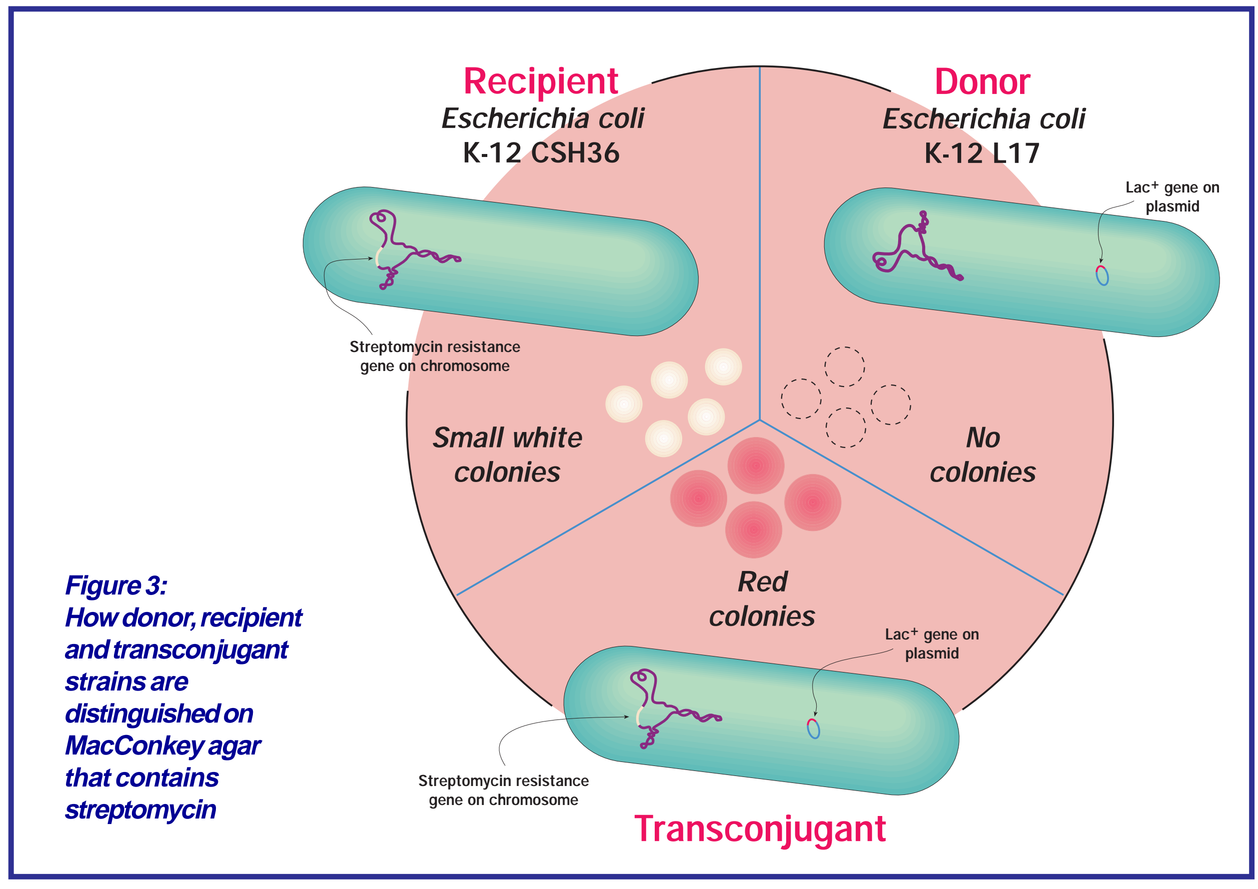


Figure 3:
How donor, recipient and transconjugant strains are distinguished on MacConkey agar that contains streptomycin

Aims

- To provide a stimulating introduction to bacterial genetics
- To provide an opportunity for students to discuss issues associated with natural gene transfer e.g. the spread of antibiotic resistance and 'risk assessment in gene technology

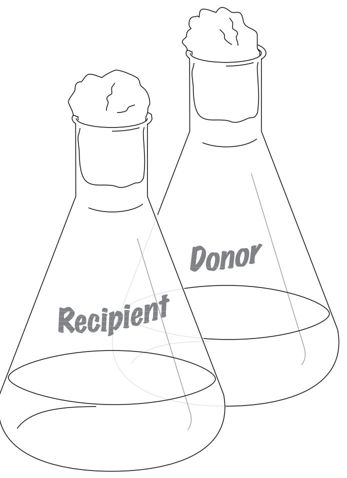
Advance preparation

The following media should be prepared and sterilised:

- 3 small (e.g. 100 ml) conical flasks, each containing 10 ml of sterile nutrient broth;
- Sterile MacConkey agar with added Streptomycin sulphate [200 mg per ml]. After the agar has cooled to 50 °C (hand hot) it should be divided between sterile Petri dishes (allow 15–20 ml per dish).

- The cultures used are:
  Donor strain:
  Escherichia coli K-12 CSH36 (DSM Number 6253);
  Recipient strain:
  Escherichia coli K-12 L17 (DSM Number 6254).

