Bio Factsheet

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Practical Techniques in Microbiology II: Measuring the growth of microorganisms

This Factsheet covers the techniques involved in measuring the growth of:

Bacteria and other unicellular organisms by:

- *Haemocytometry -using a microscope counting chamber to count the actual cells*
- *Optical density – using the effects of the micro-organism on the light absorption of the culture medium*
- *Dilution plate counting – using the growth of single cells into colonies to count number*

Filamentous organisms by:

- *Mycelial growth rate – measuring the expansion of a circular inoculum on a solid medium*
- *Dry mass – measuring the increase in dry mass of an inoculum in a liquid culture*

Introduction

Growth is commonly defined as a permanent increase in size of an organism as it undergoes development. Although individual cells of unicellular micro-organisms can be measured, they are very small, so individual growth patterns are difficult to follow. It can be measured in a number of ways. For unicellular micro-organisms like bacteria, yeast and protoctists the most usual way is to estimate the increase in population over time. Filamentous fungi are measured either by increase in mass or the rate of extension mycelium, or the tip of a hypha.

Bacteria and other unicellular organisms

Bacteria are most commonly used to investigate growth in micro-organisms. There are a number of bacteria available that are safe to use in school laboratories. Bacteria and yeast reproduce on such a way that the population doubles every generation *(Fig. 1)*. To find the growth of a population, a liquid culture is inoculated with bacteria and the number of cells counted over a period of time. The results are plotted to give a growth curve *(Fig. 2)*. As the numbers are very large, log values are frequently plotted against time. You can use a calculator to find the log number to plot.

Exam Hint: *Microbiology questions often refer to the growth curve of a bacteria population.*

Four stages (phases) can usually be recognised:

- **Lag phase.** There is no apparent increase in cell numbers. Individual bacteria may increase in size. The length of the lag phase depends on the temperature, the medium and the state of activity of the bacteria. Enzymes may need to be synthesised to utilise the nutrient medium.
- **Log (or exponential) phase.** The population doubles each generation. The expected number of bacteria can be calculated during the log phase using the relationship: $N = N_0 \times 2^n$ (N_0 = number of cells at the start, n = number of generations). The population can increase exponentially as there are no limiting factors.
- **Stationary phase.** The population remains the same as the number of new cells formed is equal to the number of cells dying. Limiting factors, such a nutrient supply, have started to influence further increase in population size. The micro-organisms also produce changes in the environment. Their metabolic products may be toxic, for example ethanol kills yeast. Other metabolic products may change the environment so it is unsuitable for further growth; for example *Lactobacillus* produces lactic acid, which lowers the pH.
- **Death phase.** Individuals unable to compete successfully die and undergo autolysis (self digestion). These may provide a limited source of nutrient for some cells, but eventually, all die as energy sources run out.

Fig.1 Bacteria reproduction

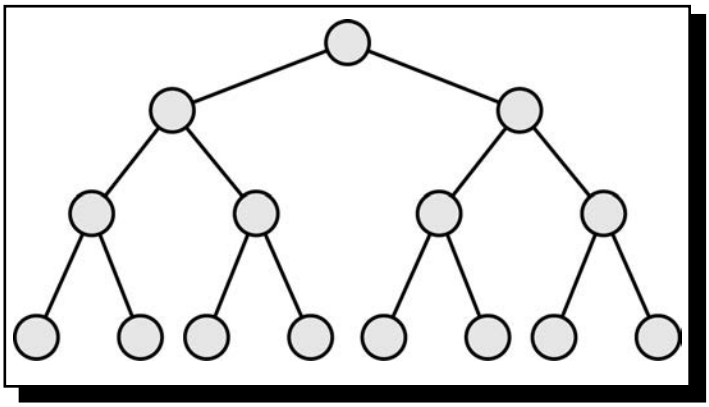
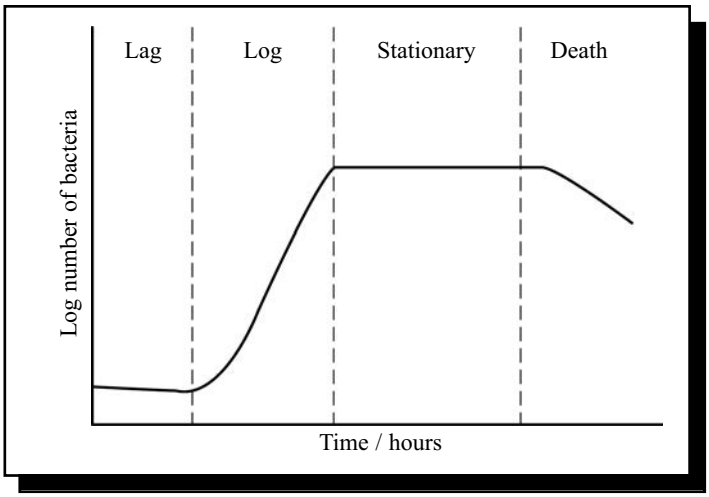


