Bio Factsheet

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Practical Techniques in Microbiology I: Culturing bacteria

This Factsheet will describe the following techniques involved in culturing bacteria in the laboratory:

- **Safety in the microbiology laboratory;**
- **Culture containers and instruments;**
- **Types of media and their preparation;**
- **Plating out;**
- **Inoculation and culture techniques;**
- **Anaerobic cultures.**

Factsheet No 71 'The control of bacteria' (April 2000) is also relevant to this topic.

Introduction

Bacteriological investigations usually involve growing pure cultures in a nutrient medium under controlled conditions of temperature (usually 37°C) and pH (usually around pH 7.4). The nutrient media must contain a source of:

- **carbon** (e.g. glucose, another simple sugar, or a salt of an organic acid, e.g. sodium ethanoate);
- **nitrogen** (usually amino acids, peptides, or ammonium salts);
- **mineral salts** (commonly required positive ions are those of calcium, potassium, sodium, and iron; commonly required negative ions are chloride, phosphate, and sulphate)
- **water**.

Also the bacteria must have a source of **energy**:

- **Heterotrophs** gain this by the oxidation of sugars.
- **Photoautotrophs** have the power of photosynthesis and require light.
- **Chemoautotrophs** gain energy by oxidising inorganic chemicals such as ammonia and nitrite.

Trace amounts of growth factors or **vitamins** (particularly some B vitamins) are often needed. Bacteria are usually cultured in a solid medium or a liquid medium. Media are solidified with **agar**, (a transparent complex polysaccharide derived from red algae), which acts as a gelling agent into which the nutrients are mixed. The agar itself has no nutritional value to the bacteria.

Safety in the microbiology laboratory

Remember that microorganisms are all around – on clothes, hands, in the air, on work surfaces, on apparatus. These microorganisms must not be inadvertently cultured during laboratory work, because they will contaminate experiments and they may be pathogenic. Also, although the organisms commonly used in schools and colleges are harmless, there is no guarantee of this – they may mutate into harmful forms or be contaminated with harmful forms. **For these reasons aseptic techniques must always be applied rigorously to minimise risks to yourself and to colleagues.**

Basic safety rules for microbiology are listed in the appendix at the end of the factsheet.

***Exam hint** – microbiology exam questions frequently test the candidate's knowledge about microbiological safety. It is important to do microbiology practical work to gain experience of the safety procedures involved.*

Culture containers and instruments

Wire loops and wires are used for inoculating cultures. Because they have to be exposed to red heat they have long metal handles (glass would break and wood would burn).

Fig 1. Inoculating instruments.

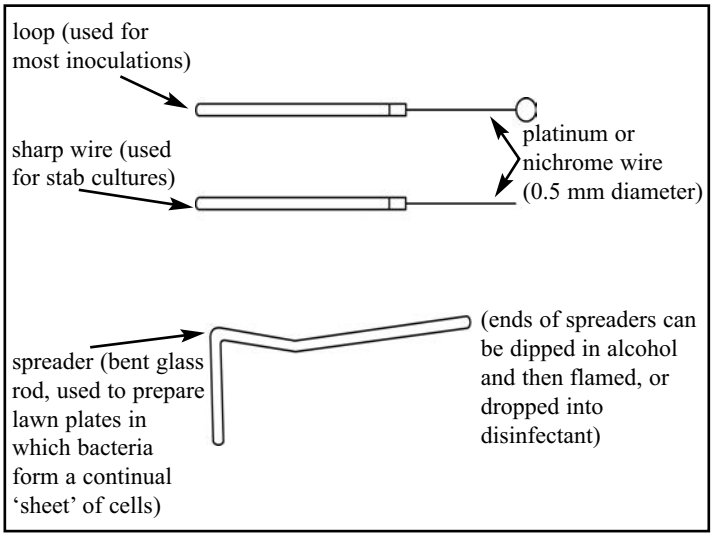
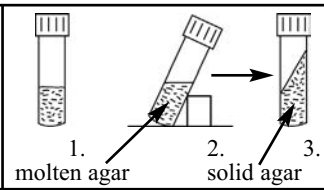