These naturally-occurring strains come from the German Collection of Microorganisms and Cell Cultures (DSM). Both cultures are supplied freeze-dried, in glass ampoules. The ampoules must be opened in the correct manner to ensure that their contents are not contaminated and that the user is not exposed to unnecessary hazards (see accompanying instructions).The donor strain is difficult to maintain on a slope, so a freshly-resuscitated culture is necessary.



Overnight cultures of the donor and recipient strains should be prepared as follows:

1. Inoculate one flask of sterile nutrient broth with the donor strain (labelled 'Donor'), and another with the recipient (labelled 'Recipient');
2. Incubate both flasks overnight at 37 °C, ideally in a shaking water bath.

'Matings' of these two cultures are performed as follows:

1. Using a sterile pipette, aseptically add 0.8 ml of the donor strain to the third conical flask, containing 10 ml of sterile nutrient broth.
2. Using a sterile pipette, aseptically add 0.2 ml of the recipient strain to the same conical flask.
3. Label the flask appropriately and incubate it at 30 °C for 16–24 hours. *Note: shake the flask only VERY GENTLY during incubation — vigorous action will break the pili which link conjugants.*

Sterile cotton wool swabs are prepared by twirling a small amount of cotton wool round the tip of a cocktail stick. Autoclave these in a McCartney bottle or loosely-wrapped in aluminium foil at 121 °C for 15 minutes.

Organisation

Preparation of media:	60 minutes
Initial incubation:	48 hours
Inoculation of plates:	15 minutes
Incubation:	24 hours
Observation of results:	20 minutes

Equipment and materials

Required by each student or group of students (It is assumed that normal laboratory equipment is also available)

- Access to an incubator, set at 30 °C
- Sterile Petri dish, containing 15–20 ml of MacConkey agar, with added Streptomycin
- The following cultures, grown in advance on nutrient broth:
  - *Donor strain*
  - *Recipient strain*
  - *Mixed mating of donor and recipient strains*
- 3 sterile, home-made cotton wool swabs
- Small beaker, containing disinfectant solution e.g. 3% *Domestos* (Lever) solution, in which to place used swabs
- Marker pen (to label Petri dish)

Procedure

1. Draw three lines on the base of one of the Streptomycin / MacConkey agar plates, dividing it into three equal segments.
2. In the middle of each segment, draw a circle about 10 mm in diameter. Label one 'D' (for the donor strain), one 'R' (for the recipient strain) and the final circle 'M' (for mixture of the two strains — forming transconjugants).
3. Use a sterile cotton swab to inoculate each marked area on the plate with the appropriate bacterial strains. Remember to use a fresh swab for each culture. Dispose of used swabs into disinfectant.
4. Allow the plates to stand for a few minutes, until liquid can no longer be seen on the surface of the agar medium. Incubate the plates, inverted, for one to two days at 30 °C.

Extension

Several quantitative versions of this work may be carried out, for example:

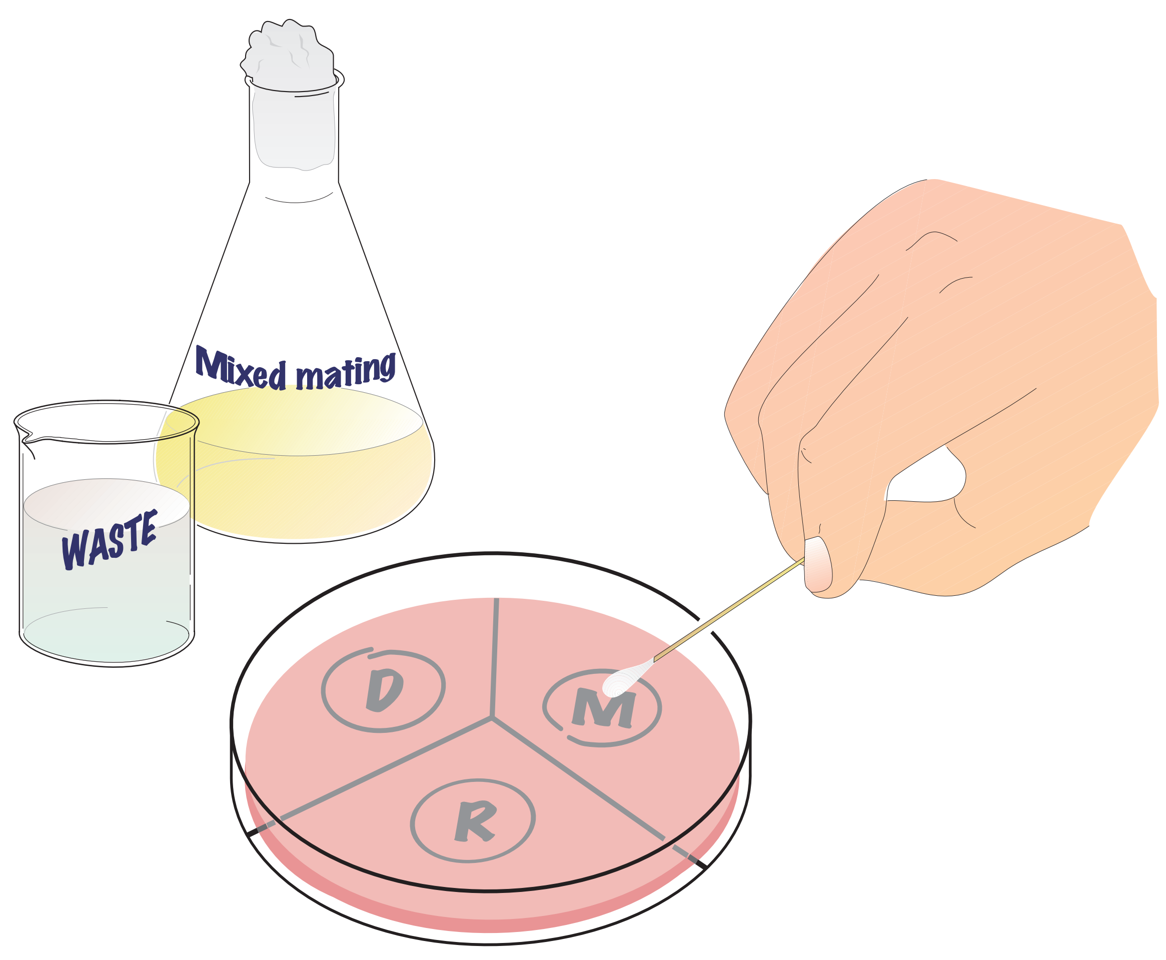
1. By diluting the donor and recipient cultures in sterile Ringer's or 10^{-3} $MgSO_4$ solution, the optimum ratio of donor to recipient may be determined.
2. The optimum mating time required for conjugation may be determined.