Fig. 2. Bacteria growth curve



Remember: *When investigating the growth rate of a population a sterile technique is essential. Any other micro-organisms from the environment will compete for resources, affecting both the growth rate and the final population size.*

Exam Hint: *You may be asked to compare growth rates in different conditions or in relation to the oxygen and nutrient supply*

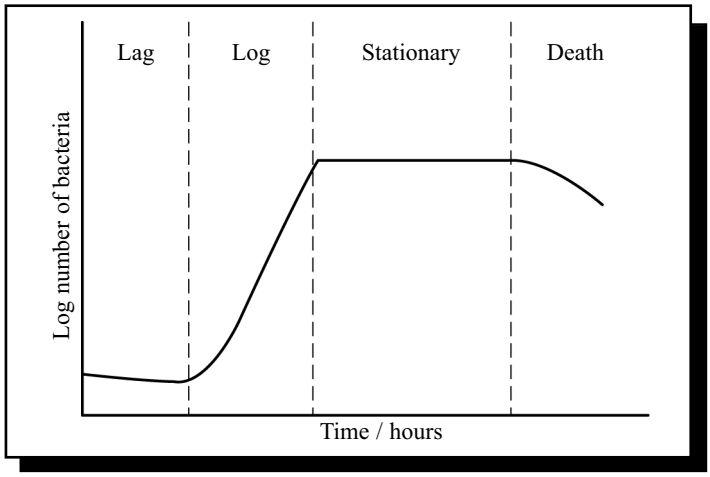
Filamentous organisms

Fungi grow by elongation of hyphae. In a liquid medium the fungus forms mats or pellets that can be removed and the dry mass obtained. In these conditions the fungus shows four phases of growth:

- **Lag phase.** This occurs for the same reasons as on a solid medium.
- **Rapid growth phase.** In some species, the cube root of the dry mass increases linearly with time. Different fungi double in mass in different times.
- **Retarded growth phase.** The growth slows down for the same reasons as for a solid medium.
- **Death phase.** The dry mass decreases as the mycelium undergoes autolysis.

The growth curve is very similar to that of bacteria.

Fig. 3 Fungal population growth in liquid medium.



On a solid medium the rate at which the fungal colony increase in diameter can be used to estimate growth. However if comparisons are being made between different media this method is unreliable. On a solid medium, three growth phases occur.

- **Lag phase.** A spore takes some time before it germinates. A mycelial disc has broken hyphae, which are repaired before growth starts. An old inoculum has a longer lag phase.
- **Rapid growth phase.** The colony expands linearly with time. The increase is constant with time.
- **Retarded growth phase.** The growth slows down. This is usually due to accumulation of toxins. Contact with the sides of the dish may also limit growth. Autolysis of hyphae at the centre of the colony may occur.

Factors affecting the growth of micro-organisms

These include temperature, pH, oxygen, nutrients and antibiotics.

Methods of measuring bacterial growth

There are two main methods used for bacteria:

- **Total counts**, in which all cells are included. They include dead cells which have not yet undergone autolysis.
- **Viable counts** include only living cells.