Table 1. Culture containers

Petri dishes (for agar plates)	Sterile plastic dishes are used to grow microbes on solid agar before disposal by autoclaving. Glass dishes must be autoclaved before reuse.	
Bacteriological tubes	These can hold 10 – 20 cm^3 of solid or liquid media. They have a loose metal cap or cotton wool bung for easy removal and replacement when transferring inoculum. 1. is an agar deep-medium allowed to solidify in an upright position. 2. and 3. are solid agar slopes.	
Screw capped glass bottles (McCartney bottles)	These have 20 – 30 cm^3 capacity. They have plastic screw caps which require some dexterity when removing and replacing when transferring inoculum.	
Medicinal flats	These are flat glass medicine bottles of 100 cm^3 and 250 cm^3 capacity. They are mainly used to store large volumes of prepared sterile agar media. They may take about an hour for the agar to melt but a large number of plates can then be poured.	

Sterile (autoclaved) pasteur pipettes and 1.0, 5.0 and 10.0 cm^3 may also be required. They should have a small cotton wool plug pushed into the mouth before autoclaving, and are preferably sterilised and stored in a metal container.

If your school does not possess an autoclave a good pressure cooker can be used instead. You will also need a constant temperature incubator to grow the bacteria efficiently. An inoculating cabinet is also helpful (but not essential). This protects the operator from the bacteria being handled and minimise the risk of contamination from air-borne organisms.

Types of media and their preparation

A wide variety of nutrient media capable of supporting bacterial growth are commercially available in a ready-prepared, dehydrated state. The ingredients can be dissolved in water in a large beaker, and then dispensed into suitable containers ready for autoclaving. Medical flats must only be partly filled, leaving an air-gap of 20-30 cm^3. 10–20 cm^3 volumes are dispensed into bacteriological tubes or McCartney bottles. The liquid media used in bacteriology are known as broths, and are prepared as follows:

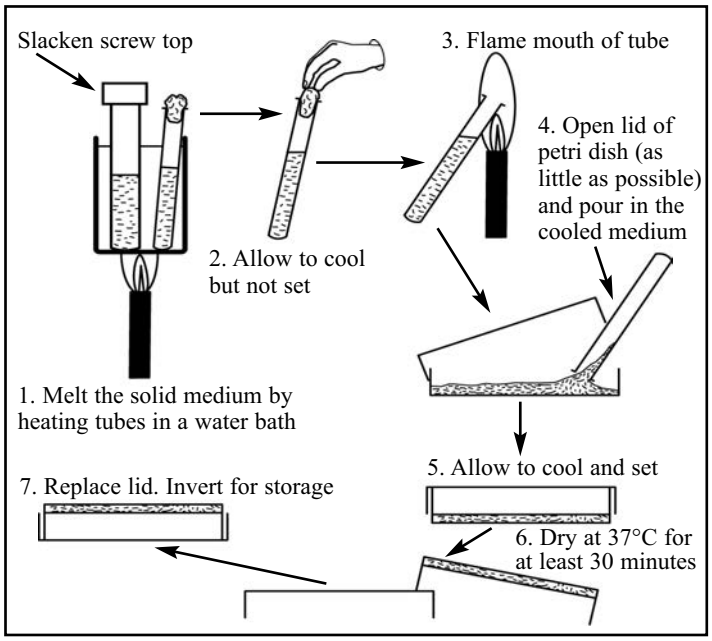
- Dissolve 10g meat extract, 10g of peptone and 5g of sodium chloride in 1dm^3 of tap water. Heat gently if necessary to dissolve the ingredients.
- Adjust the broth to pH 7.4 using drops of 1M NaOH or 1M HCl. Dispense the broth into suitable containers (McCartney bottles).
- Autoclave at 103.4 kPa (15 psi), 121°C for 15 minutes. Ensure that bottle caps are only lightly screwed on before autoclaving to avoid bursting.
- After autoclaving and cooling, screw down the bottle caps firmly.

Nutrient agar is prepared in the same way as nutrient broth but 20g of plain agar is dissolved into the mixture before autoclaving. After sterilization, when the medium is still molten, agar deeps, agar slopes or agar plates can be prepared, as required.

Plating out

Stored media must be melted down prior to dispensing into sterile petri dishes. Small volumes of medium, enough for one plate, will melt quickly, but 250 cm^3 medical flats of media will require about an hour for melting. However, they will contain sufficient medium to pour 15 to 20 plates.

Fig 3. Pouring an agar plate.