Safety precautions

This work must be done in a laboratory. Standard microbiology safety procedures, including aseptic technique, should be followed when carrying out this work and when disposing of cultures.

Acknowledgements

This investigation is a simplified version of one devised by Professor Patricia Nevers of the University of Hamburg. In turn, Professor Nevers's protocol was based on that of E. Härle and R. Hausmann at the University of Freiburg.



APPENDIX

Opening an ampoule

CAUTION!
Safety glasses must be worn, as glass splinters may scatter when the ampoule is opened.

1. Heat the pointed tip of the ampoule in a Bunsen burner flame. Rotate the ampoule as you heat it (see diagram).
2. With a pipette, drop, at most, two or three drops of cold water onto the heated tip of the ampoule. *The glass should shatter.*
3. Tap the shattered tip of the ampoule firmly yet carefully with a pair of forceps, catching the shards of glass in a Petri dish base. Ensure that the broken glass is properly disposed of.
4. With a pair of forceps, remove the glass wool plug which retains the inner glass tube within the ampoule.
5. Carefully tip the inner tube out into a sterile Petri dish, replacing the lid on the dish as you do so.

Resuscitating a freeze-dried culture

1. With forceps, remove the cotton wool plug and briefly flame the opening of the inner glass tube.
2. Aseptically add about 1 ml of sterile nutrient broth to the contents of the inner tube.
3. Reflame the opening of the tube and replace the cotton wool plug. Leave the tube for 20 minutes to allow the dried culture to revive.
4. Using a flamed inoculation loop, mix the contents of the tube well and pour them into a sterile test tube containing about 5 ml of nutrient broth solution.
5. Incubate the culture at 30 °C overnight.

The next day...

6. Use a flamed inoculation loop to streak a drop of the prepared suspension onto the surface of a nutrient agar plate. *This is done to check whether the culture has been contaminated—only one type of colony should grow on the plate.*

1

2

3

4

5

Opening an ampoule

Natural gene transfer by *Agrobacterium tumefaciens*

★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★ ★

Plant tumours are a serious nuisance in agriculture, horticulture and viticulture. They often develop after tiny injuries occur to plants through soil cultivation, following frost damage or during grafting.

At the turn of the century it was noticed that certain tumours were always associated with bacterial infection. The bacteria involved were named *Agrobacterium tumefaciens* (latin: *agar* = ground; *tumour* = swelling; *facere* = to do). Only towards the end of the 1970s was light finally shed upon the complex interrelationship between plants and *Agrobacterium*.

The infective bacteria introduce a small piece of their genetic information (a plasmid) into the genome of the plant cells, forcing the infected cells to follow a bacteria-friendly programme. This causes each affected plant cell to divide. A tumour develops which serves as a habitat and provides unusual amino acid derivatives that are required by the uninvited bacterial guests.

The methods of gene transfer used by *Agrobacterium* have been adapted and are used by plant breeders to transfer desirable genes without time-consuming cross-breeding. As a result, modified forms of *Agrobacterium* have become an important tool in gene technology.