Remember: *All of the methods involve removing samples from cultures at different points in time. Sterile technique is used throughout the measuring technique being used.*

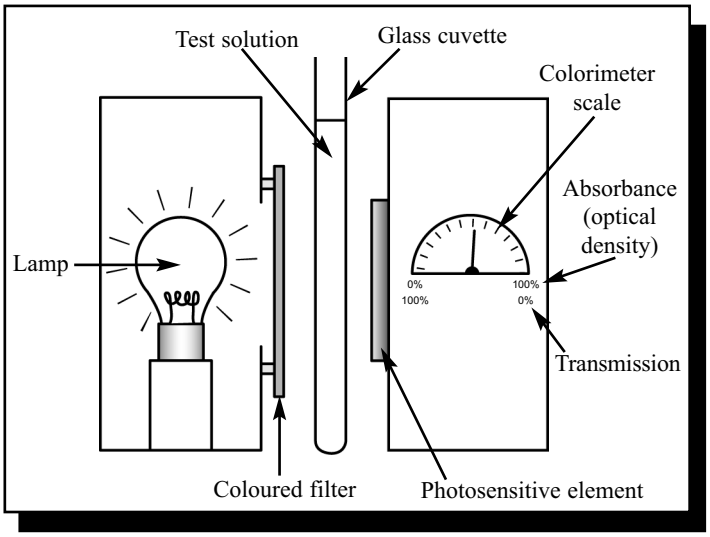
- ***Indirect total count:***

In this case the effects of the micro-organism growing in a liquid culture medium is used. One method is to measure changes in **optical density**.

As a microbial culture grows, the medium changes and the additional cells make the culture more **turbid**. The more cells present, the more turbid the culture and the greater the optical density i.e. the less light can pass through.

Turbidity is measured using a colorimeter. A colorimeter passes a beam of light through a liquid. The cells and the culture solution absorb some of the light (absorbance) and the rest passes through (transmission) and is detected by a photo-electric cell. The current from the photo-electric cell depends on the intensity of light reaching it and thus the greater the turbidity of the sample, the smaller will be the current. The current is shown as a reading on the colorimeter scale as the % transmission and the % absorbance.

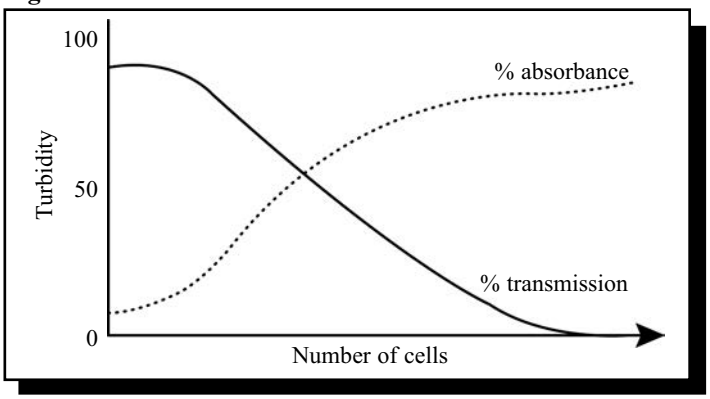
Fig. 4 A colorimeter.



To use a colorimeter place a sample of the nutrient growth medium in a special container, called a **cuvette**. These are made of a special type of plastic or glass that does not interfere with the light passing through. A red filter is used. Place the cuvette in the colorimeter. Switch on the light and set the absorbance to 0 (or transmission to 100%). Keep this cuvette and use it between every reading to reset the colorimeter. This is called a colorimeter blank and removes the effects of the culture medium form the readings. Place a sample of the culture being measured in a different cuvette and read the % transmission. Take samples at fixed time intervals to find changes in the turbidity.

Measurements of turbidity have no meaning in themselves. To find actual cell numbers a calibration curve is needed. To produce a calibration curve plot turbidity readings against either a haemocytometer reading or a viable count reading of the same culture.

Fig. 5. A calibration curve.