***Remember** - in the above technique, the plates should be protected from atmospheric contamination as much as possible. The plates are stored inverted so that any condensation falls into the lid, not onto the agar.*

Inoculation and culture techniques

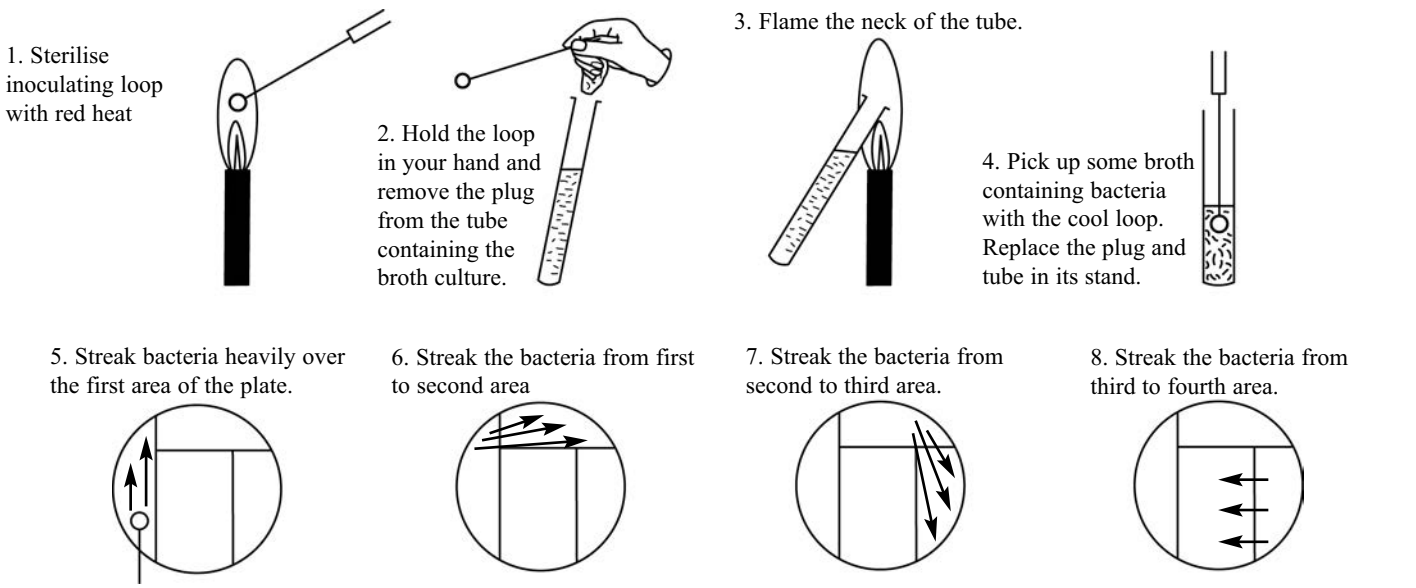
It is important to inoculate from young cultures, preferably in the exponential phase of growth. If you use an inoculum from old cultures growth may be poor and atypical.

Various types of culture may be used, for example, streak plates, pour plates, confluent plates, agar slopes, agar deeps, and broth cultures. Bacterial cultures are usually incubated overnight at 37°C.

(a) Streak plates

This technique is used to separate mixed cultures into pure cultures. The individual bacterial colonies that are formed can be used to inoculate fresh agar plates or broth tubes.

Fig. 2. Preparing a streak plate

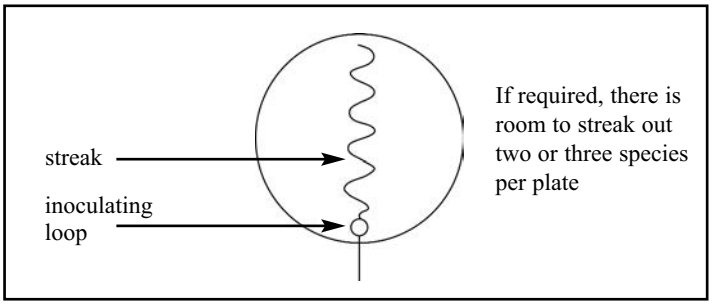


Use the cool inoculating loop to streak the bacteria in actions 5, 6, 7 and 8, being careful not to scratch or dig up the agar surface. The loop must be sterilised by red heat and allowed to cool between each of these actions. To reduce the risk of contamination avoid breathing on the plates and do not expose them unduly to the atmosphere. The streaking sequence should 'thin out' the bacteria so that single colony growth can be achieved.