***Agrobacterium* is rod-shaped and about the same size as *E. coli*; 1 to 3 µm in length. *Agrobacterium tumefaciens* retains its virulence in the soil, where it lives aerobically in the upper layers. It is a saphrophyte, although it can utilize inorganic nitrogen. *Agrobacterium* invades only dicotyledonous plants and can only enter and infect the plants through wounded areas. This is because the bacteria are unable to penetrate intact plant cell walls. Sap from damaged cells 'lures' nearby bacteria and activates the gene transfer. They multiply in the area around the wound and penetrate the intercellular area, attaching themselves to the plant's cell walls. The *Agrobacterium's* plasmid is transferred and eventually integrated into the plant's own chromosome, from where it directs the production of opines, which the *Agrobacterium* uses as a source of carbon and nitrogen.**

Aims

- In the following investigation *Bryophyllum* = *Kalanchoe* sp. (which is both easy to grow and propagate) is infected under various conditions with the naturally-occurring form of *Agrobacterium*. Within 4 weeks, tumour growth can be observed. The following variations in the experimental protocol can be investigated:

A. Mode of infection:
- scratch a wound and infect immediately with *Agrobacterium*;
- scratch a wound and coat with *Agrobacterium* the following day;
- scratch a wound and infect with *Agrobacterium*. Cover with a damp piece of paper tissue;
- scratch a wound but DO NOT infect it with bacteria;
- apply *Agrobacterium* to unwounded areas of plants.

B. Site of infection:
- stem;
- leaf surface;
- shoot tip.

Advance preparation

1. Propagation of the host plants (students can do this — at home if appropriate);
2. Preparation of nutrient medium and cultures of *Agrobacterium tumefaciens*. fresh cultures must be prepared at least 2 weeks before they are to be used.

Organisation

Growing the host plants (if necessary): 4 months

Preparing the *Agrobacterium* culture: 2 weeks

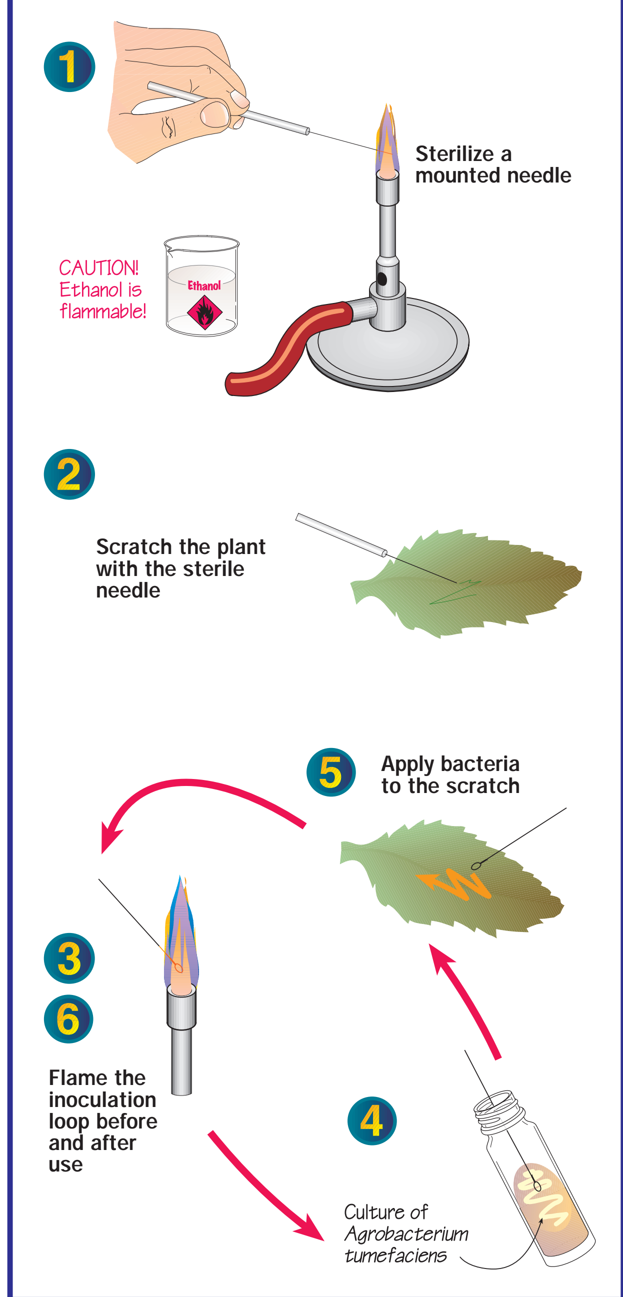
Infecting the plants with *Agrobacterium*: 20 minutes

Observing the tumour growth: 3 to 4 weeks

Equipment and materials

Required by each student or group of students (It is assumed that normal laboratory equipment is also available)

- Sterile water (dispensed into a McCartney bottle)
- Inoculation needle
- Inoculation loop
- Binocular microscope
- Scissors
- Tie-on labels and marker pen
- Paper towels
- Adhesive tape
- Ethanol, for flaming instruments
- Culture of *Agrobacterium tumefaciens* (on nutrient agar)
- *Kalanchoe* (*Bryophyllum*) plants, roughly 3 to 4 months old



Procedure

The plants used in the trial are treated in various ways (see introductory notes, above and the instructions below).

1. Label each plant with the date and what treatment it has received. If necessary, identify the infected parts of the plants *e.g with tie-on labels.*
2. After treatment, place the plants in a well-lit area and keep them damp (but do not overwater them!).
3. Observe and record the development of the tumours during the following 4 to 6 weeks. Examine a piece of the tumour tissue under a binocular microscope and compare it to a piece of tissue from a normal leaf.