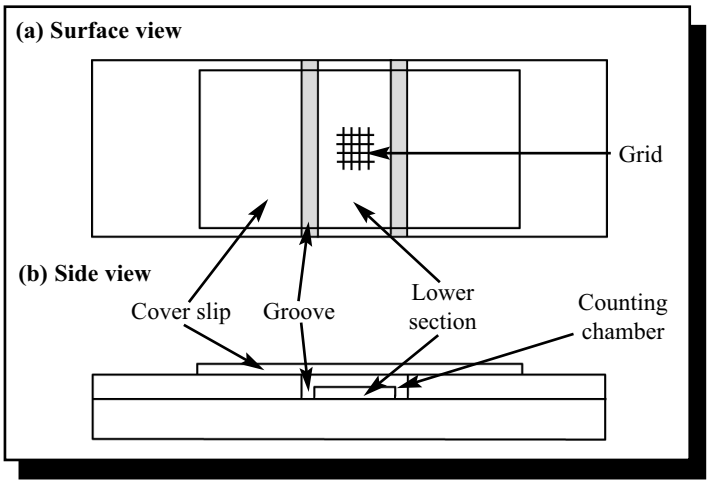
Use the calibration curve to covert turbidity readings of further samples of the same species to actual cell.

Hint: *Don't forget to multiply the number by the dilution factor and to express the result as the number of bacteria per cm³ of the original sample.*

- ***Direct total count:***

Cells growing in liquid culture are counted using a special type of slide called a haemocytometer *(Fig. 6)* and a microscope.

Fig. 6 A haemocytometer slide



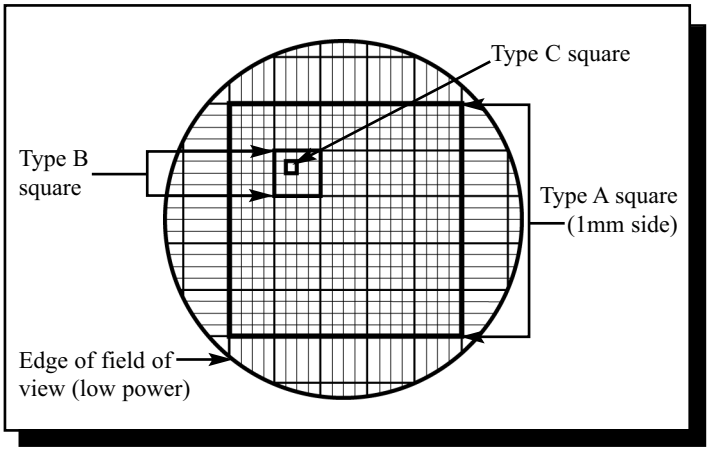
Before using a haemocytometer slide, spend some time with the slide to familiarise yourself with the grid and the different types of square. Use x10 or x8 eyepiece lens and x10 objective lens.

How to use a haemocytometer:

- Gently press a coverslip onto the slide until it adheres to the haemocytometer slide. A halo effect, called Newton's ring, will appear.
- It sometimes helps to slightly moisten the surface of the central platform around the grid and rotate the coverslip slightly.
- Use a pipette to remove a drop of the culture and release it at the edge of the coverslip. Capillarity will draw the culture under the coverslip and into the grid area.
- The number of cells can then be counted.

A haemocytometer has a central chamber of known depth with a grid etched on it. The grid is 1mm^2 and is divided into 400 small squares *(Fig. 7)*. Notice that there are three types of square. The largest, type A, square is the 1mm square containing the whole grid. This is divided into 25 smaller, type B squares which each have triple lines as a border. Each type B square is divided into 16, type C squares, with a single line border.

Fig. 7 Enlargement of a haemocytometer grid.



Care: *Haemocytometers should be handled with great care. To clean, wash and blot dry with lens tissue. Do not rub the grid area.*