After incubation at 37°C for about 12 hours, the plate should show heavy continuous growth cover in areas 1 and 2, less growth cover in area 3, but individual colonies in area 4. The individual colonies may have differing appearances due to their consisting of different single species.

Using a flamed, cooled inoculating loop, a single colony can be sampled and streaked out on an agar plate. When this grows it should be a pure culture of the organism. Alternatively the single colony may be inoculated into a broth culture for incubation and growth.

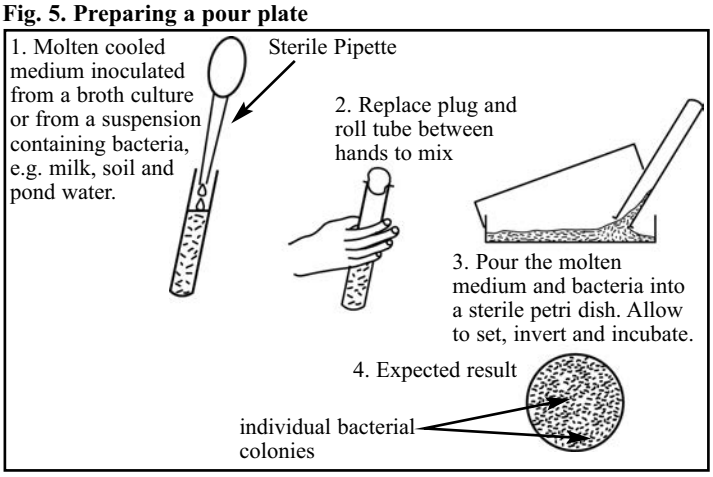
Fig. 4. Streaking a plate for pure cultures



(b) Pour plates

Each colony formed grows from a single live bacterium in the inoculum. Thus the method can be used to count the number of viable bacteria in a liquid sample.

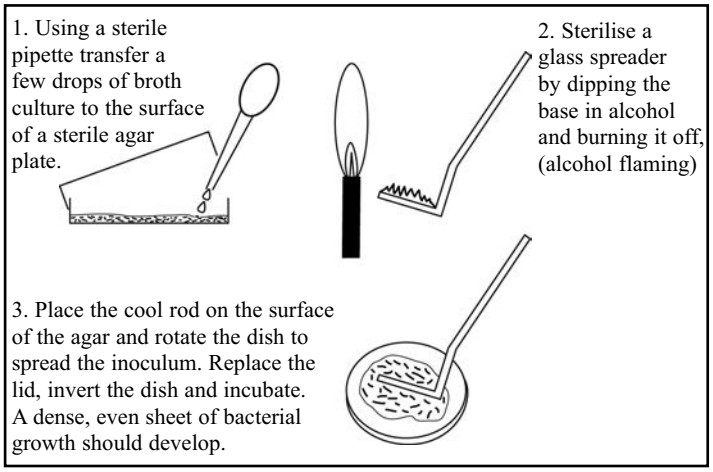
Fig. 5. Preparing a pour plate



(c) Confluent plates

In these, a dense sheet of bacterial growth is formed over the surface of the medium.

Fig. 6. Preparing a confluent plate

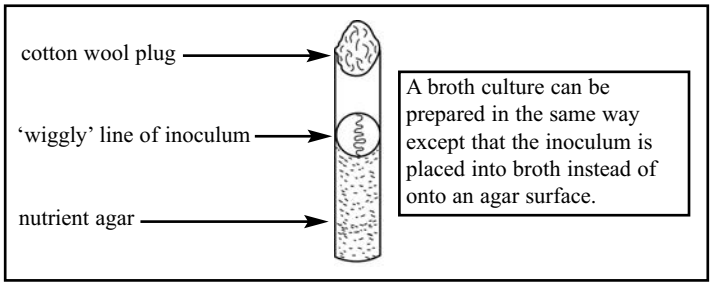


(d) Agar slopes

These are used to grow aerobic bacteria and are useful to maintain 'stock cultures'. This is because they do not occupy much space and are easy to subculture weekly.