How to infect the plants with *Agrobacterium*

Method 1 *(the wounds are infected immediately)*

1. Dip an inoculation needle into alcohol and then ignite the alcohol and allow it to burn off. Scratch the plant's surface one or more times.
2. Infect the wounds with *Agrobacterium* from the culture. To do this, flame an inoculation loop and cool it briefly. Lift up a little of the whitish bacteria culture with the loop and spread it over the wound. Reflame the loop.

Method 2 *(the plants are infected 24 hours after they have been wounded)*

1. Wound the plant (as in Method 1).
2. Spread the wounded area with *Agrobacterium* the next day.

Method 3 *(the wounds are infected, then covered with damp paper tissue)*

1. Wound and infect the plant as described above (Method 1).
2. Cut pieces of paper from towels and dampen them with sterile tap water. Place the strips on the wound and fasten them with adhesive tape. Keep the paper damp for two days.

Method 4 *(wounds are not infected)*

1. Make wounds at the same sites as in Method 1, then on a second site — cover the latter with damp paper. Do **not** treat the wounds with *Agrobacterium.*

Method 5 *(infection without wounding)*

1. Do not wound the plant.
2. Apply use a wire loop to aseptically apply *Agrobacterium* to one or more parts of the unwounded surface.

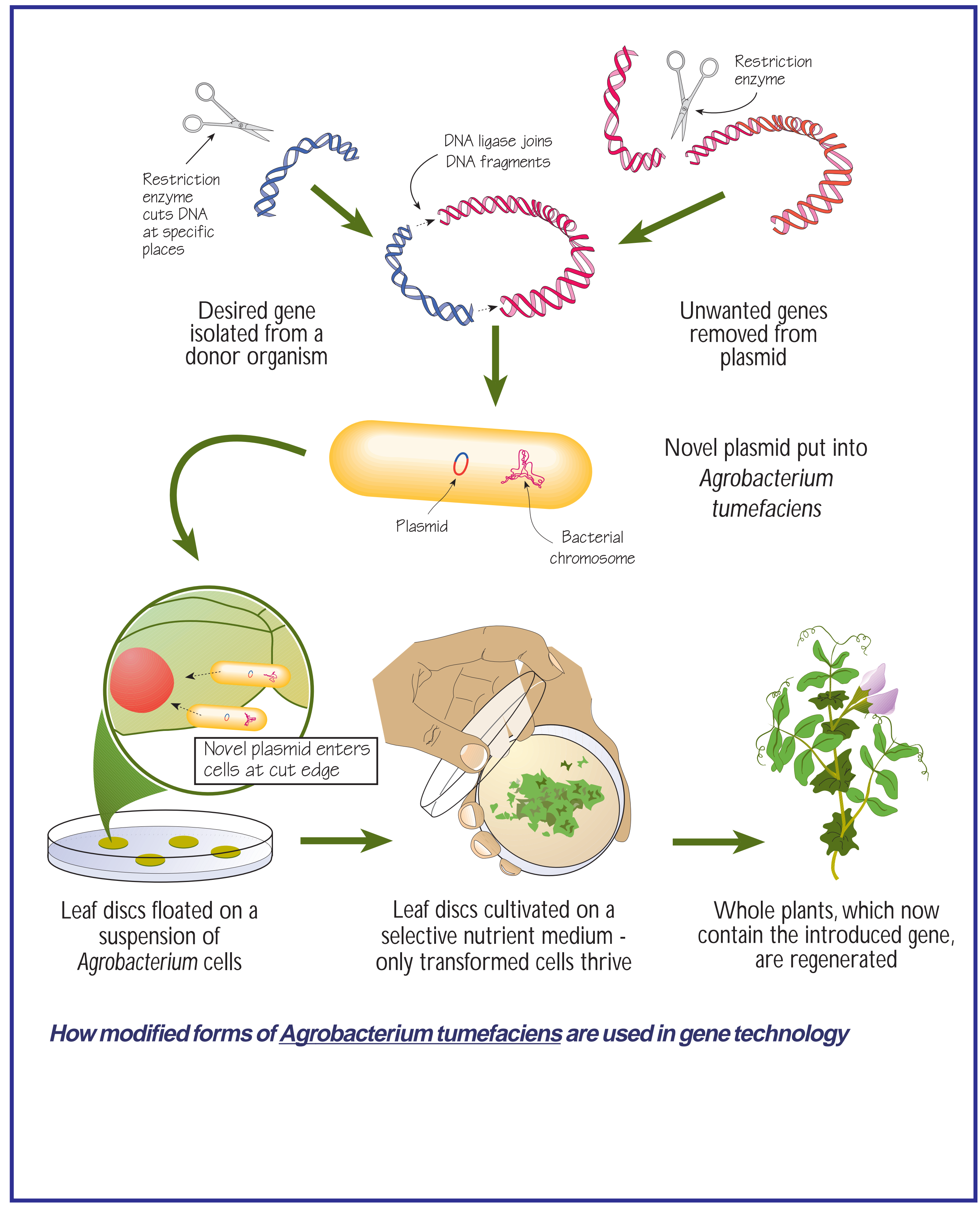
Safety precautions

This work must be done in a laboratory. Standard microbiology safety procedures, including aseptic technique, should be followed when handling microbial cultures.