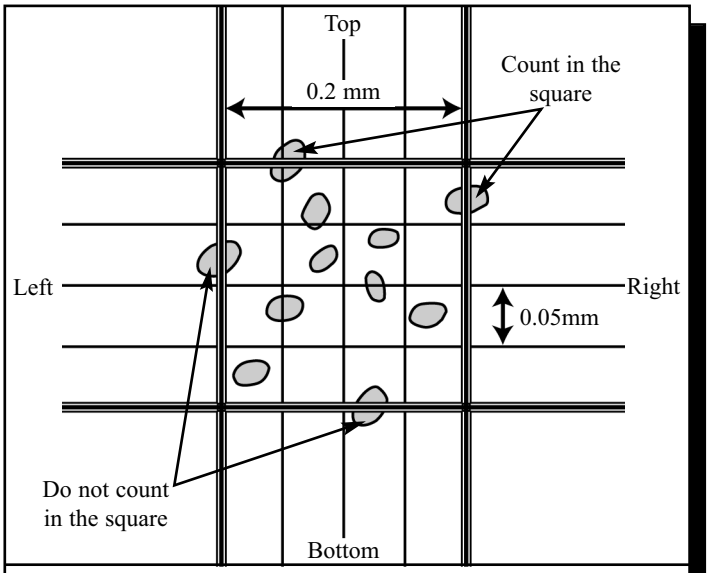
To estimate the size of a population of bacteria or other unicellular microorganism, the number of cells is counted. Ideally the whole grid should be counted. In practice, the four corner type B squares and the central type B square are often counted. This means that 80 of the type C squares will be counted.

Exam Hint: *You should be able to work out population sizes from the both type B and type C square counts.*

If the original sample is from a culture that has been diluted, the number of bacteria in the dilution factor has to be taken into account. For example, a 10^4 dilution would contain $2 \times 10^6 \times 10^4 = 2 \times 10^{10}$ bacteria per cm^3.

The enlargement of a single type B square illustrates how to count the cells in one square *(Fig. 8)*. The procedure prevents the same cell from being counted twice. To work out the population size you need to use the dimensions of the grid.

Fig. 8 Enlargement of one type B cells.



The sides of each type B square $= \frac{1}{5} = 0.2\text{mm}$

The area of each type B square $= \frac{[\ 1\ ]^2}{[5]^2} = 0.04\text{mm}^2$

When the coverslip is correctly positioned the distance between the slide and the coverslip is 0.1mm.

The volume of each type B square = 0.04 x 0.1 = 0.004mm^3

There are 8 bacteria in a type B square.
0.004mm^3 contains 8 bacteria.

1mm^3 contains $\frac{8 \times 1}{0.004} = 2000$ bacteria

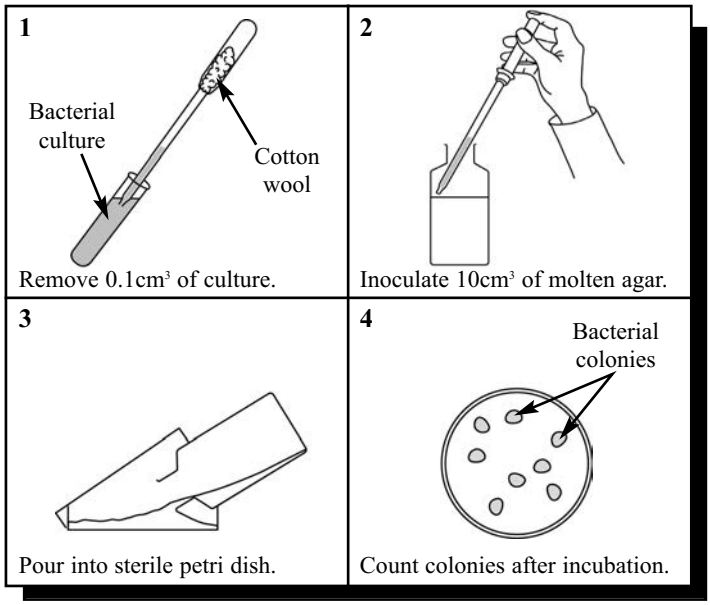
1cm^3 contains $2000 \times 1000 = 2{,}000{,}000 = 2 \times 10^6$ bacteria

Tip: *Make a copy of the grid and record the number of cells in each type B cell counted*

- ***Viable counts:***

The most common method is a **plate count**. A 0.1cm³ sample is removed from a culture and inoculated into a bottle of melted agar and used to make a pour plate. The plates are incubated at 30°C until colonies are easily visible. The number of bacterial colonies growing on the plate is counted.

Fig. 9 The preparation of a plate for counting.