- Follow steps 1 to 4 described above under 'preparing a streak plate' to obtain a loop charged with inoculum.
- Remove the stopper from the agar slope tube and flame the mouth of the tube.
- Touch the surface of the agar with the charged loop and pull it upwards slowly, streaking out a 'wiggly' line.
- Replace the stopper, replace the tube in its stand and incubate it.

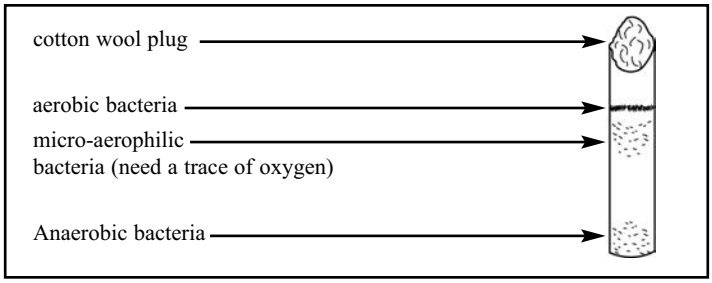
Fig 7. An agar slope.



(e) Agar deeps

These are used to grow anaerobic organisms or to separate aerobic and anaerobic organisms. They are prepared in a similar way to the agar slope described above, except that instead of using an inoculation loop an inoculation wire is used to stab deep into the agar medium. After incubation the following types of organism may grow.

Fig. 8. An agar deep.



Anaerobic cultures

Any of the culture techniques described above can be used to grow anaerobic organisms, but the cultures must be grown in the absence of oxygen, particularly in the case of obligate anaerobes. In these organisms growth will be inhibited by the presence of oxygen.

The culture plates (or bottles) can be kept in anaerobic jars. These are evacuated of air (containing oxygen) and the air is replaced by an atmosphere usually consisting of 80% nitrogen, 10% carbon dioxide and 10% hydrogen. These gases are generated by packets of chemicals placed in the jar before sealing and clamping the lid. Initially the lid valves are open to enable the generated gases to push out the air (oxygen). When this has happened the valves are closed.

An alternative way of providing an anaerobic atmosphere is to vent nitrogen from a gas cylinder in through the open valves, closing the valves when air is evacuated.

Anaerobic jars are available to contain 9, 12 or 48 petri dishes.

Exam Hint *– exam questions are often set to test the practical experience of candidates. You may be asked to describe media preparation or inoculation and culture techniques.*

Practice Questions

1. (a) Distinguish 'nutrient broth' from 'nutrient agar' 2
   (b) What are the essential basic contents of any bacteriological nutrient media? 6
   (c) What are the main aims when growing bacteria on a streak plate? 3
   (d) What is the minimum recommended pressure, temperature and time for sterilisation in an autoclave? 3
   (e) Disinfectants must be applied 'strong enough for long enough'. Suggest why. 3

2. (a) Describe how you would obtain pure broth cultures of the two aerobic organisms, *Escherischia coli* and *Staphylococcus aureus* from a mixed broth culture of the two organisms. ***5***
   (b) Outline how you would prepare pure plate cultures of a soil water mixture of obligate anaerobic bacteria species obtained from waterlogged clay soil. 4

3. (a) Suggest how you can reduce the cross-infection risks involved when red-heat flaming inoculation loops. 3
   (b) Why are agar plates and plate cultures incubated and stored upside down? 2
   (c) What do you understand by the terms 'stock culture' and 'subculturing'? 3

Answers

1. (a) nutrient broth remains in liquid form, nutrient agar sets as a gel/is solid;
   bacteria grow in nutrient broth but usually on the surface of nutrient agar: 2
   (b) a source of carbon/glucose/simple sugar/salt of organic acid;
   a source of nitrogen/peptides/peptone/amino acids/ammonium salts;
   mineral salts/any two correct cations and any two correct anions stated: water; energy source; vitamins/growth factors/ B-vitamins; 6
   (c) to separate two or more species of bacteria from mixed culture/source;
   streaking technique reduces the density of bacterial growth resulting in isolated colonies;
   these can then be subcultured/harvested and grown in pure culture; 3
   (d) 15 psi/103.4 kPa; 121°C; 15 minutes; 3
   (e) not all bacteria will be equally susceptible to the disinfectant; thus it must be strong enough to kill/inhibit the most resistant individuals; and be allowed to act long enough to reduce the bacterial population to a safe level;; 3