IMPORTANT: *A. tumefaciens* is a serious plant pathogen. Its use in experimental work is strictly controlled in some countries and regions. Those wishing to carry out such work should ensure that they comply with local regulations.

Acknowledgements

Uta Nellen at the Centre for School Biology and Environmental Education in Hamburg devised this investigation. EIBE is grateful for her permission to use this material.



How modified forms of Agrobacterium tumefaciens are used in gene technology

Appendix 1
Microbiological media

Nutrient agar medium and nutrient broth should be prepared from commercially-available sources, following the directions of the manufacturer. They should be autoclaved before use for 15 minutes at 121 °C. Prepared media may in general be stored at ~4 °C for several months.

Starch agar medium

Nutrient agar	20.7 g
Starch, soluble	2.0 g

Make up to 1 litre with distilled water then autoclave for 15 minutes at 121 °C.

MacConkey agar with streptomycin

MacConkey agar	50.0 g
Distilled water	990 ml

After autoclaving for 15 minutes at 121 °C , allow to cool to 50 °C, then add:

Streptomycin sulphate solution	200 mg/10 ml

These plates must be freshly-prepared. They may be stored for no more than a few days in a refrigerator at ~4 °C.

Appendix 2

Microbiological techniques

UNIT 1

European Initiative for Biotechnology Education

Aerosols

Aerosols are small microbe-laden droplets which may be released into the air by accident, persist for half an hour or more, and be inhaled. They are the major potential source of infection in laboratories. Aerosols from spilt cultures may result in skin and eye infections. Ingestion of microbes is also very likely if cultures are pipetted by mouth.

General laboratory practice

The following precautions should be taken.

No hand-to-mouth operations should occur (e.g. chewing pencils, licking labels, mouth pipetting). Eating, drinking and smoking must not be allowed in the laboratory.

It is strongly recommended that students should wear laboratory coats. Any exposed cuts and abrasions should be protected with waterproof dressings before the practical work starts.

Benches should be swabbed with a laboratory disinfectant before and after each practical session. Suitable disinfectants are available from school science suppliers.

Teachers, technicians and students should always wash their hands after practical work.

Spills and breakages

Accidents involving cultures should be dealt with as follows:

Disposable gloves should be worn. The broken container and/or spilled culture should be covered with a cloth soaked in disinfectant. After not less than 10 minutes, it must be cleared away using paper towels and a dustpan. The contaminated material must be placed in an infected waste container or disposal bag. This must be autoclaved before disposal. The dustpan should also be autoclaved or placed in disinfectant for 24 hours.

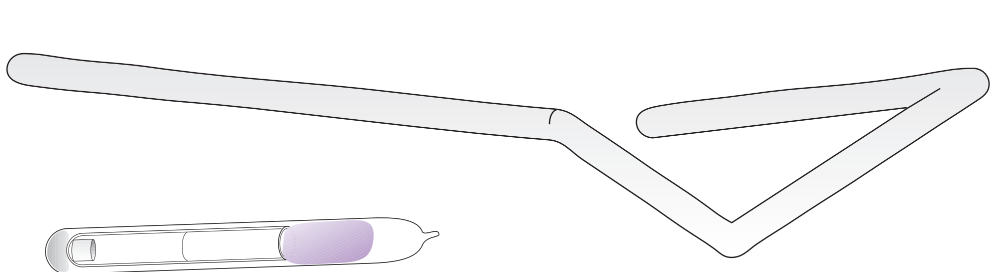
Accidental contamination of skin or clothing

As soon as possible, anyone who has been splashed should wash. Severely contaminated clothing should be placed in disinfectant before it is laundered.

Contaminated cleaning cloths should be autoclaved or soaked in disinfectant.

Sources of microbes

All micro-organisms should be regarded as potentially harmful. The organisms used in the investigations in this Unit present minimum risk given good practice. They should only be obtained from recognised suppliers.



Aseptic techniques

The aims of aseptic techniques are:

a) To obtain and maintain pure cultures of microorganisms;
b) To make working with microorganisms safer.

A 'pure culture' contains only one species of microorganism,whereas a 'mixed culture' contains two or more species.

Contamination of cultures is always a threat because microbes are found everywhere; on skin, in the air, and on inanimate objects. To obtain a pure culture, sterile growth media and equipment must be used and contaminants must be excluded. These are the main principles of aseptic techniques.

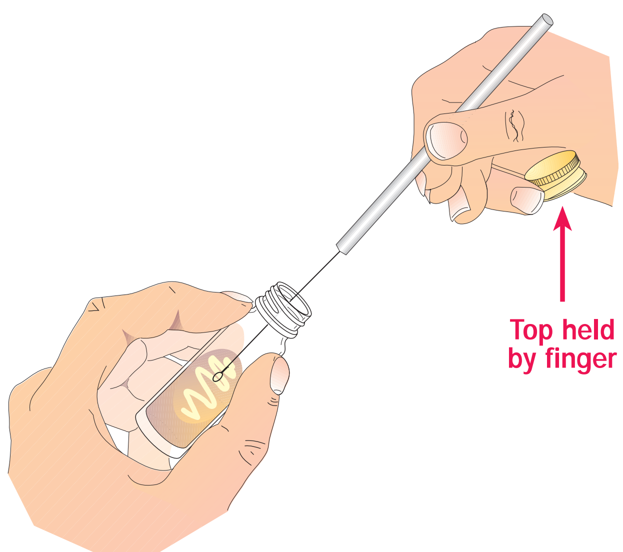
It is unrealistic to expect younger students to be fully accomplished at aseptic techniques. However, some of the activities in this Unit require students to transfer cultures aseptically. In such instances, the following procedures should be adopted.