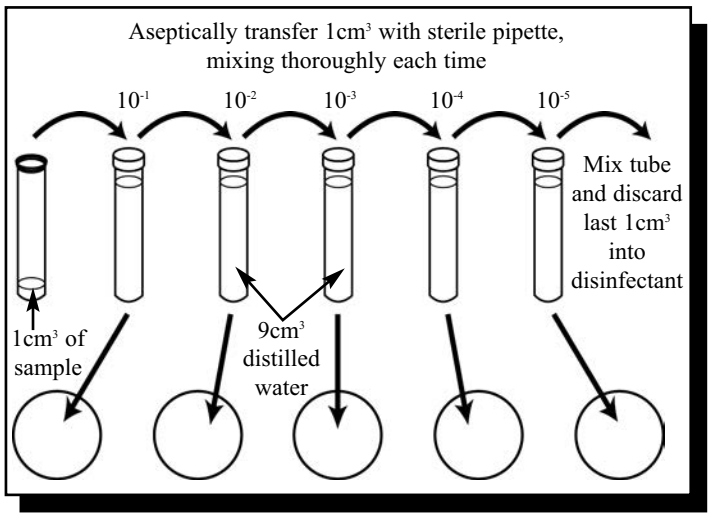


Tip: *You will find it easier to count if you draw lines with a marker pen on the underside of the dish to divide it into quarters and to put a small mark on each colony counted. Ideally you should take the average count of at least 3 plates.*

Each living bacterium in the culture will grow to produce a colony, so the number of colonies is equal to the number of bacteria in the culture. Most cultures contain a very large numbers of cells so the number of colonies forming would also be large. Crowded colonies tend to grow across each other making it impossible to count accurately. For this reason, a sample under is first diluted a number of times (a serial dilution) before plating.

After incubation, discard any plates showing more than 300 colonies or fewer than 30 colonies. If there are more than 300 errors will arise because colonies will overlap and it will be difficult to count accurately. If there are fewer than 30 sampling errors are likely due to the nature of the dilution and plating procedure.

Fig. 10 Making a dilution plate series.

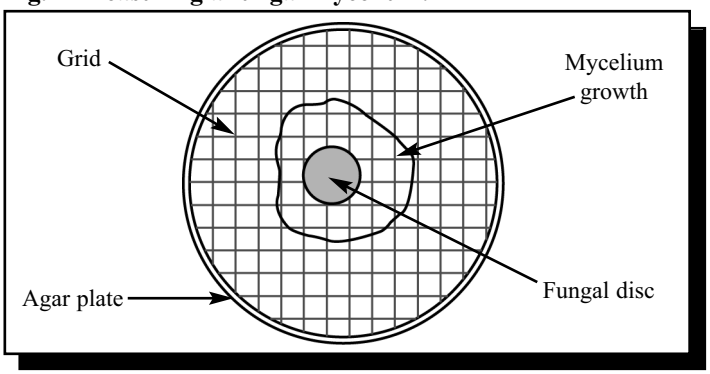


Methods of measuring filamentous growth

To measure growth of a filamentous fungus on a solid medium, the extension of the mycelium is measured at timed intervals. In a liquid culture the increase in dry mass is measured. As for bacteria, sterile technique must be used throughout the procedure.

To measure the growth of a mycelium, use a cork borer to obtain a sample of the mycelium from a culture that is actively growing. Avoid cultures that have spores as they can contaminate the sample. Cut a disc from the edge of a fungal mycelium and transfer to the centre of a suitable nutrient agar plate. Make a photocopy of a graph grid onto transparent acetate. Cut a shape to fit the underside of the petri dish and tape the grid to the petri dish. Draw the shape of the disc of mycelium onto the grid using a waterproof pen. Incubate the plate at 25°C and draw around the mycelium at 24 hour intervals. Use the grid to measure the increase in diameter of the colony. The rate of increase of the mycelium can be worked out by dividing the distance by the time.

Fig.11 Measuring a fungal mycelium.