2. (a) prepare a streak plate from an inoculum of the mixed broth culture;
   correct details of how this is done;; *(2 marks – details of inoculation /streaking technique/ incubation)*
   use isolated S. aureus colonies as inoculum for a broth culture and isolated E. coli colonies as inoculum for the other broth culture;
   some correct practical detail of inoculating/incubating/aseptic technique involved in producing a broth culture; 5
   (b) ref to the need to grow all cultures in anaerobic jars/in the absence of oxygen;
   prepare a broth culture using the soil water mixture as inoculum (to increase the population density of the bacteria);
   prepare a streak plate from an inoculum of this broth culture;
   subculture distinct isolated colonies onto sterile agar plates and incubate anaerobically; 4

3. (a) never overload inoculation loop because residual material could be dispersed as an aerosol (particularly when nearing the flame);
   always draw the loop through the flame from behind so that the flame is between you and the bacteria/any formed aerosol;
   work under a safety hood/do not allow other people near to the flame when flaming/flame contaminated loops promptly before they touch anything else; 3
   (b) because condensation may occur (from water in the agar);
   it is safest if the drops of water collect in the lid rather than on the bacterial growth; 2
   (c) a 'stock culture' is a culture that is maintained to provide a supply of a particular bacterial species/microorganism over a long period of time;
   it is usually an agar slope culture and needs regular subculturing for continuity;
   'subculturing' is the term used to describe the inoculation of fresh medium using bacteria/microorganism from an old culture; 3

Appendix

1. Always wear a laboratory coat when working in the laboratory. This will protect your clothes from contamination with the cultures you handle and will protect your clothes from splashes which may occur. Laboratory coats should not be taken out of the laboratory because of the risk of spreading contamination (unless in a sealed laundry bag).
2. Cover any minor cuts on exposed parts of your body with a surgical plaster before working with microbes.
3. Always wipe down the top of your bench with disinfectant at the beginning and end of each laboratory period. All used cotton-wool or absorbent paper must be discarded for burning.
4. Hand-to-mouth operations such as eating, smoking and licking of labels are forbidden. Dampen sticky labels with water, (not with your tongue), or mark containers with a felt pen or wax pencil.
5. Never pipette cultures by mouth. Always use a teat pipette for transferring small volumes of liquid cultures. Do not forcefully squeeze the liquid from the pipette because this can produce an aerosol.
6. If a culture is spilt tell your supervisor at once so that appropriate remedial action can be taken. If an accident occurs, always report it to your supervisor.
7. When transferring micro-organisms, properly sterilise the inoculating instrument before and after the transfer. The inoculating wire should be raised to red heat on each occasion. Be careful when loops are contaminated with inoculum because splattering and aerosol formation can occur. (It is safest, for the operator, to bring the loop into the flame on the side furthest from the operator – move the loop through the flame towards the operator).
8. The opening of any container containing a culture can produce an aerosol. Only open containers if necessary. Never sniff the cultures unless you are told that it is safe to do so.
9. Never remove cultures from the laboratory.
10. Used cultures, petri dishes or other glassware should be autoclaved after the experiment. Microscope slides with live mounts must be immersed in disinfectant immediately after use.
11. Thoroughly wash and dry your hands before leaving the laboratory. Hot water, soap and disposable towels must be available.

All used cultures must be disposed of immediately, because they are a hazard to other workers and may contaminate other cultures:

1. Plastic petri dishes containing agar cultures must be autoclaved, in a small metal bucket or in an autoclave bag and then disposed of via the refuse bin.
2. Cultures grown in glassware must be autoclaved and the glassware then washed for further use. After autoclaving, the warm liquid medium can be flushed away down a sink with hot water.
3. Immerse used microscope slides in a beaker containing freshly prepared 2% Chloros.
4. Immediately place used pipettes in jars with freshly prepared 2% Chloros. Wash and sterilise the pipettes later.
5. Treat spillages of cultures on clothing with a non-bleaching, non-staining disinfectant, for example 1% Cetavlon
6. Spillages of cultures are best dealt with by swabbing the contaminated area with a strong sporicidal disinfectant, for example, 10% Chloros. (Lysol is not recommended as it is not sporicidal, and it is also a strong irritant).

Acknowledgements
This Factsheet was researched and written by Martin Griffin.
Curriculum Press. Unit 305B, The Big Peg, 120 Vyse Street, Birmingham B18 6NF