Growth media should be sterilized before use by autoclaving. Sterile containers (flasks, Petri dishes, etc.) should be used. Lids must be kept on containers to prevent contamination.

Work should be done near a Bunsen burner. Rising air currents from the flame will carry away any microbes that could contaminate growth media and pure cultures.

When cultures are transferred, tops and lids should not be removed for longer than necessary. After a lid has been taken from a bottle, it should be kept in the hand until it is put back on the bottle. This prevents contamination of the bench and the culture. After removal of the top, the neck of the culture bottle should be flamed for 1–2 seconds. This will kill any microbes present there and cause convection currents which will help to prevent accidental contamination of the culture from the atmosphere.

With practice, it is possible to hold the bottle in one hand and the wire loop in the other in such a way that the little finger is free to grip the bottle top against the lower part of the hand. (In this case, it is important that the bottle top should be loosened slightly before the inoculation loop is picked up.) If necessary, two students may work together to perform these operations.



Inoculation loops should be heated until they are red hot along the entire length of the wire part. This should be done both before and after transfer of cultures takes place. They should be introduced slowly into the Bunsen burner flame to reduce sputtering and aerosol formation.

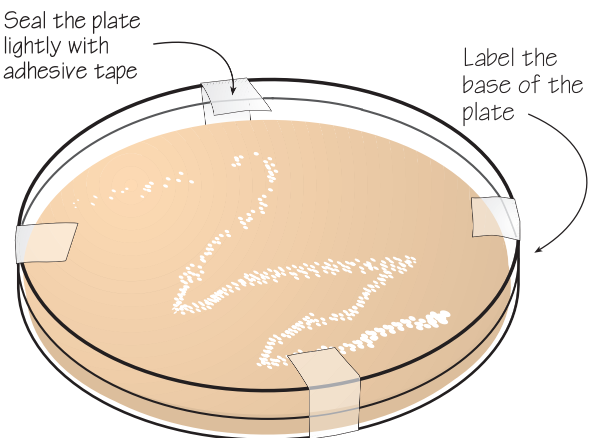
When the Bunsen is not in use it should be kept on the yellow flame, so that it can be seen easily. A blue flame about 5 cm high should be used for sterilizing loops or wires and flaming the necks of bottles.

Avoid contaminating the work area. Instruments should be sterilized immediately after use and used pipettes should be placed directly into a jar of fresh disinfectant solution.

Incubation of cultures

Label the Petri dish on the base, before inoculation. A name, date and the name or source of the organism used will allow the plate and its contents to be identified.

Where appropriate, use adhesive tape to seal Petri dishes as shown below:



The seal will ensure that the plates are not accidentally opened or tampered with. Note: do not seal plates completely round the edges as this could create anaerobic growth conditions within the dish.

Bacteria
Bacterial cultures in Petri dishes should usually be incubated with the base uppermost, so that any condensation that forms falls into the lid and not on the colony. (If there is heavy condensation in the sterile Petri dish before inoculation, it should be dried before use.)

After 2–3 days of incubation at 25–30 °C, bacterial colonies will be seen.

Fungi
Petri dishes containing fungi do not need to be inverted. Fungal cultures should be incubated for 7 days or so. Room temperature (~21 °C) is sufficient to allow their growth, although an incubator gives greater control.

Disposal and sterilization

It is very important to dispose of all the materials used in a practical class properly to avoid contamination of the laboratory and people. All containers used for storing and growing cultures must be autoclaved, then washed in disinfectant and rinsed, before re-use.

Two autoclave bags should be available in the laboratory: one for reusable glassware and another for disposable materials. There should be a large and a small discard jar near each work area for pipettes and microscope slides. A metal bucket should be available for disposal of any broken glassware.

Disposable plastic pipettes, microscope slides and any liquid from cultures should be put into the small disinfectant pot. Plastic pipettes are then autoclaved and disposed of, microscope slides should be soaked in disinfectant for 24 hours then washed and rinsed before re-use.

Glass pipettes should be put in the larger pot. Do not take the pipette filler off the end of the pipette until the tip is in the disinfectant, otherwise aerosols can be created. Dirty pipettes should be autoclaved, washed and rinsed before they are used again.

Contaminated paper towels, cloths and plastic Petri dishes should be put into the disposable items autoclave bag.

Any contaminated glassware (including used glass Petri dishes) should be put into the autoclave bag for glassware.

Glassware that is not contaminated can be washed normally. Broken glassware should be put in a waste bin reserved exclusively for that purpose. If the glassware is contaminated it must be autoclaved before disposal. Uncontaminated glassware can be disposed of immediately.