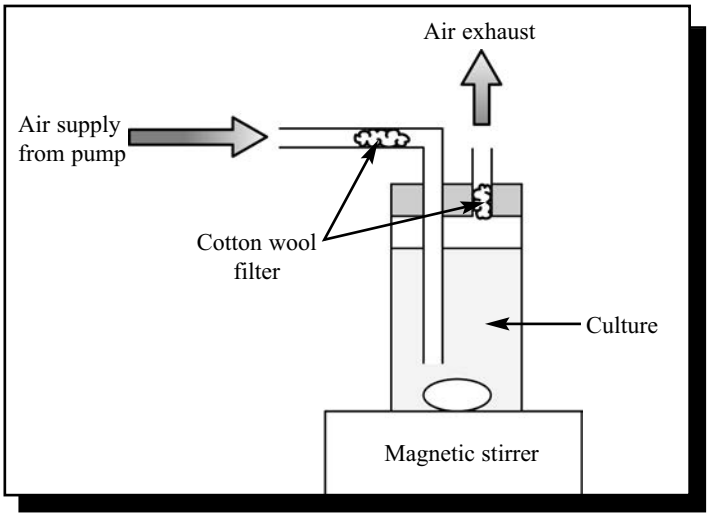


To measure the dry mass obtain a sample of an actively growing mycelium as in the previous procedure. Blend the mycelial disc with 5cm³ of liquid culture medium using sterile mortar and pestle or a blender. Add the blended mixture to 195cm³ of liquid culture medium and mix thoroughly. Divide the mixture into 10 equal sized samples. Incubate at 25°C and at 24 hour intervals find the dry mass of one of the cultures.

Static culture will form a mat of fungus at the surface. Ideally, the culture should be stirred constantly during incubation and supplied with air. In this case the fungus will form small pellets throughout the culture medium.

To obtain the dry mass, filter the sample through a pre-weighed filter paper. Dry the filter paper to a constant mass in an oven at 100°C. Any increase in mass is due to the growth of fungus.

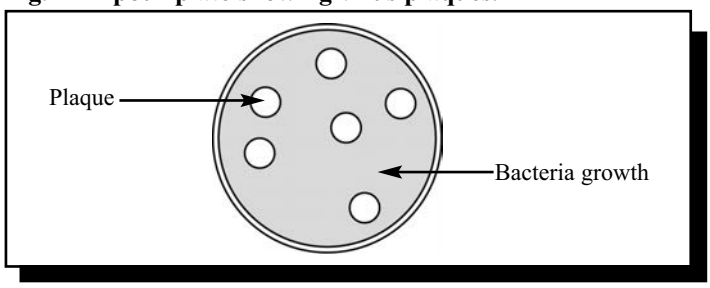
Fig. 12 Incubating the culture.



Measuring viruses

As viruses do not grow outside cells they are counted using a **plaque assay**. When a virus infects a cell it causes the cell to burst, or **lyse**. Viruses than spread to surrounding cells and cause more lysis. This leaves holes or plaques in cell culture that can be counted. Most viruses are unsuitable for classroom investigations. Some bacteriophage viruses that infect bacteria can be used. A serial dilution of a virus suspension is made in the same way as a bacteria serial dilution, 0.1cm^3 of each dilution is mixed with host bacterial cells, which are then used to make a pour plate. After incubation, bacteria will be visible throughout the plate, except where viruses are present. At these points, plaques will be visible as clear spaces in the agar.

Fig. 12 A pour plate showing virus plaques.



Practice questions

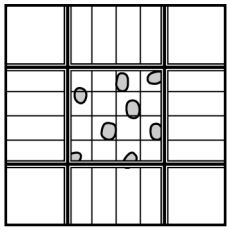
1. (a) What is the difference between total count and viable count? **1**
   (b) There are 7 x 10^6 organisms per cm^3 in a suspension. How many organisms would you expect in 1cm^3 of a 10^{-4} dilution? **2**
   (c) You have a culture of bacteria. Outline a method you could use to find the number of living cells per cm^3 in the culture. **8**

2. Investigations were made into the growth of *E.coli* in fermentation vessels containing a single sugar source (glucose or lactose) or a double sugar source (glucose and lactose). The concentration of sugar added was the same in each vessel and the cultures were incubated at the same temperature and aeration. Samples were removed at intervals and used for viable plate counts. The table shows the results.

Sampling time following inoculation (min)	Viable plate count per mm^3 medium		
	Glucose only	Lactose only	Glucose & lactose
0	28	27	28
20	32	30	33
40	64	32	60
60	80	47	78
80	115	62	112
100	133	80	127
120	135	88	132
140	135	94	150
160	135	99	180
260	121	101	230

   (a) (i) Plot, on the same axes, a growth curve for *E.coli* on each of the three media. **4**
   (ii) Describe and explain the similarities and differences in the growth curves. **12**
   (b) Explain how the growth curves would differ if a total count had been used to measure the population. **3**

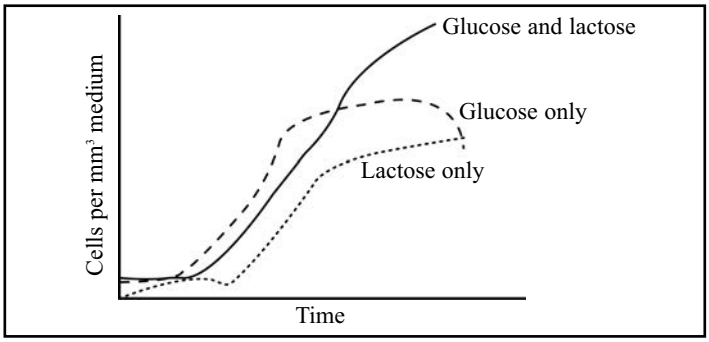
3. The number of cells from a 10^5 dilution of a culture in one type B square of a aemocytometer is shown in the diagram.
   (a) How many cells are present in 1cm^3 of the culture? **3**
   (b) Describe how the haemocytometer slide would have been prepared. **4**



Answers

1. (a) Total count includes dead cells, viable is only cells capable of growing **1**
   (b) 700 (7 x 10^6 – 10^{-4} = 10^2 x 7) **2**
   (c) Make a serial dilution by adding 1cm^3 of culture to 9cm^3 sterile water to give 10^{-1} dilution; Mix and transfer 1cm^3 to 9 cm^3 of sterile water to make 10^{-2} dilution; Mix and repeat to 10^{-6} dilution; Melt 6 x 10 cm^3 agar in separate containers; To each melted agar, add 1cm^3 of a different dilution and make pour plates for each dilution; Incubate for 24 hours at 30°C; Count the number of colonies discarding any plates below 30 or above 300; Each colony represent some bacteria in 1cm^3 of the dilution added. **8**

2. (a) (i) Correct axes **1;**
   3 correctly; plotted lines (1 mark deducted for each incorrect plot – approximate shape shown) **3;**



   (ii) Similarities:
   Lag phase. Little increase in cell number; Individual bacteria may be increasing in size; Enzymes may be being synthesised to utilise the nutrient medium; **max.2**
   Log phase. The population shows exponential phase; Nutrient supply is not a limiting factor; **2**
   Stationary phase. The population growth slows down; The number of new cells formed is balanced by the number of cells dying; Limiting factors, such a nutrient supply and metabolic wastes have started to influence further increase in population size; **max 2**
   Differences:
   Lag phase for lactose alone longer; nutrient less readily available; Growth of population less than with glucose; fewer cells and longer to increase; **max.2**
   Growth with lactose and glucose gives a greater population; Has a second lag; around 100-120 minute and then increases again; Uses glucose first as growth curve similar to glucose alone; and then uses lactose; **max 3**
   Death phase is occurring for glucose alone as all the energy source/glucose has run out. **1**
   (b) as dead cells are included in the count; there would be higher numbers;; there would be no death phase evident for glucose alone; **3**

3. (a) 6 are in the square; volume of the square is 0.004mm^3; number of cells I 1mm^3 = 6/0.004 = 1500; Number of cells in 1cm^3 = 1500 x 1000 = 15 x 10^5 (1.5 x 10^6); number of cell in original culture = 15.10^5 x 10^5 = 15 x 10^{10} **3**
   (b) Fix coverslip to haemocytometer slide; make a serial dilution of the culture; add 1 drop to edge of coverslip; sterile technique used through out; **4**

Acknowledgements
This Factsheet was researched by Chris Lea.
Curriculum Press. Unit 305B, The Big Peg, 120 Vyse Street, Birmingham B18 6NF