Autoclaving

Sterilization means the complete destruction of all microorganisms, including their spores.

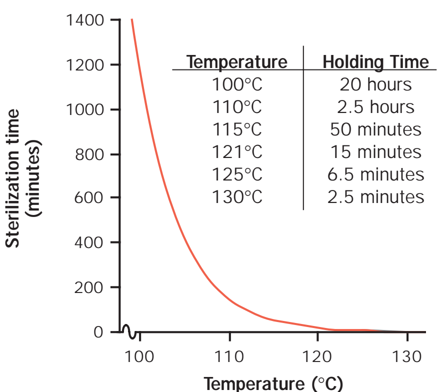
All equipment should be sterilized before starting practical work so that there are no contaminants. Cultures and contaminated material should also be sterilized after use for safe disposal.

Autoclaving is the preferred method of sterilization for culture media, aqueous solutions and discarded cultures. The process uses high pressure steam, usually at 121°C. Microbes are more readily killed by moist heat than dry heat as the steam denatures their protein. Autoclaving can be done with a domestic pressure cooker or a purpose-built autoclave. Domestic pressure cookers can be used in school laboratories but their small capacity can be a disadvantage when dealing with class sets of material.

Principles of autoclaving

Two factors are critical to the effectiveness of the process. Firstly, all air must be removed from the autoclave. This ensures that high temperature steam comes into contact with the surfaces to be sterilized: if air is present the temperature at the same steam pressure is lower. The materials to be sterilized should be packed loosely so that the air can be driven off. Screw-capped bottles and jars should have their lids loosened slightly to allow air to escape and to prevent a dangerous build-up of pressure inside them.

Secondly, sufficient time must be given for heat to penetrate (by conduction) to the centre of media in Petri dishes or other containers. The times for which media or apparatus must be held at various temperatures for sterilization are shown below:



Notice that just a small difference in temperature can result in a great difference in the time required for sterilization. It is also important that these temperatures are reached by all materials to be sterilized for the specified time e.g. the broth in the very centre of a fermenter vessel.

Hence three factors contribute towards the duration of the autoclaving process:

- **penetration time**
  the time taken for the centre-most part of the autoclave's contents to reach the required temperature;
- **holding time**
  the minimum time in which, at a given temperature, all living organisms will be killed;
- **safety time**
  a safety margin; usually half the holding time.

Domestic pressure cookers operate at 121°C. Thus the total sterilization time might typically be: penetration time, say 5 minutes; plus 15 minutes holding time; plus a safety margin of 5 or so minutes, giving a total time of 25 minutes.

Vessel	Volume	Holding time
Test tube	20 ml	12–14 minutes
Flask	50 ml	12–14 minutes
Flask	200 ml	12–15 minutes
Fermenter	1 litre	20–25 minutes

Caramelization

Purpose-built autoclaves sometimes operate above 121 °C, and whilst the savings in time they offer can seem beneficial, it should be remembered that higher temperatures are detrimental to certain media. Glucose solution, for example, is caramelized at high temperatures, forming compounds which may prove toxic to microbes. In the case of glucose this reaction can be avoided by adjusting the pH of the medium to 4. After sterilization the pH can be re-adjusted as necessary.

Maillard reactions

A browning reaction (the Maillard reaction) can also be caused by the interaction of nitrogenous compounds and carbohydrates in the medium at high temperatures. Here too the compounds formed are toxic to some microbes, so it may in some circumstances be necessary to autoclave the carbohydrate and the remainder of the medium separately e.g. in the preparation of milk agar.

Use and routine care of autoclaves

The manufacturer's instructions should always be followed when using a pressure cooker or an autoclave. Particular care should be taken to ensure that there is enough water in the autoclave so that it does not boil dry during operation. A domestic pressure cooker requires at least 250 ml of water - larger autoclaves may need considerably greater volumes. The use of distilled or deionised water in the autoclave will prevent the build-up of limescale. Autoclaves should be dried carefully before storage. 'Pitting' of the autoclave base may occur if this is not done, seriously weakening the vessel and so causing it to 'bow' outwards under pressure.

When the autoclave is used, before the exit valve is tightened, steam should be allowed to flow freely from the autoclave for about one minute to drive off all the air inside. After the autoclave cycle is complete, sufficient time must be allowed for the contents to cool and return to normal atmospheric pressure. The vessel or valves should not be opened whilst under pressure as this will cause scalding. Premature release of the lid and the subsequent reduction in pressure will cause any liquid inside the autoclave to boil. The agar or broth will froth up and it may boil over the outside of the containers.

Chemical sterilization

Many different chemicals are used for sterilization. The most commonly used disinfectants in laboratory work are clear phenolics and hypochlorites.

Clear phenolics are effective against bacteria and fungi but inactive against spores and some types of virus. They are inactivated to some extent by contact with rubber, wood and plastic. Laboratory uses include discard jars and disinfection of surfaces.

Hypochlorites (such as bleaches) are not ideal for sterilization of used Petri dishes etc. as they can be inactivated by protein and plastic materials. However, a 5% solution of *Domestos* (Lever) or Chlorate I solution is suitable for use in discard jars.